POSTMITOCHONDRIAL REGULATION OF APOPTOSIS IN NEURONS AND CANCER CELLS

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ABSTRACT

ALLYSON EVANS VAUGHN
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(Under the direction of Mohanish Deshmukh, Ph.D.)

The apoptotic pathway is a genetically conserved, energetic process that is essential for the development and homeostasis of organisms. Aberrant apoptosis, however, can result in variety of diseases including neurodegeneration and cancer. Apoptotic stimuli ultimately converge at the mitochondria, where cytochrome *c* is released into the cytosol to trigger formation of the apoptosome complex. The active apoptosome then goes on to activate the caspase family of proteases which cleave a myriad of substrates, resulting in cell death.

The core apoptotic components are ubiquitously expressed in cells, however some cell types (such as neurons and cancer cells) have an increased need to strictly regulate the apoptotic pathway. Unlike normal mitotic cells, postmitotic neurons have little regenerative potential and must often last the lifetime of the organism. Here, I identify novel ways in which neurons and cancer cells inhibit the apoptotic pathway at points downstream of cytochrome *c*. Specifically, I describe mechanisms by which postmitotic neurons of the PNS and CNS posttranslationally inactivate cytochrome *c* and Apaf-1, respectively, in order to ensure their longterm survival. In addition, my work suggests

that cancer cells may use mechanisms similar to those adapted by neurons in order to evade apoptosis.

Despite a neuron's ability to inhibit programmed cell death, these cells must still be able engage the apoptotic pathway during development or in the event of extreme stress. Here, I uncover the mechanism by which cytochrome *c* becomes reactivated in sympathetic neurons during developmental apoptosis. In addition, I examine how XIAP's inhibition of caspases is overcome to allow neuronal apoptosis in response to DNA damage.

Together, these results not only illustrate the importance of a strict regulation of apoptosis in differentiating neurons, but also identify the mechanisms by which these blocks are overcome to allow death. I hope that a deeper knowledge of how the apoptotic pathway is reactivated in dying neurons could eventually lead to therapeutics to inhibit cell death in the context of neuronal pathologies, or to promote cell death in cancer.

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6-AN:	6-anicatimamide
BIR:	Bacculoviral IAP Repeat
BH:	Bcl-2 Homology
CARD:	Caspase activation and recruitment domain
CGN:	Cerebellar granule neuron
CNS:	Central nervous system
DEM:	Diethylmaleate
DHEA:	Dehydroepiandrosterone
dADP:	Deoxy adenosine diphosphate
dATP:	Deoxy adenosine triphosphate
DIABLO:	Direct IAP binding protein with low pI
EGFP:	Enhanced green fluorescent protein
ER:	Endoplasmic reticulum
G6PDH:	Glucose-6-phosphate dehydrogenase
GAPDH:	Glyceraldehyde phosphate dehydrogenase
GSH:	Reduced glutatione
HDF:	Human dermal fibroblasts
HSP27/70/90:	Heat shock proteins
IAPs:	Inhibitor of Apoptosis Proteins
IBM:	IAP Binding Motif
MEF:	Mouse Embryonic Fibroblast
NGF:	Nerve growth factor

P5,10.13.19:	Postnatal day…
PC12 cell:	Pheochromocytoma cell
PKC:	Protein Kinase C
PKA:	Protein Kinase A
PNS:	Peripheral Nervous System
RING:	Really Interesting New Gene domain
SMAC:	Second mitochondrial activator of caspases
SOD:	Superoxide Dismutase
XIAP:	X-linked Inhibitor of Apoptosis Protein

I. CHAPTER ONE:

Introduction to Apoptosis

Although the first record of programmed cell death was likely made by Carl Vogt as early as 1842, observations in 1972 by Drs. J.F.R. Kerr, A.H. Wyllie, and A.C. Currie mark the birthdate of the term, apoptosis (Clarke and Clarke 1996). As this team of scientists examined the tiny ovoid cytoplasmic fragments within sections of fixed tissue, they were surely unaware that within just forty years, their observations would lead to over 130,000 journal articles and a Nobel Prize.

Kerr and colleagues noticed that the phenotype of cells dying spontaneously within organisms were distinct from the swollen cells seen after injury. While the latter cells would eventually burst, spilling their contents into surrounding tissue, this new form of death was characterized by cell shrinkage, chromatin condensation and the engulfment of these corpses by surrounding cells. They deemed this new death process "apoptosis," from the Latin meaning "dropping or falling off," as of petals from flowers or leaves from trees (Kerr, Wyllie et al. 1972). Apoptosis was quickly appreciated for its essential place in organismal development, from nematodes to mammals, and its role in countless human disease conditions ranging from cancer to neurodegeneration.

A. Apoptosis : a history of the key players

While the implications for apoptosis as an important part of development and disease were easily recognized, more slowly to emerge from the scientific community has been an understanding of the genetic and biochemical processes that control it. In the mid 1980s, studies from Dr. Bob Horvitz's lab mapped the fate of every cell in the nematode *C. elegans*, leading to the identification of two genes, *ced-3* and *ced-4*, required for programmed cell death. Loss of function mutations in these genes allowed for the survival of the 131 cells

fated to die during the nematode's development (Ellis and Horvitz 1986; Yuan and Horvitz 1990). Shortly after, the Horvitz lab identified the first antiapoptotic gene, *ced-9,* mutations of which cause excessive cell death in the nematode (Hengartner, Ellis et al. 1992). These discoveries not only earned Dr. Horvitz the Nobel Prize, but paved the way for other researchers to look for conserved death machinery in the mammalian cell.

The search for mammalian gene sequence homology to *ced-3* uncovered an entire family of cysteine proteases, called caspases, which, following their activation, are responsible for cleavage of a myriad of cellular substrates within the cell (Yuan, Shaham et al. 1993; Hengartner and Horvitz 1994). Of the many caspases, caspase-3 and caspase-9 are critical for developmental cell death, as mice deficient in both caspase-9 and caspase-3 are unable to undergo the extensive apoptosis that occurs during brain development (Kuida, Haydar et al. 1998).

In an assay designed to identify proteins capable of cleaving and activating caspase-3, the mammalian homologue of *ced-4* was discovered and named apoptotic protease-activating factor-1 (Apaf-1) (Liu, Kim et al. 1996; Zou, Henzel et al. 1997). Although *ced-4* and Apaf-1 share sequence homology, mammalian Apaf-1 is more complex than its nematode precursor, with an additional regulatory element (a WD-40 repeat motif) at its C-terminus. This WD-40 domain renders Apaf-1 incapable of inducing activation of caspase-3 in the absence of an additional cytosolic partner (Hu, Ding et al. 1998). This activator of Apaf-1, which was identified concomitantly with Apaf-1, turned out to be cytochrome *c*, a well studied and critical component of the mitochondrial respirator chain (Liu, Kim et al. 1996).

With the discovery of *ced-9* in the worm, it was realized that members of the mammalian Bcl-2 (B-cell lymphoma 2) family share significant sequence homology to this

antiapoptotic *C elegans* gene. The importance of Bcl-2 as an inhibitor of apoptosis was soon appreciated because deletion of this protein or a family member, Bcl-xL, from mice results in massive apoptosis in the hematopoetic system and in the developing nervous system (Nakayama, Nakayama et al. 1993; Motoyama, Wang et al. 1995). In addition, overexpression of Bcl-2 prevents mammalian cells from undergoing apoptosis. Bcl-2-mediated inhibition of apoptosis blocks caspase activation (Reed 1994), indicating that it functions upstream of the caspase cascade. Bcl-2 is localized primarily to the outermembrane of the mitochondria (De Jong, Prins et al. 1992; Monaghan, Robertson et al. 1992; Krajewski, Tanaka et al. 1993), and although this was curious at the time of its discovery, the implications of the mitochondrial protein, cytochrome *c,* in cell death suggested that Bcl-2 may play a role in the apoptotic regulation of cytochrome *c* (Chao and Korsmeyer 1998).

1. Bcl-2 and family members: gatekeepers of the mitochondria

Bcl-2 was identified at an interchromosomal breakpoint in B-cell lymphoma, and was the first oncogene described to promote cancer survival not by enhancing proliferation, but by prohibiting cell death (Chao and Korsmeyer 1998). Multiple proteins bearing sequence homology to Bcl-2 through Bcl-2 homology (BH) domains have since been identified, with members being divided into two groups: 1) The antiapoptotic Bcl-2 members include, but are not limited to, Bcl-2, Mcl-1 and Bcl-xL, which typically contain BH1 through BH4 domains and are often anchored to membranes through their hydrophobic carboxy-terminus (Martinou and Green 2001); and 2) the proapoptotic members of the Bcl-2 family (e.g., Bax, Bak, Bid, Bim, Puma, Noxa, et al.), which often only localize to mitochondrial membranes following a

death signal. The proapoptotic members are further classified into: 1) "multidomain" members such as Bax and Bak which generally have sequence homology in BH1, BH2, and BH3 domains; and 2) "BH3-only" members including, but not limited to Bid, Bim, Bad, and Puma (Scorrano and Korsmeyer 2003). These domains allow members of the Bcl-2 family to interact with each other, forming heterodimers or homodimers (Kelekar and Thompson 1998). It is thought that the BH3 domain of proapoptotic members of the Bcl-2 family can interact with a hydrophobic cleft formed by anti-apoptotic members, thus allowing the strict regulation of these protein (Harris and Thompson 2000).

a. Activation of the Bcl-2 family

The activation of the apoptotic death pathway can occur in response to a wide variety of physiological as well as pathological cell death insults including growth factor deprivation, DNA damage, and ER stress (Vaux and Korsmeyer 1999). In mammalian cells, cytosolic cytochrome *c* can trigger the activation of caspases and cellular death, thus the regulation of cytochrome *c* release by the Bcl-2 family is for many cells, the critical step in determining whether it will live or die. Cell death insults engage BH3-only proteins such as Bid, Bim, and Puma which then localize to the mitochondria where they bind and displace anti-apoptotic molecules like Bcl-2 and Mcl-1. Through mechanisms that are not fully understood, the displacement of Bcl-2 members allows Bax and Bak to oligimerize at the mitochondria and initiate the release of cytohchrome *c* (Fig. 1.1), (Willis and Adams 2005).

Although it is Bax and Bak which directly cause the release cytochrome *c* from the mitochondria, it is often the activation of upstream BH3-only members that are tightly regulated in response to apoptotic stimuli in a cell. BH3-only members can be activated by a

variety of means both posttranscriptionally and transcriptionally. For example, both Bim and Bad activity depend, in part, on their localization. Upon growth factor stimulation, Bad is phosphorylated, allowing it to bind to the protein 14-3-3, which holds it harmlessly in the cytosol. However, in the absence of growth factor stimulation, unphosphorylated Bad localizes to the mitochondria where it can interact with and inhibit the antiapoptotic protein, Bcl-xL (Zha, Harada et al. 1996; Puthalakath and Strasser 2002). In addition, the activity of Bim is also inhibited by sequestration. Whereas inactive Bim is tethered to the microtubule-associated dynein motor complex, it can become unleashed and translocate to the mitochondria to inhibit Bcl-2 (Puthalakath, Huang et al. 1999). The activity of the BH3 protein, Bid, is regulated by cleavage. In response to certain apoptotic stimuli that activate the extrinsic pathway of apoptosis, such as TNFα or Fas, full length Bid is cleaved to truncated Bid (tBid) which then can initiate cytochrome *c* release at the mitochondria (Esposti 2002). BH3-only proteins are also often regulated via transcription (Freeman, Burch et al. 2004). During embryogenesis, the BH3 only protein DP5/HRK is induced in neuronal tissues that undergo extensive apoptosis (Harris and Johnson 2001). Bim is also transcriptionally induced in response to growth factor withdrawal in neuronal and hematopoetic cell types (Dijkers, Medema et al. 2000; Putcha, Moulder et al. 2001). In addition, transcriptional upregulation of both Puma and Noxa by the tumor suppressor p53 are important for DNA damage-induced apoptosis (Oda, Ohki et al. 2000; Nakano and Vousden 2001; Puthalakath and Strasser 2002).

2. Apoptosome formation and caspase activation

While control of Bcl-2 family members and cytochrome *c* release often dictates the outcome of survival, caspases are the true executioners of programmed cell death. Active caspases are responsible for the cleavage of a broad spectrum of cellular targets which results in the classical apoptotic morphology and the ultimate death of the cell. In healthy cells, caspases are present as catalytically inactive zymogens, and can become activated in response to cytochrome *c* release from the mitochondria. Caspase activation occurs in a cascade, with initiator caspases, such as caspase-9, -8, -2 and -10 cleaving and exposing the catalytic domain of effector caspases (caspase-3 and caspase-7), which go on to kill the cell (Shi 2002). The activation of caspase-9 occurs by autoactivation, and requires the assembly of a large complex known as the apoptosome. Apoptosome formation can be triggered in response to the release of cytochrome *c* from the mitochondria into the cytosol (Shi 2002; Shi 2002).

The main function of the apoptosome is to initiate the enzymatic activity of caspase-9. The primary component of this 700-kD complex is the *ced-4* homolog, Apaf-1 (Liu, Kim et al. 1996; Cain, Bratton et al. 2000). Apaf-1 is comprised of an N-terminal CARD domain, a CED-4 Nucleotide Binding homology domain, and 13 WD-40 repeats at the C-terminus (Shi 2002), (Fig. 4.4). In healthy cells, Apaf-1 is in an auto-inhibitory conformation whereby the WD-40 domain renders the N-terminal domains inaccessible. However, in response to an apoptotic insult, cytosolic cytochrome *c* binds to the WD-40 domain, exposing Apaf-1's caspase recruitment domain (CARD) and Nucleotide Binding Domain (NBD) (Hu, Benedict et al. 1998; Acehan, Jiang et al. 2002; Yu, Acehan et al. 2005). In addition to the requirement of cytochrome *c* binding, the oligimerization of Apaf-1 into the apoptosome requires dATP binding to the NBD (Liu, Kim et al. 1996). In response to cytochrome *c*

binding, dATP hydrolysis to dADP may occur followed by exchange to dATP (Kim, Du et al. 2005; Yu, Acehan et al. 2005). This exchange seems to be important for defining a stable conformation of Apaf-1 to allow it to assemble with six other subunits into an active apoptosome. This arrangement of oligomerized Apaf-1 exposes its CARD domains at the central hub of the wheel, which are responsible for caspase-9 recruitment and binding via CARD-CARD interactions.

While effector caspases must be cleaved to become active, the activation of initiator caspase-9 does not require cleavage, but becomes activated after dimerization on the apoptosome (Boatright, Renatus et al. 2003; Boatright and Salvesen 2003). In fact, the activity of processed caspase-9 is enhanced more than 1000-fold upon association with the apoptosome (Rodriguez and Lazebnik 1999). However, this theory, known as the "induced proximity model," may not explain caspase-9 activation entirely because constitutively dimeric caspase-9 remain considerably less active than those associated with Apaf-1 (Chao, Shiozaki et al. 2005). In any case, the requirement for the apoptosome in activating caspase-9 is clear as Apaf-1-/- animals exhibit phenotypes similar to caspase-9-/- animals, in that the absence of either inhibits cell death during development (Kuida, Zheng et al. 1996; Cecconi, Alvarez et al. 1998; Hakem, Hakem et al. 1998; Yoshida, Kong et al. 1998).

B. Regulators of apoptosome assembly and caspase activation

Cytochrome *c,* Apaf-1, caspase-9, and dATP, are together sufficient for caspase activation and apoptosis to occur *in vitro*. Yet, in most cell types, these dangerous components are maintained at high levels even in the absence of an apoptotic stimulus. This suggests that other factors in an intact cell must regulate this core apoptotic machinery to

ensure that apoptosis occurs only at the right time and in the right place. Over the last two decades, several such modifiers have been identified. While controversy remains as to whether the release of cytochrome *c* is a point of no return for a cell, it is clear that neurons in which caspases are blocked downstream of cytochrome *c* release are able to recover the disruption of the mitochondria, and continue to function longterm (Neame, Rubin et al. 1998; Deshmukh, Kuida et al. 2000). This finding indicates that the cell's ability to regulate the apoptotic pathway downstream of cytochrome *c* release may be important in deciding whether a cell lives or dies. Here, I will introduce several ways in which cytochrome *c* itself, the formation of the apoptosome, and the activity of caspases can be regulated.

1. Regulators of cytochrome *c* function

In healthy cells, cytochrome *c* functions to transfer electrons between integral mitochondrial membrane protein complexes in the respiratory chain of eukaryotes. However, in response to many apoptotic stimuli, cytochrome *c* relocates from the mitochondria into the cytosol where it is able to bind and activate the apoptosome, leading to caspase activation and death. Cytochrome *c* is synthesized in the nucleus and is then imported into the mitochondria where a heme is attached. Holocytochrome *c* (heme-attached) is capable of activating the apoptosome, however, apocytcohrome *c*,which lacks the heme, has no proapoptotic activity, and when overexpressed can even act as a dominant negative (Yang, Liu et al. 1997; Martin and Fearnhead 2002). While it is unclear whether apocytochrome *c* affects the proapoptotic activity of cytochrome *c* in living cells, it may prove a viable target to inhibit apoptosis in pathological situations (Martin and Fearnhead 2002).

The expression level of cytochrome *c* may also determine a cell's apoptotic potential. Several groups have shown that in response to an apoptotic stimulus, cytochrome *c* mRNA and protein are induced, enhancing its ability to induce apoptosis (Sanchez-Alcazar, Ault et al. 2000; Chandra, Liu et al. 2002). Levels of cytcohrome *c* protein can also be regulated strictly at the translational level. Overexpression of Smooth Muscle-20 (SM20), a prolyl hydroxylase that is induced upon growth factor mediated apoptosis in neurons, promotes a massive accumulation of cytochrome *c* protein with no apparent increase in cytochrome *c* mRNA (Straub, Lipscomb et al. 2003). In addition, binding proteins can also affect the ability of cytochrome *c* to function. One group has shown that Heat Shock Protein 27 (HSP27) negatively regulates cytochrome *c* by binding to it and sequestering it away from Apaf-1 (Bruey, Ducasse et al. 2000).

Posttranslational modifications of cytochrome *c* itself may also play a role in its ability to induce apoptosis. For example, cytochrome *c* purified from yeast is unable to promote apoptosis, in part due to tri-methylation of Lysine 72. Similarly, if mammalian cytochrome *c* is methylated on this residue, it loses its apoptotic activity (Kluck, Ellerby et al. 2000). Whether regulation of cytochrome *c* by methylation occurs in mammalian cells to control the apoptotic pathway is unknown. The redox status of cytochrome *c* has also been implicated in its ability to promote cell death. Some groups suggest that *in vitro*, oxidized cytochrome *c* is more apoptotically active than reduced cytochrome *c* (Pan, Voehringer et al. 1999; Hancock, Desikan et al. 2001; Suto, Sato et al. 2005). This, however, is controversial, as other groups suggest there is no difference (Kluck, Martin et al. 1997; Hampton, Zhivotovsky et al. 1998). Chapter Two of this dissertation examines the role that cytochrome *c* redox plays in

apoptosis of intact cells. In addition, Appendix B of this dissertation explores a novel way in which cytochrome *c* degradation may regulate its apoptotic activity.

2. Regulators of apoptosome activity

In addition to regulation of cytochrome *c* itself, caspase activation and apoptosis can be controlled at the point of apoptosome assembly and activity. Like cytochrome *c*, Apaf-1 and the activity other apoptosome components can be regulated by expression at the transcriptional level, as well as by various binding partners that either inhibit or enhance the activity of the apoptosome.

Our lab and others have recently shown that as dividing cells differentiate and become postmitotic, they lose their ability to undergo cytochrome *c*-mediated apoptosis. This increased resistance to apoptosis allows for survival of these postmitotic cells which must often last the lifetime of the organism. In cardiomyocytes, myotubes, and neurons of both the CNS and PNS, this loss in sensitivity to cytochrome *c* is due, at least in part, to the dramatic decrease in Apaf-1 protein at the transcriptional level (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005) (Chapter Four, and unpublished data). In these cells, low levels of Apaf-1 render the apoptosome unable to activate sufficient caspases to overcome endogenous inhibitors of caspase activation (such as XIAP) found in the cell. XIAP and its regulation are discussed in more detail below. In addition, alternative splicing of Apaf-1 could regulate apoptosome function, as splice variants encoding fewer WD-40 repeats have reduced activity (Benedict, Hu et al. 2000).

The ability of Apaf-1 to assemble into the apoptosome and recruit caspase-9 can also be influenced by proteins and molecules which bind to and inhibit Apaf-1. For example,

Heat shock protein-90 (HSP90) has been shown to bind Apaf-1 and inhibit apoptosome assembly, perhaps by restricting binding of cytochrome *c* to Apaf-1 (Pandey, Saleh et al. 2000). HSP70 may also regulate the apoptosome, but by binding to Apaf-1 and preventing caspase-9 processing (Beere, Wolf et al. 2000; Saleh, Srinivasula et al. 2000; Beere 2004). In addition, the proteins Aven and ProT can prevent apoptosome assembly (Chau, Cheng et al. 2000; Jiang, Kim et al. 2003), whereas TUCAN, APIP, JNK, and multiple truncated forms of caspase-9 can all prevent apoptosome activity by inhibiting caspase-9 recruitment and activation (Srinivasula, Ahmad et al. 1999; Angelastro, Moon et al. 2001; Pathan, Marusawa et al. 2001; Cao, Xiao et al. 2004; Tran, Andreka et al. 2007). Apoptosome activity is also potentially negatively regulated by non-protein interactors such as nitric oxide donors, high levels of potassium, and intracellular nucleotides (Cain, Langlais et al. 2001; Zech, Kohl et al. 2003; Chandra, Bratton et al. 2006).

In contrast, some binding partners of Apaf-1 are able to increase apoptosome activity. NAC and hepatacellular carcinoma antigen 66 (HCA66) were shown to enhance caspase-9 recruitment and activation through their association with Apaf-1 (Chu, Pio et al. 2001; Piddubnyak, Rigou et al. 2007). Recently, putative HLA-DR-associated protein 1 (PHAP1) has been shown to increase activity of the apoptosome through unknown mechanisms, and its increased expression in breast cancer cells sensitizes them to cytochrome *c*-mediated apoptosis (Jiang, Kim et al. 2003; Schafer, Parrish et al. 2006).

Although to date no known sites of posttranslational modifications have been identified on Apaf-1, Apaf-1 phosphorylation has been seen in correlation with regulation of caspase-9 processing on the apoptosome in response to PKA and Bcr-Abl (Deming, Schafer et al. 2004; Martin, Allan et al. 2005). In Chapter Four of this dissertation, I provide

evidence suggesting that Apaf-1 phosphorylation can regulate apoptosis in the developing cerebellum.

3. XIAP and regulators of caspase activation

The ability to regulate caspase activity is critical for a cell, because caspase activation can lead to a feed forward cascade that results in massive caspase processing, cleavage of a multitude of substrates and eventual cell death (Fischer, Janicke et al. 2003). Caspase activity can be controlled by modification of caspases themselves, such as by phosphorylation or nitrosylation (Kim, Kwon et al. 2002; Allan, Morrice et al. 2003; Brady, Allan et al. 2005). However, to date, the most potent and well characterized endogenous caspase regulators are members of the Inhibitor of Apoptosis Protein (IAP) family.

The first IAP was discovered in a screen to identify regulators of host-cell viability during virus infection. This screen uncovered the first IAP motif from baculovirus, now known as the baculovirus IAP repeat (BIR) domain, present in all IAP family members. In mammalian cells, IAPs include XIAP, cIAP1, cIAP2, ML-IAP, NAIP, Survivin, and Apollon/Bruce (Salvesen and Duckett 2002). While overexpression of many of the IAP family members are able to suppress apoptosis induced by a variety of stimuli (Duckett, Nava et al. 1996; Liston, Fong et al. 2003), XIAP is by far the most potent inhibitor of caspases (Salvesen and Duckett 2002; Scott, Denault et al. 2005; Eckelman and Salvesen 2006; Eckelman, Salvesen et al. 2006). Although the XIAP knockout mouse shows no overt phenotype (Harlin, Reffey et al. 2001), recently endogenous XIAP was identified as a critical regulator of caspase activation in cytochrome *c*-mediated apoptosis in neurons as well as cardiomyocytes. While cytosolic cytochrome *c* alone is incapable of inducing apoptosis in

these postmitotic cells, XIAP deficient neurons and cardiomyocytes are susceptible to cytochrome *c*-mediated apoptosis (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). This suggests that XIAP may act as a safety brake for caspase activation, only becoming important in situations of mitochondrial stress or accidental spillage of cytochrome *c* from the mitochondria.

XIAP's inhibition of caspases is direct. XIAP contains three BIR domains, the third which can bind directly to cleaved and activated caspase-9, inhibiting its ability to interact with downstream substrate (Sun, Cai et al. 2000; Srinivasula, Hegde et al. 2001). XIAP's inhibition of caspase-3 and 7 is mediated by the small linker region of XIAP lying immediately N-terminal of the BIR2 domain, resulting in the reversible steric occlusion of caspase substrates (Chai, Shiozaki et al. 2001; Huang, Park et al. 2001; Riedl, Renatus et al. 2001). While the BIR domains at the N-terminus of XIAP have the ability to inhibit caspases, XIAP also contains a C-terminal RING finger domain which is known to have E3 ubiquitin ligase activity (Fig. C1), (Yang, Fang et al. 2000). Although XIAP has been shown to ubiquitinate several target proteins in the apoptotic pathway (Silke, Ekert et al. 2001; Suzuki, Nakabayashi et al. 2001; MacFarlane, Merrison et al. 2002; Sun 2003; Morizane, Honda et al. 2005), whether this domain plays a role in regulating apoptosis in intact cells is less understood. Appendix C of this dissertation suggests that the RING domain of XIAP may play an antiapoptotic role in neurons.

a. Regulators of XIAP

Unlike for mammalian cells, the deletion of the XIAP homologue, DIAP1 in *Drosophila* leads to massive apoptosis and early lethality. In *Drosophila*, DIAP1 activity is

inhibited by the DIAP1 binding proteins, Reaper, Grim, HID, and Sickle (Martin 2002). Immunoprecipitation experiments looking for similar regulators of XIAP in mammalian cells identified two XIAP binding proteins, SMAC/DIABLO, and Omi/HtrA2 which could enhance caspase activation in biochemical assays (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000; Suzuki, Imai et al. 2001; Hegde, Srinivasula et al. 2002; Martin 2002; van Loo, van Gurp et al. 2002; Verhagen and Vaux 2002). Unlike their *Drosophila* counterparts, both SMAC and HtrA2 are localized to the mitochondria in healthy cells where their mitochondrial targeting sequence is cleaved off, exposing an N-terminal AVPI (SMAC) or AVPS (HtrA2) sequence. Upon apoptotic stimulation, SMAC and HtrA2 are released into the cytosol where they interact with XIAP. SMAC inhibits XIAP through the binding of SMAC's N-terminal AVPI motif with a groove made by the BIR2 and BIR3 domain of XIAP (Salvesen and Duckett 2002). This interaction occurs at the same region as caspase-9-XIAP binding, and at high enough ratios, can result in exclusion of caspase-9 from XIAP (Sun, Cai et al. 2000; Srinivasula, Hegde et al. 2001; Shi 2002). SMAC binding to XIAP at the BIR2 domain also destabilizes and inhibits caspase 3/7 binding (Verhagen and Vaux 2002).

Although SMAC is a powerful XIAP inhibitor *in vitro,* the endogenous role of SMAC is less understood. We would anticipate that SMAC would be important as an XIAP inhibitor exclusively in cell types where XIAP is a critical inhibitor of caspase activation, such as neurons and cardiomyocytes (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000; Ekert, Silke et al. 2001). Indeed, ectopic expression of SMAC in these cell types inhibits XIAP, allowing cytochrome *c* to induce cell death (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). Interestingly, however, data presented in Chapter Two of this dissertation suggest

that endogenous SMAC does not inhibit XIAP in neurons undergoing cytochrome *c*-mediated apoptosis (Vaughn and Deshmukh 2007). Despite the failure to identify a role of endogenous SMAC in apoptosis, understanding the mechanism by which SMAC inhibits XIAP may prove useful in cancer thereaputics, as expression of SMAC mimetics induces cell death in many cancer cells lines as well as mouse tumor models (Li, Thomas et al. 2004; Schimmer, Welsh et al. 2004; Bockbrader, Tan et al. 2005).

The activity of XIAP can also be regulated by mechanisms independent of SMAC or HtrA2. In sympathetic neurons undergoing apoptosis in response to Nerve Growth Factor (NGF) deprivation, caspase activation proceeds due to the degradation of XIAP itself (Potts, Singh et al. 2003). Although the mechanism by which this occurs remains elusive, the RING finger domain of XIAP has been shown to function as an E3 ligase, targeting itself for ubiquitylation (Yang, Fang et al. 2000). As described earlier, the effectiveness of XIAP can be indirectly influenced by levels of Apaf-1, and hence, apoptosome activity. When Apaf-1 is low, caspase activation is minimal, making XIAP an affective inhibitor of cell death. However, high levels of Apaf-1 lead to massive caspase activation which overwhelms the ability of XIAP to inhibit apoptosis (Fig. 1.2) (Wright, Linhoff et al. 2004; Vaughn and Deshmukh 2007). The regulation of XIAP activity via Apaf-1 levels is an crucial mechanism by which differentiating neurons and cardiomyocytes develop resistant to cytochrome *c* mediated apoptosis, and is also necessary for neuronal apoptosis in response to DNA damage (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005; Vaughn and Deshmukh 2007), This latter aspect of apoptotic regulation is examined in detail in Chapter Two.

C. ROS and Glucose Metabolism as Regulators of Apoptosis

Reactive Oxygen Species (ROS) are generated as a result of normal cellular processes such as aerobic respiration, and can also be readily taken up by the cell from its environment. In a cell, ROS can cause damage to lipids, proteins, as well as DNA. Gone unchecked, intracellular ROS accumulation causes oxidative insults that often result in cell death (Chandra, Samali et al. 2000). ROS has been linked to apoptotic cell death in multiple scenarios including TNF, Fas, p53, myc, and virus-induced apoptosis (Polyak, Xia et al. 1997; Chen, Willis et al. 2005). In addition, well known endogenous inhibitors of apoptosis, such as Bcl-2, can act, in part, by modulating ROS in the cell (Mirkovic, Voehringer et al. 1997). Despite these links to apoptosis, whether ROS plays a direct mechanistic role in activating the apoptotic pathway is has not been extensively examined.

Some research suggests, however, that ROS may directly influence cytochrome *c* release. For example, ROS-mediated oxidation of cardiolipin on the mitochondrial membrane has been shown to facilitate its detachment from cytochrome *c*, and subsequent release of cytochrome *c* into the cytosol (Bayir, Fadeel et al. 2006; Orrenius, Gogvadze et al. 2007). This may play a role in disease conditions such as ischemia, where increases in ROS can result in cardiolipin oxidation and subsequent apoptosis (Polyak, Xia et al. 1997). In addition, neuronal glutamate toxicity leads to cytochrome *c* release in a ROS dependent fashion (Orrenius, Gogvadze et al. 2007). ROS signaling may also regulate physiological forms of cell death. Neurons undergoing developmental apoptosis induced by NGF deprivation release cytochrome *c* in a ROS dependent manner (Dugan, Creedon et al. 1997; Kirkland and Franklin 2001). In these neurons, ROS enhances the proapoptotic conformation of Bax, suggesting that ROS acts as a proapoptotic signal at multiple points in the apoptotic pathway. In Chapter Two of this dissertation, I describe a novel mechanism by

which ROS regulates the apoptotic pathway via oxidation, and thus activation of cytochrome *c* itself.

The disruption of cellular metabolism has long been associated with apoptotic cell death. Many cell types require growth factors for their survival, and in the absence of these growth factors, cell metabolism becomes dysregulated, and apoptosis is induced. Recently, enzymes as well as kinases required for glucose metabolism have been directly linked to regulation of the apoptotic pathway. For example, the proapoptotic member of the Bcl-2 family, Bad, has recently been identified in a complex containing glucokinase. Not only can glucokinase influence the phosphorylation and apoptotic activity of Bad, but mice deficient in Bad exhibit defects in glucose homeostasis (Danial, Gramm et al. 2003) Additionally, some growth factor regulated signaling kinases are intimately tied to both glucose metabolism and apoptosis. In neurons, NGF promotes survival through the activation of AKT, whose activity is involved in glucose uptake and metabolism. In response to NGF deprivation, glucose uptake and glycolysis are markedly reduced, and neurons undergo apoptosis (Deckwerth and Johnson 1993; Deckwerth and Johnson 1993). Glucose metabolism through the pentose phosphate pathway may also be an important regulator of cellular apoptosis. Inhibition of this pathway as a result of nutrient depletion has been shown to activate caspase-2 and subsequent death of *Xenopus* oocytes (Nutt, Margolis et al. 2005). Another mechanism by which glucose metabolism through the pentose phosphate pathway can regulate cell death and survival is by modulation of the oxidative state of the cell. Flux through the pentose phosphate pathway supplies the cell with reducing equivalents of glutathione (GSH), one of the cell's most abundant weapons against ROS (Chandra, Samali et al. 2000). In situations of cellular stress such as DNA damage, cells attempt to control

ROS and apoptosis by increasing reduced glutathione (GSH) levels through the pentose phosphate pathway (Bensaad, Tsuruta et al. 2006). In Chapter Three of this dissertation, I show that glucose metabolism through the pentose phosphate pathway inhibits ROS mediated activation of cytochrome *c* in neurons and cancer cells.

D. Apoptosis in the Animal:

1. Apoptosis in mammalian disease

Not only is apoptosis critical for mammalian development and homeostasis, it also plays a role in the pathologies of many diseases including cancer, cardiomyopathy, autoimmune disease, inflammatory diseases and a wide variety of neuronal pathologies (Siegel, Chan et al. 2000; Fadeel and Orrenius 2005; Foo, Mani et al. 2005; Garg, Narula et al. 2005). In this section I will discuss the role of apoptosis in cancer and neurodegeneration in more detail.

a. Apoptosis in cancer

Cancer is a disease involving the expansion of dysregulated somatic cells and their invasion into normal tissues. It is clear that cellular mutations that cause unchecked proliferation and invasion of cells are hallmarks of cancer, however, additional mutations that arise soon after to ensure that these cancer cells survive are critical for the progression of the disease (Evan and Vousden 2001). Tumor cells that resist the response to activate apoptosis can arise through mutations that either inactivate pro-apoptotic genes, or activate prosurvival genes. For example, deregulated expression of survival factors like insulin-like growth factor I and II (IGF-I, II) and activating mutations in the survival kinase, AKT, help confer

resistance of cancer cells to apoptosis (Datta, Brunet et al. 1999; Stambolic, Mak et al. 1999; Yu and Rohan 2000). In addition, mutations that affect the core apoptotic machinery, including mutations that elevate the anti-apoptotic proteins Bcl-2 and Bcl-xL, can also aid in cancer cell survival (Seto, Jaeger et al. 1988; McDonnell, Deane et al. 1989).

For most mitotic mammalian cells, inhibition of the apoptotic pathway downstream of cytochrome *c* release is unable to protect cells longterm, as release of cytochrome *c* from the mitochondria and subsequent loss of mitochondrial membrane potential results in a caspase-independent commitment to death (McCarthy, Whyte et al. 1997; Brunet, Gunby et al. 1998; Ekert, Read et al. 2004). Despite this, many cancer cells have acquired mutations affecting the apoptotic pathway at points downstream of cytochrome *c*. For example, melanomas and leukemias tend to acquire mutations that cause loss of function or diminished expression in the proapoptotic gene, Apaf-1, or increased expression of IAPs (Vucic, Stennicke et al. 2000; Jia, Srinivasula et al. 2001; Soengas, Capodieci et al. 2001; Altieri 2004; Nachmias, Ashhab et al. 2004; Zangemeister-Wittke and Simon 2004; Wright and Duckett 2005). The fact that these mutations downstream of cytochrome *c* release are advantageous to the survival of cancer cells, suggests that in some situations, cells can survive disruption of the mitochondria and cytochrome *c* release. In fact, sympathetic neurons in which apoptosis is arrested after the point of cytochrome *c* release are able to recover upon removal of the apoptotic stimulus (Martinou, Desagher et al. 1999; Deshmukh, Kuida et al. 2000). Recently, it was suggested that activity of the glycolytic enzyme GAPDH may play a role in the ability of cells to recover from mitochondrial membrane permeability, as GAPDH overexpressing cells are protected from caspase independent cell death following cytochrome *c* release (Colell, Ricci et al. 2007).

While all cancers must develop mutations that allow them to inhibit the apoptotic pathway, the mechanisms by which cancers do this are as diverse as the cancers themselves. A better understanding of how the apoptotic machinery is regulated across cancers lines could give us insight into specialized thereapeutics for these diseases. For example, unlike the surrounding postmitotic neural tissue which is resistant to cytochrome *c,* many brain tumors such as glioblastoma and medulablastoma remain sensitive to cytosolic ytochrome *c* (Huang, YY et al., currently in revision for PNAS). This differential sensitivity may allow us to induce apoptosis in cancer cells, while sparing the surrounding tissue. In contrast, other cancers have developed mechanisms similar to those used by postmitotic neurons to evade apoptosis and ensure their longterm survival (Wright and Deshmukh 2006). A novel mechanism by which both neurons and cancer cells inhibit the apoptotic pathway is the subject of Chapter Three of this dissertation.

b. Apoptosis in neuronal pathologies

Unlike the rapid turnover of cells in proliferating tissues, postmitotic cells of the nervous system have limited regenerative potential and often survive the lifetime of the organism. Thus, if excessive apoptosis occurs in these cells, the result can be devastating. Although apoptotic cells have been observed in tissues from patients with numerous neurodegenerative disorders and brain injuries, it is nevertheless difficult to demonstrate a role of apoptosis in these diseases. First, apoptosis is a process which occurs rapidly, making quantitation of apoptotic cells in post-mortem tissues a difficult task. Second, experiments to determine whether blocking apoptosis can prevent neuronal death cannot be easily done in

humans. For such reasons, much of the evidence for apoptosis in neuronal disease comes from animal and cell-culture studies.

Although the immediate cell death occurring after focal ischemic stroke or traumatic injury is most likely a result of necrosis, cells in the surrounding penumbra of injury die more slowly and in an apoptotic manner. In animal models of brain ischemia, cytochrome *c* is released from the mitochondria followed by activation caspases (Cheng, Deshmukh et al. 1998; Fujimura, Morita-Fujimura et al. 1998). In addition, patients with brain injury exhibit evidence of apoptosis such as caspase activation and increased Bax and p53 activation (Clark, Kochanek et al. 1999). Interestingly, mice in which caspases are pharmacologically inhibited prior to stroke or traumatic brain injury show reduced cell death and improved symptoms (Hara, Fink et al. 1997; Yakovlev, Knoblach et al. 1997).

Alzheimer's disease is correlated with death of neurons in the hippocampus, amygdala, and parts of the cortex. Key characteristics of Alzheimer's are accumulation of hyperphosphorylated tau protein and aggregates of amyloid-β peptide. The importance for apoptosis in the pathology of Alzheimer's is supported by the fact that cultured neurons exposed to amyloid-β undergo apoptosis, and exhibit an increased sensitivity to other stresses (Loo, Copani et al. 1993; Mattson 1998). In addition, neurons deficient for the apoptotic proteins Bax, caspase-2, or caspase-12 show decreased sensitivity to amyloid-β (Nakagawa, Zhu et al. 2000; Selznick, Zheng et al. 2000; Troy, Rabacchi et al. 2000).

Huntington's disease is an inherited neurodegenerative disorder resulting in degeneration of the striatum. It is caused by an expansion of the CAG sequence in the gene *Huntington,* producing a protein with increased polyglutamine repeats (Brandt, Bylsma et al. 1996). In huntington patients, there is an increase in caspase activation and apoptosis of

lymphblasts (Sawa, Wiegand et al. 1999). In addition, mouse models of Huntington show symptoms similar to those seen in humans, and have increased apoptosis in degenerating brain regions (Reddy, Williams et al. 1998). In cultured cells, mutant Huntington is able to induce caspase-8 activation, and can itself be cleaved by active caspases, perhaps promoting neurotoxicity (Sanchez, Xu et al. 1999; Wellington and Hayden 2000). Apoptosis has also been implicated in patients with amyotrophic lateral sclerosis (ALS). Signs of apoptotic nuclei have been observed in these patients, and levels of the proapoptotic protein, Bax, are increased in ALS patients. In mice, overexpression of Bcl-2 is able to inhibit the death and degeneration seen in mouse models of ALS (Kostic, Jackson-Lewis et al. 1997; Li, Ona et al. 2000).

While it is clear that apoptosis occurs in many neuropathologies, it is possible that apoptosis is not a primary abnormality in the disease, but acts as a secondary affect of the disorder. If this is the case, we must keep in mind that neuronal dysfunction could precede the apoptotic cell death seen in neurodegenerative disorders, making treatments that inhibit the apoptotic pathway unlikely to cure all symptoms.

2. Apoptosis in mammalian homeostasis and development

Early work by Dr. Kerr and others observed apoptotic cells not only in the context of pathologies, but also in healthy adult tissues, suggesting a role for apoptosis in the normal regulation of cell populations. In humans, as many as 100,000 cells are produced every second by mitosis, creating a need for an equal number to be removed by apoptosis to maintain organismal homeostasis (Vaux and Korsmeyer 1999). The ability to undergo apoptosis quickly and efficiently may be more critical for certain cell types than others. For

example, epithelial cells, which are constantly exposed to damaging agents such as ultraviolet light and reactive oxygen species (Truong-Tran, Grosser et al. 2003; Van Laethem, Claerhout et al. 2005) have a high turnover rate, and rapidly undergo apoptosis in order to eliminate the possibility of passing on debilitating mutations to future generations of cells (Green and Evan 2002). In contrast, postmitotic neurons and cardiomycocytes have limited regenerative potential and therefore must strictly regulate the apoptotic pathway in order to last the lifetime of the organism (Wright and Deshmukh 2006).

In addition to its role in maintaining homeostasis in the adult, apoptosis is a crucial part of development. It has been implicated in processes from embryogenesis onwards, for the sculpting of tissues and organs, the removal of unwanted structures, and the elimination of damaged cells (Jacobson, Weil et al. 1997). For example, the separation of digits requires programmed cell death, as inhibiting this process results in loss of digit formation (Milligan, Prevette et al. 1995; Jacobson, Weil et al. 1996). Apoptosis is also required to remove unwanted cells such as those in the thymus which fail to rearrange their antigen receptors or recognize self-antigens (Marsden and Strasser 2003). Importantly, extensive apoptosis occurs during development to ensure the proper set up of a functioning nervous system.

a. Apoptosis in neuronal development

Mature mammalian neurons of the CNS and PNS are some of the longest lived cells in the body. However, during neuronal development, as many as 50% of neurons must undergo apoptosis in order to lay down a functional nervous system (Oppenheim 1991; Pettmann and Henderson 1998). The most striking evidence for the importance of caspase-dependent apoptosis in mammalian neuronal development comes from genetic deletion studies of

caspase-3, caspase-9, and Apaf-1. Although strain specific, these mice show massive defects in CNS development due to expansion of neuronal precursors and inhibition of immature neuronal death (Kuida, Zheng et al. 1996; Cecconi, Alvarez et al. 1998; Kuida, Haydar et al. 1998). In addition, mice deficient in the antiapoptotic protein, Bcl-xL show extensive apoptosis in immature postmitotic neurons of the central and peripheral nervous system (Motoyama, Wang et al. 1995). These data indicate that while both mitotic neuronal precursors and postmitotic immature neurons undergo apoptosis in development, the exact mechanisms by which they do so may differ (Kuan, Roth et al. 2000; Roth, Kuan et al. 2000).

Sympathetic neurons of the superior cervical ganglia have proved an excellent model to study apoptosis in response to both developmental and pathological apoptotic insults. Apoptosis of sympathetic neurons occurs during the developmental period when the neuron is innervating its target. Competition for the trophic factor NGF, secreted by target cells, results in death of unsuccessful neurons, thus matching the number of target cells to innervating neurons (Deshmukh and Johnson 1997). Much of what we know about the mechanisms controlling neuronal apoptosis have been discovered by studying sympathetic neurons deprived of NGF *in vitro* (Levi-Montalcini and Booker 1960; Gorin and Johnson 1979; Crowley, Spencer et al. 1994; Smeyne, Klein et al. 1994; Putcha, Deshmukh et al. 2000).

The removal of NGF from cultured sympathetic neurons results in cell atrophy, condensation of the nuclei, fragmentation of neurites, and loss of structural integrity (Deshmukh and Johnson 1997). Early signaling events that lead up to to these morphological changes have been well characterized. NGF promotes survival by phosphorylating the TrkA receptor tyrosine kinase to activate the survival PI-3-kinase pathway, and in its absence, the

JNK/c-Jun pathway results in the increased expression and activation of the BH3 only protein, Bim (Putcha, Moulder et al. 2001; Whitfield, Neame et al. 2001; Putcha, Le et al. 2003). It is clear that protein translation, in part by inducing expression of Bim, is critical for apoptosis to proceed in these neurons, as cycloheximide (CHX) can potently block apoptosis after NGF deprivation (Martin, Ito et al. 1992). At the mitochondria, Bcl-2 family proteins, including Bax initiate the release of cytochrome *c* from the mitochondria into the cytosol where the apoptosome forms. Unlike apoptosis of mitotic cells, neuronal apoptosis requires two events. The first, which triggers cytochrome *c* release, and a second, Bax-independent "competence pathway" to relieve XIAP's inhibition on caspases. NGF deprivation induces competence by targeting XIAP for degradation to allow cytochrome-*c* mediated cell death (Deshmukh, Du et al. 2002; Potts, Singh et al. 2003). The events involved in neuronal death by NGF deprivation are summarized in Fig 1.3.

b. Increased regulation of apoptosis in neurons

Despite the substantial apoptosis that occurs in neurons during development, these cells have developed multiple restrictions in the cell death pathway in order to meet the demand for their longterm survival. These "checks" on the apoptotic pathway occur both upstream and downstream of cytochrome *c* release from the mitochondria, and have recently been reviewed (Wright and Deshmukh 2006).

i. Elimination of Bax/Bak redundancy

The proapoptotic members of the Bcl-2 family, Bax and Bak, can both induce the release of cytochrome *c* by acting directly on the mitochondria. In most mitotic cells, these

proteins play redundant roles, with either one being sufficient to promote apoptosis in the event of an apoptotic signal (Wei, Zong et al. 2001). However, in a variety of neuronal cell types, the deletion of Bax alone is sufficient to block cytochrome *c* release and inhibit apoptosis (Deckwerth, Elliott et al. 1996; Miller, Moulder et al. 1997; Johnson, Xiang et al. 1998; White, Keller et al. 1998; Xiang, Kinoshita et al. 1998; Bar-Peled, Knudson et al. 1999; Cregan, MacLaurin et al. 1999; Gibson, Han et al. 2001; Besirli, Deckwerth et al. 2003; Sun, Gould et al. 2003; Wyttenbach and Tolkovsky 2006), while neurons derived from Bak deficient mice acquire no additional protection from apoptosis. It was later discovered that neurons express a neuronal-specific splice variant of Bak (N-Bak) resulting in a premature truncation and elimination of the BH3 domains necessary for any direct proapoptotic activity (Sun, Yu et al. 2001; Uo, Kinoshita et al. 2005). Thus, neurons have eliminated the redundancy of the proapoptotic activity of Bax/Bak, most likely to restrict apoptosis.

ii. Restriction of cell death by maintenance of mitochondrial membrane potential

In addition to the regulation of apoptosis upstream of cytochrome *c* release, neurons have adapted to strictly inhibit the apoptotic pathway even after the point of cytochrome *c* release. In many cell types, the release of cytochrome *c* is accompanied by a rapid loss of mitochondrial membrane potential which represents a point of commitment to death even in the absence of caspase activation (McCarthy, Rubin et al. 1997; Brunet, Gunby et al. 1998; Ekert, Read et al. 2004). In sympathetic neurons, however, the mitochondrial membrane potential is retained for some time after cytochrome *c* release, and in the presence of caspase inhibitors, neurons can recover from NGF deprivation induced cytochrome *c* release if NGF

is restored prior to loss of membrane potential (Martinou, Desagher et al. 1999; Deshmukh, Kuida et al. 2000).

iii. Restriction of apoptosis by XIAP and Apaf-1 levels

Although XIAP can readily inhibit caspase activation *in vitro*, the function of endogenous XIAP seems to be restricted to postmitotic cells such as neurons and cardiomyocytes (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). Introduction of cytochrome *c* into the cytosol of intact mitotic cells or addition of cytochrome *c* to extracts of these cells results in caspase activation and apoptosis (Liu, Kim et al. 1996; Li, Srinivasan et al. 1997; Brustugun, Fladmark et al. 1998; Juin, Hueber et al. 1999; Wright, Linhoff et al. 2004). However, injection of cytochrome *c* into the cytosol of intact neurons or addition of cytochrome *c* to cerebellar extracts is only able to induce caspase activation and apoptosis if XIAP is first removed either by genetic deletion or by the addition of excess SMAC (Potts, Singh et al. 2003), (Chapter Four).

Recently, our lab determined that the differential ability of XIAP to inhibit apoptosis in postmitotic cells as compared to mitotic cells is actually mediated by levels of Apaf-1, and thus, apoptosome function. In mitotic cells, Apaf-1 expression and apoptosome activity are very high, and XIAP is unable to inhibit the massive resulting caspase activation. However, in postmitotic neurons of the CNS, PNS, and in cardiomyocytes, levels of Apaf-1 are dramatically reduced, making endogenous XIAP an efficient inhibitor of caspase activation and cell death (Fig 1.2) (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005; Vaughn and Deshmukh 2007),(Chapter Four). The regulation of apoptosis at the point of apoptosome activity and caspase activity is not inherent, but develops as neuronal precursors differentiate

into postmitotic neurons. While naïve PC12 cells, sympathetic neurons derived from E16 mice, and mitotic cerebellar granule neuron precursors have high levels of Apaf-1 and are sensitive to cytochrome *c,* differentiated PC12 cells, sympathetic neurons derived from P1 mice, and mature cerebellar neurons have low levels of Apaf-1 and develop an XIAP-mediated resistance to cytochrome *c* (Wright, Linhoff et al. 2004), (Chapter Four).

Once neurodevelopment is complete, neurons adopt an even more stringent control over the apoptotic pathway. For example, sympathetic neurons gradually lose their dependence on NGF for survival (Angeletti, Levi-Montalcini et al. 1971; Goedert, Otten et al. 1978; Gorin and Johnson 1980; Ruit, Osborne et al. 1990; Easton, Deckwerth et al. 1997). Consistent with this, our lab has shown that in mature sympathetic neurons, Apaf-1 levels are even further decreased, resulting in a resistance to cytochrome *c* that is now XIAP independent (Wright, KM et al., currently in revision for JCB). In neurons of the mature cortex and cerebellum, a similar XIAP-independent resistance to cytochrome *c* occurs, accompanied by a reduction in Apaf-1 as well as caspase-3 levels (Yakovlev, Ota et al. 2001), (Chapter Four). The molecular mechanisms underlying the resistance of cerebellar neurons to cytochrome *c* at different developmental stages will be examined in Chapter Four of this document.

Figure 1.1 Bcl-2 family regualation of cytochrome *c* release. The BH3 only family of proteins typically act through binding and sequestering the anitapoptotic Bcl-2 proteins from Bax and Bak. Bax and Bak act can directly at the mitochondria to induce the release of cytochrome *c*

Figure 1.1

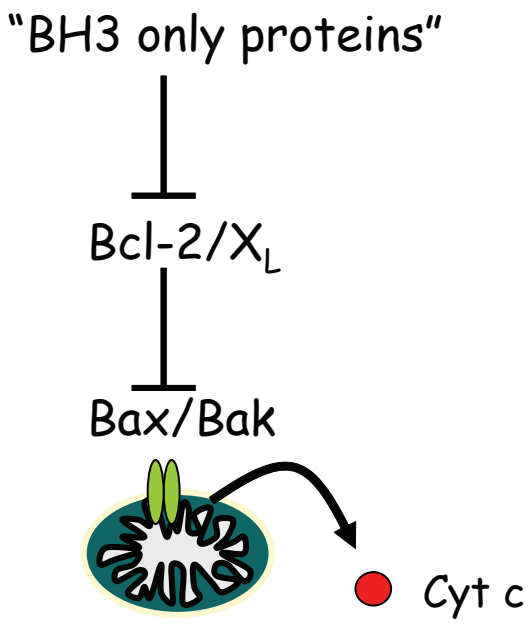
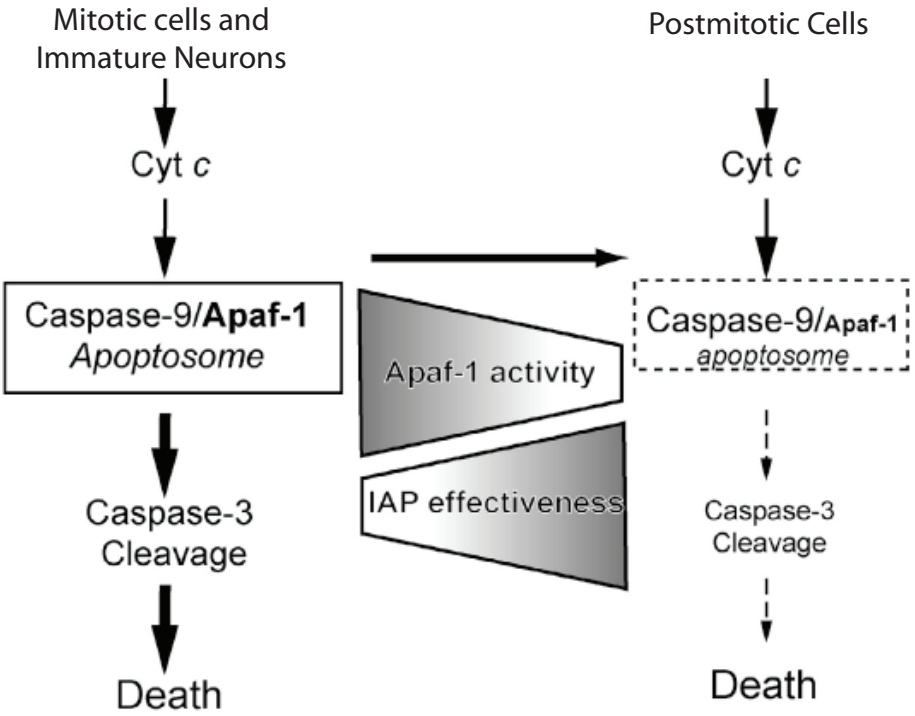


Figure 1.2 Model showing how a marked reduction in Apaf-1 expression/activity in postmitotic cells engages XIAP as an affective inhibitor of caspases.

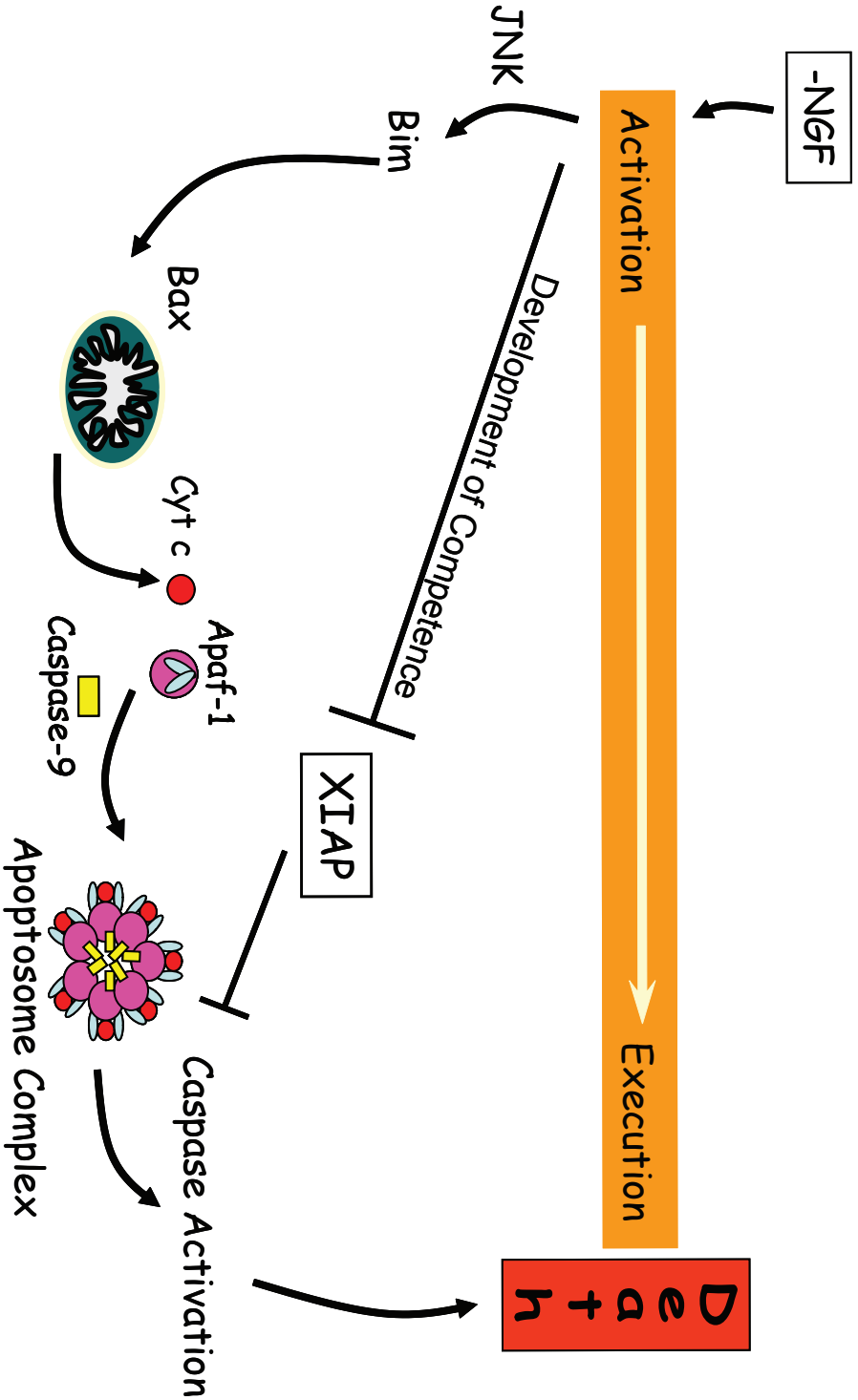
Figure 1.2



modified from Wright, KM et al., JCB 2004

Figure 1.3 Model of NGF deprivation-induced apoptosis in sympathetic neurons. NGF deprivation signals the activateion of JNK, which can activate proapoptotic members of the Bcl-2 family to induce cytochrome c release from the mitochondria into the cytosol. Cytosolic cytochrome c engages Apaf-1 and caspase-9 to form the apoptosome and initate cspase activation. In addition to this signal to release cytochrome c, NGF deprivation also activates the "Development of Competnence" pathway which is necessary to overcome XIAP's inhibition of caspases and allow apoptosis.

Figure 1.3


-NGF
Activation
Execution
Death
JNK
Bim
Development of Competence
Bax
Cyt c
Apaf-1
Caspase-9
XIAP
Apoptosome Complex
Caspase Activation

II. CHAPTER TWO:

Essential Postmitochondrial Function of p53 Uncovered in DNA Damage-Induced Apoptosis in Neurons

A. Abstract

In postmitotic sympathetic neurons, unlike most mitotic cells, death by apoptosis not only requires the release of cytochrome *c* from the mitochondria, but an additional step to relieve XIAP's inhibition of caspases. Here, we examine the mechanism by which XIAP is inactivated following DNA damage and find that it is achieved by a completely different mechanism than following apoptosis by NGF deprivation. While NGF deprivation relieves XIAP by selectively degrading it, DNA damage overcomes XIAP *via* a p53-mediated induction of Apaf-1. Unlike wildtype neurons, p53-deficient neurons fail to overcome XIAP and remain resistant to cytochrome *c* after DNA damage. Restoring Apaf-1 induction in p53-deficient neurons is sufficient to overcome XIAP and sensitize cells to cytochrome *c*. While a role for p53 in apoptosis upstream of cytochrome *c* release has been well established, this study uncovers an additional, essential role for p53 in regulating caspase activation downstream of mitochondria following DNA damage in neurons.

B. Introduction

Apoptosis is a highly regulated genetic process that is crucial for the development of the entire organism, including the nervous system. Yet, apoptosis also plays an important role in neuronal injury and disease. Aberrant apoptosis can result in too little cell loss as seen in cancer progression, as well as too much cell loss such as following stroke, spinal cord injury and in many neurodegenerative diseases (Yuan and Yankner 2000).

A crucial event of apoptosis is the activation of caspase proteases. In mammalian cells, including neurons, caspase activation can be triggered by the release of cytochrome *c* from the mitochondria (Deshmukh and Johnson 1998; Wang 2001). Once released, cytochrome *c* binds to Apaf-1, inducing its oligomerization and subsequent recruitment of the initiator caspase, procaspase-9, to form the apoptosome complex. This complex results in activation of caspase-9, which then can cleave and activate downstream caspases such as caspase-3 which are responsible for cell death (Wang 2001).

These major components of the apoptotic pathway have been well characterized through the use of cell free biochemical studies and in intact cells using mitotic cell lines. However, recent evidence suggests that apoptosis is regulated very differently between mitotic and postmitotic cells. For example, in many mitotic cells such as HeLa cells, HEK 293 cells, primary fibroblasts, and naïve PC12 cells, the addition of cytochrome *c* to cytosolic extracts, or injection of cytochrome *c* into the cytosol of these cells induces rapid caspase activation and apoptosis (Liu, Kim et al. 1996; Li, Srinivasan et al. 1997; Brustugun, Fladmark et al. 1998; Juin, Hueber et al. 1999; Wright, Linhoff et al. 2004). In contrast, cytochrome *c,* while necessary, is not sufficient to induce apoptosis in postmitotic cells such as sympathetic neurons, differentiated PC12 cells and cardiomyocytes (Deshmukh and

Johnson 1998; Vyas, Juin et al. 2004; Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005). Recently, the X-linked Inhibitor of Apoptosis Protein (XIAP) was identified as the critical regulator of caspase activation in these postmitotic cells (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). XIAP is a member of a family of Inhibitor of Apoptosis Proteins (IAPs) that have been shown to regulate caspases by directly binding to and inhibiting their function (Salvesen and Duckett 2002). Whereas cytosolic microinjection of cytochrome *c* is insufficient to induce apoptosis in wildtype neurons and cardiomyocytes, it is capable of doing so in XIAP-deficient neurons and cardiomyocytes (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005).

While sympathetic neurons are resistant to injection of cytochrome *c* into their cytosol, they are capable of undergoing cytochrome *c*-dependent apoptosis when induced by insults such as NGF deprivation and DNA damage (Deshmukh and Johnson 1997; Besirli, Deckwerth et al. 2003; Wyttenbach and Tolkovsky 2006). Therefore, in order to undergo apoptosis, neurons must activate signaling events that cause cytochrome *c* release as well as inactivate XIAP. The mechanism by which neurons overcome XIAP's inhibition of caspases to become sensitive to cytochrome *c* has been termed 'development of competence' and has been examined in the model of NGF deprivation (Deshmukh and Johnson 1998). In NGF deprivation-induced death, the 'development of competence' is dependent neither on Bax function nor protein synthesis, but occurs by the selective degradation of XIAP (Potts, Singh et al. 2003). Whether other apoptotic stimuli, most importantly those involved in pathological situations that engage the cytochrome *c*-mediated apoptosome pathway, use the same or different mechanisms to overcome XIAP remains unknown.

Previous studies have shown that DNA damage can activate the intrinsic apoptotic pathway leading to cytochrome *c* release and caspase-dependent cell death in many cell types including sympathetic neurons (Besirli, Deckwerth et al. 2003; Morrison, Kinoshita et al. 2003). While DNA damaging agents are commonly used as chemotherapeutics in the treatment of many cancers, they can also cause widespread neurotoxicity in patients (Lazarus, Herzig et al. 1981). DNA damage has also been observed in neurodegenerative diseases (Morrison, Kinoshita et al. 2003; Culmsee and Mattson 2005). Therefore, understanding how apoptosis is regulated in neurons in response to DNA damage has clinical significance.

The tumor suppressor p53 has been implicated in the regulation of apoptosis in response to DNA damage in mitotic cells as well as neurons (Miller, Pozniak et al. 2000; Schuler and Green 2001; Morrison, Kinoshita et al. 2003). p53 has been shown to regulate cytochrome *c* release from the mitochondria both directly (Marchenko, Zaika et al. 2000; Chipuk, Kuwana et al. 2004), and through the transcriptional induction of proapoptotic Bcl-2 proteins such as Bax, Puma, and Noxa (Schuler and Green 2001). In this report, we find that p53 has an additional function to regulate apoptosis even after the point of mitochondrial cytochrome *c* release in neurons.

We find that the mechanisms by which sympathetic neurons overcome XIAP's inhibition of caspases are distinct between NGF deprivation and DNA damage. Specifically, whereas NGF deprivation induces competence to relieve XIAP by targeting it for degradation, XIAP's inhibition of caspases after DNA damage is overcome by a p53-dependent increase in Apaf-1. Thus, in the absence of p53, DNA damaged neurons are unable to execute apoptosis in response to cytochrome *c* because they are incapable of

relieving XIAP. These results identify an essential postmitochondrial role of p53 in regulating apoptosis in mammalian neurons.

C. Results

1. Etoposide induces cytochrome *c* release and apoptosome-dependent death

Sympathetic neurons undergoing apoptosis mediated by the apoptosome pathway require not only the release cytochrome *c* from mitochondria, but must also overcome XIAP's strict inhibition of caspases. While the mechanism by which XIAP is relieved has been previously studied in the model of NGF deprivation-induced neuronal death (Potts, Singh et al. 2003), here we examined how XIAP is overcome in sympathetic neurons undergoing apoptosis with DNA damage.

First we established that sympathetic neurons treated with DNA damaging agents undergo a cytochrome *c* and apoptosome-mediated cell death. Sympathetic neurons were treated with the topoisomerase II inhibitor, etoposide, and cell death was assessed at various time points. In response to etoposide, greater than 70% of sympathetic neurons undergo cell death by 48 hours. This death was apoptotic as it could be blocked by the pan-caspase inhibitor, zVAD-fmk (Fig. 2.1A), and dying neurons exhibited Annexin V positive staining (Supplemental Fig. 2.1). Furthermore, this etoposide induced death resulted in the release of cytochrome *c* (Fig. 2.1B) and engaged the apoptosome pathway. Unlike wildtype neurons, sympathetic neurons isolated from Apaf-1 deficient mice were resistant to etoposide (Fig. 2.1C). Likewise, caspase-3 deficient sympathetic neurons have also recently shown to be resistant to etoposide-induced apoptosis (Wright, Vaughn et al. 2006).

In sympathetic neurons undergoing apoptosis *via* the cytochrome *c*-mediated apoptosome pathway, the removal of XIAP's inhibition on caspases is necessary to permit death. Whereas wildtype neurons are resistant, XIAP deficient neurons rapidly undergo apoptosis following injection of cytochrome *c* (Fig. 2.1D) (Potts, Singh et al. 2003). The observation that etoposide engages death *via* the apoptosome pathway indicates that etoposide signaling must relieve XIAP's inhibition of caspases in order for cells to die. Indeed, etoposide treatment for 24 hours (a time point at which cytochrome *c* release and cell death is minimal) is able to relieve inhibition of caspases as it sensitizes wildtype neurons to cytochrome *c* (Fig. 2.1D). These results indicate that etoposide activates a mechanism that makes neurons permissive for cytochrome *c*-induced apoptosis before the neurons reach the point of cytochrome *c* release.

2. Unlike NGF deprivation, etoposide-induced death requires translation to overcome XIAP

In the model of NGF deprivation-induced neuronal death, XIAP is inactivated by its selective degradation (Fig. 2.2A). We were surprised to find, however, that neurons treated with etoposide underwent apoptosis yet maintained their XIAP levels (Fig. 2.2A, B). These results indicate that the mechanisms by which NGF deprivation and DNA damage overcome XIAP are distinct.

The development of competence can be directly determined by assessing the sensitivity of neurons to microinjection of cytochrome *c*. Only after neurons develop competence (overcome XIAP activity) do they become sensitive to cytosolic cytochrome *c*. While cytochrome *c* release is dependent on Bax and protein synthesis, in previous studies we have

shown that neither Bax nor protein synthesis is required for the development of competence *via* degradation of XIAP after NGF deprivation. Therefore, NGF-deprived neurons, both Bax deficient and cycloheximide (CHX) treated, are unable to release cytochrome *c*, yet develop competence and die with microinjected cytochrome *c* (Fig. 2.2C), (Deshmukh and Johnson 1998).

As the 'development of competence' pathway in etoposide-treated neurons appeared distinct and did not involve the degradation of XIAP, we asked whether this pathway was dependent on Bax or protein synthesis. Bax-deficient or wildtype cycloheximide-treated sympathetic neurons were exposed to etoposide for 24 hours, followed by microinjection of cytochrome *c* and assessment of cell survival. Like the NGF-deprived condition, etoposide treatment induced competence in the Bax-deficient neurons (Fig. 2.2D). However, unlike NGF-deprived neurons, etoposide-treated neurons failed to develop competence in the presence of cycloheximide and remained resistant to cytochrome *c* (Fig. 2.2D). These results indicate that the mechanism by which XIAP is overcome to develop competence in etoposide-treated neurons requires protein synthesis.

3. Apaf-1 is markedly induced in etoposide-treated sympathetic neurons

We have shown previously that Apaf-1 levels and the ability of XIAP to inhibit apoptosis are inversely coupled in cells (Wright, Linhoff et al. 2004). Under conditions where Apaf-1 levels are low, such as in postmitotic sympathetic neurons and cardiomyocytes, endogenous XIAP is an effective inhibitor of caspases (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). In contrast, in mitotic cells where Apaf-1 levels are high,

equivalent levels of XIAP are unable to strictly block caspase activation in response to cytosolic cytochrome *c* (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005).

Based on this observation, we speculated that an increase in Apaf-1 levels could be a mechanism by which sympathetic neurons disengage XIAP's brake on caspase activation in response to DNA damage. We examined whether Apaf-1 levels are increased in sympathetic neurons treated with etoposide. Indeed, Western analysis shows that Apaf-1 protein levels were elevated by 24 hours following treatment with etoposide (Fig. 2.3A). In contrast, no significant increase in Apaf-1 was seen after NGF deprivation. This increase in Apaf-1 with DNA damage was also seen at the transcriptional level (Fig. 2.3B). Similar increases in Apaf-1 have also been reported in cortical neurons following treatment with camptothecin or after traumatic brain injury (Fortin, Cregan et al. 2001; Yakovlev, Ota et al. 2001). These data suggest that Apaf-1 induction could be necessary for disengaging XIAP's brake on caspase activation to permit apoptosis in neurons.

4. p53 deficient neurons are unable to develop competence in response to DNA damage

In the context of cell death, two functions of p53 have received much attention. First, p53 transcriptionally regulates proapoptotic Bcl-2 family members such as Bax, Puma and Noxa in response to DNA damage, which are important upstream activators of cytochrome *c* release (Miyashita and Reed 1995; Oda, Ohki et al. 2000; Yu, Wang et al. 2003). Second, a transcriptional-independent role has been described for p53 in which it acts directly at the mitochondria to induce cytochrome *c* release (Marchenko, Zaika et al. 2000; Mihara, Erster et al. 2003; Chipuk, Kuwana et al. 2004; Chipuk, Bouchier-Hayes et al. 2005).

Recently, p53 has also been identified as a transcriptional activator of Apaf-1, but the importance of this activity has remained unclear (Fortin, Cregan et al. 2001; Moroni, Hickman et al. 2001).

Since the increase in Apaf-1 could be a potential mechanism by which XIAP is relieved to permit neuronal apoptosis, we investigated the specific importance of p53 in this process. First, we examined whether p53 deficiency blocks apoptosis in sympathetic neurons in response to etoposide. While less than 10% of wildtype sympathetic neurons remained viable 60 hours after etoposide treatment, greater than 80% of p53 deficient neurons survived at this time (Fig. 2.4A). Since p53 has been shown to induce Puma, an event which is required for cytochrome *c* release in response to DNA damage in sympathetic neurons (Wyttenbach and Tolkovsky 2006), we anticipated that p53 deficient neurons might be resistant to apoptosis at multiple points in the apoptotic pathway. Consistent with this known pre-mitochondrial function of p53 in neurons, while the majority of wildtype neurons had released cytochrome *c* by 36 hours of etoposide treatment, p53 deficient neurons retained cytochrome *c* in the mitochondria (Supplemental Fig. 2.2).

To specifically examine whether p53 is also important downstream of cytochrome *c,* we investigated whether p53 deficient neurons fail to become sensitive to cytochrome *c* following DNA damage. Wildtype and p53 deficient neurons were treated with etoposide for 24 hours (a timepoint at which the majority of neurons have not released cytochrome *c*; Supplemental Fig. 2.2), followed by microinjection with cytochrome *c.* Activating the apoptotic pathway directly at the point of cytosolic cytochrome *c* is necessary as it allows one to bypass the known defect of cytochrome *c* release found in p53 deficient neurons (Supplemental Fig. 2.2). Unlike etoposide-treated wildtype neurons which underwent

apoptosis after cytochrome *c* injections, etoposide-treated, p53 deficient neurons remained resistant to cytochrome *c* injection unless XIAP was removed by co-injection of SMAC protein (Fig. 2.4B). Injections with rhodamine dextran alone did not induce death in wildtype or p53 deficient etoposide-treated neurons.

To determine whether the inability of p53 deficient neurons to develop competence was specific to DNA damage, we asked whether p53 deficient neurons were able to develop competence in response to NGF deprivation. Wildtype or p53 deficient sympathetic neurons were deprived of NGF in the presence of cycloheximide (CHX) (to prevent cytochrome *c* release) for 24 hours. Consistent with our expectations, both wildtype and p53-deficient neurons developed competence and were sensitive to microinjection of cytochrome *c* (Fig. 2.4C). Thus, p53 is selectively important for the development of competence in response to DNA damage, which involves upregulation of Apaf-1, but not NGF deprivation, which involves the degradation of XIAP.

To confirm that the inability of p53-deficient neurons to undergo apoptosis in response to cytochrome *c* was due to XIAP's inhibition of caspases, we asked whether p53-deficient neurons became sensitive to cytochrome *c* following direct inactivation of XIAP. Indeed, injection of cytochrome *c* along with SMAC (an inhibitor of IAPs), but not cytochrome *c* alone nor SMAC alone, induced apoptosis in p53-deficient neurons, indicating that all the necessary apoptosomal components are present and capable of activation in the p53-deficient neurons if XIAP is removed (Supplemental Fig. 2.3).

Thus, in sympathetic neurons, p53 is required to overcome XIAP (develop competence) to permit cytochrome *c*-mediated apoptosis following DNA damage.

5. p53 links Apaf-1 induction with XIAP inactivation during DNA damage-induced apoptosis in neurons

To investigate whether p53 is necessary for the induction of Apaf-1 protein in response to DNA damage, neurons from p53 heterozygous or p53-deficient mice were either left untreated or treated with etoposide. Western analysis revealed that while levels of Apaf-1 protein increased following etoposide treatment in wildtype and p53-heterozygous neurons (Fig. 2.3A; 2.5A), Apaf-1 was not induced in p53-deficient neurons under these conditions (Fig. 2.5A). Thus, etoposide-mediated induction in Apaf-1 in sympathetic neurons was p53-dependent. We also note that p53 deficiency did not affect basal levels of Apaf-1, as Apaf-1 levels were comparable between p53-heterozygous and p53-deficient neurons.

If the resistance of p53-deficient neurons to develop competence is due to the specific inability of these cells to upregulate Apaf-1 in response to etoposide, then restoration of Apaf-1 induction in p53-deficient neurons would be expected to restore the ability of these neurons to develop competence following DNA damage. To test this, p53-deficient sympathetic neurons were injected with plasmid DNA encoding either Apaf-1 or vector alone, along with EGFP to mark injected cells. After 24 hrs, neurons were treated with etoposide for 24 hours, followed by microinjection of cytochrome *c*. Injection of cytochrome *c* into Apaf-1-expressing p53-deficient neurons resulted in complete apoptosis by 6 hours. However, injection of rhodamine alone into Apaf-1 expressing cells, or cytochrome *c* into cells expressing empty vector did not induce death (Fig. 2.5B). These data show that by restoring the induction of Apaf-1 by overexpression, DNA damaged, p53-deficient sympathetic neurons regained the ability to develop competence and overcome XIAP's inhibition of caspases.

Together, these data show that a p53-mediated induction of Apaf-1 is necessary to overcome XIAP and permitting cytochrome *c* to induce apoptosis in neurons in response to DNA damage.

D. Discussion

In this study, we have examined how the strict inhibition of caspases by endogenous XIAP is relieved when neurons undergo apoptosis following DNA damage. We find that the mechanisms by which XIAP is overcome in sympathetic neurons undergoing developmental apoptosis by NGF withdrawal and pathological apoptosis by DNA damage are distinct. Whereas NGF deprivation relieves XIAP by its selective degradation (Potts, Singh et al. 2003), XIAP levels remained unchanged after DNA damage (Fig. 2.2A,B). In contrast to NGF deprivation, the development of competence after DNA damage required protein synthesis. Consistent with this requirement, we found that Apaf-1 was markedly induced in neurons undergoing apoptosis with DNA damage (Fig. 2.3A). Our results identify an important function of p53 in the development of competence, as p53-deficient neurons failed to induce Apaf-1 after DNA damage (Fig. 2.5A). We examined the specific importance of Apaf-1 upregulation in relieving XIAP during DNA damage-induced apoptosis, and found that in the absence of an Apaf-1 induction, p53-deficient neurons failed to develop competence in response to DNA damage (Fig. 2.4B). Importantly, restoring Apaf-1 induction by overexpression of Apaf-1 in p53-deficient neurons restored their ability to relieve XIAP, permitting cytochrome *c* to induce apoptosis (Fig. 2.5B).

These results validate our previous model which proposed that the effectiveness of endogenous XIAP to inhibit apoptosis is coupled to Apaf-1 activity in cells. Mitotic cells,

which express high levels of Apaf-1, readily undergo apoptosis in response to cytochrome *c* , without the need to eliminate endogenous XIAP. In contrast, sympathetic neurons have low levels of Apaf-1, allowing endogenous XIAP to effectively inhibit cytochrome *c*-dependent caspase activation (Wright, Linhoff et al. 2004). As Apaf-1 is the limiting component of the apoptosome in these neurons, this model predicts that elevating levels of Apaf-1 alone would be sufficient to increase the number of functional apoptosomes and overcome XIAP. In this study we describe a pathological situation in which the coupling of Apaf-1 and XIAP becomes a critical regulator of caspase activation. In response to DNA damage, sympathetic neurons overcome XIAP and develop sensitivity to cytochrome *c* by specifically upregulating Apaf-1. Induction of Apaf-1 was the only step required by etoposide to sensitize p53-deficient neurons to cytochrome *c*. p53-deficient cells in which Apaf-1 induction had been restored by overexpression underwent apoptosis in response to cytochrome *c* even in the absence of etoposide treatment (Supplemental Fig. 2.4).

Strict control of caspase activation by XIAP in neurons presumably functions as a safety brake that would prevent apoptosis from occurring in the event of accidental mitochondrial damage resulting in cytochrome *c* release. However, in situations where death is inevitable, such as during development or after irreparable DNA damage, the efficient removal of this brake is important as it would permit apoptosis thereby avoiding the negative side effects of necrosis. Indeed, both NGF deprivation and DNA damage activate specific pathways that efficiently relieve XIAP at a time that precedes the release of cytochrome *c* from mitochondria (Fig. 2.1D, 2.4B), (Potts, Singh et al. 2003). Thus, apoptosis in XIAP-deficient neurons, while no longer needing the pathway to relieve XIAP, is still dependent on

the release of cytochrome *c* and therefore occurs with a time course that is indistinguishable from wildtype neurons (Supplemental Fig. 2.5), (Potts, Singh et al. 2003).

Although the major components of the intrinsic pathway of apoptosis are conserved in both developmental (NGF deprivation) and pathological (DNA damage) apoptosis in sympathetic neurons, this study has identified unique differences in the way this pathway is regulated between these two models. While removal of NGF in these neurons selectively targets XIAP for degradation, DNA damage overcomes XIAP by a p53-mediated induction of Apaf-1 (model in Fig. 2.6A). We note that biochemical and overexpression studies have shown that mature SMAC can also directly bind XIAP and inhibit its activity (Verhagen and Vaux 2002). However, endogenous SMAC does not function to relieve XIAP following DNA damage in neurons, as both wildtype and SMAC-deficient sympathetic neurons undergo apoptosis with a similar timecourse following etoposide treatment (Fig. 2.6B). Thus, neurons appear to have evolved multiple SMAC-independent pathways that are sufficient to relieve XIAP and permit cytochrome *c*-dependent apoptosis. Similar pathways may also be engaged in other postmitotic cells such as cardiomyocytes where relieving XIAP is necessary for apoptosis.

Apaf-1 has been shown to be induced in a p53-dependent manner in response to camptothecin in cortical neurons (Fortin, Cregan et al. 2001). However, whether the p53-mediated induction of Apaf-1 was necessary for neuronal apoptosis was not specifically examined. Indeed, p53 deficiency blocks apoptosis in response to DNA damage in many cells including neurons (Morrison, Kinoshita et al. 2003; Michalak, Villunger et al. 2005). However, studies investigating the mechanism of p53 action have primarily focused on events preceding cytochrome *c* release. For example, proapoptotic members of the Bcl-2

family proteins such as Bax, Noxa, and Puma are transcriptional targets of p53, and their upregulation can promote cytochrome *c* release in cells (Schuler and Green 2001; Wyttenbach and Tolkovsky 2006). In addition, recent studies show that p53 can directly induce cytochrome *c* release by localizing to mitochondria (Marchenko, Zaika et al. 2000; Mihara, Erster et al. 2003; Chipuk, Kuwana et al. 2004; Chipuk, Bouchier-Hayes et al. 2005). Our results now uncover an essential postmitochondrial function of p53 in apoptosis. While p53-mediated induction of Apaf-1 is likely to occur in multiple cell types exposed to DNA damage, we expect that it is only required in cells where the inactivation of XIAP is necessary for cytochrome *c*-mediated apoptosis, such as in neurons and cardiomyocytes. In addition, as overexpression of XIAP is seen in many cancers (Liston, Fong et al. 2003), our results predict that p53 activity could also overcome XIAP's inhibition of caspases by elevating Apaf-1 in such cancers.

Several studies show that p53 is induced following a variety of neuronal injury paradigms such ischemia, hypoxia, and excitotoxicity (Morrison, Kinoshita et al. 2003; Culmsee and Mattson 2005) as well as in neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (Martin 2000). Importantly, mice deficient in p53 are protected against glutamate or kainic acid-excitotoxic injury (Morrison, Wenzel et al. 1996) and ischemic brain damage (Crumrine, Thomas et al. 1994). Our results predict that in these models, p53 deficiency would inhibit neuronal apoptosis not only by blocking cytochrome *c* release, but also by preventing the ability of these neurons to overcome XIAP, thus regulating apoptosis at both pre- and post-mitochondrial steps.

Having multiple checkpoints in the regulation of apoptosis, and being able to precisely regulate these checkpoints, is critical for balancing a neuron's long-term survival with the

need to undergo apoptosis when physiologically appropriate. An understanding of how these checkpoints are differentially regulated between mitotic and postmitotic cells is clinically significant as it allows for the development of therapeutics that could inhibit or activate apoptosis in selective cells in the context of neurodegenerative diseases or cancers.

E. Materials and Methods

Reagents

All reagents were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical Corporation (Freehold, NJ), and cycloheximide was purchased from Tocris Cookson (Ellisville, MO). The pan-caspase inhibitor, zVAD-fmk, was purchased from Enzyme Systems Products. The Apaf-1 cDNA was a gift from Dr. Gabriel Nunez (University of Michigan, Ann Arbor). Recombinant SMAC protein was purified from bacteria as described previously (Potts, Singh et al. 2003).

Sympathetic Neuronal Cultures

Primary sympathetic neurons were dissected from the superior cervical ganglia of postnatal day zero to one mice (P0-P1), and maintained in culture as described previously (Deshmukh, Vasilakos et al. 1996). Cells were plated on collagen-coated dishes at a density of 60,000 cells per well for Western or RT-PCR analysis, or 10,000 cells per well for microinjection, survival counts, or immunofluorescence experiments. Sympathetic neurons were grown for 4-5 days in NGF contained media before treating them with experimental conditions. For treatments, etoposide was used at a concentration of 20 μM. For NGF

deprivation, cultures were rinsed three times with medium lacking NGF, followed by the addition of goat anti-NGF neutralizing antibody to this media. Other conditions required the addition of 1 ug/ml cycloheximide or 50 μM zVAD-fmk.

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, p53-deficient, and SMAC-deficient mice is C57BL/6; wildtype littermates were used as controls in these experiments. Apaf-1-deficient mice were generated by Dr.Joachim Herz (UT Southwestern) (Honarpour, Du et al. 2000) and were kindly provided by the laboratory of Dr. Susan Ackerman (Jackson Laboratories). Heterozygous p53 mice were obtained from Jackson Laboratories (Maine). SMAC-deficient mice were provided by the laboratory of Dr. Tak Mak (University of Toronto). Our methods for breeding and genotyping Bax-deficient mice are described previously (Deckwerth, Elliott et al. 1996). The specific primers used to genotype Apaf-1, p53, or SMAC deficient mice are listed in the online supplemental materials and methods section.

Immunofluorescence analysis

The status of cytochrome *c* (whether intact in the mitochondria or released) was examined by immunofluorescence. Briefly, sympathetic neurons were cultured for 4 days following which they were left untreated or treated with 20 uM of etoposide for various time points. Neurons were then fixed in 4% paraformaldehyde and incubated overnight in anti-cytochrome *c* primary antibody (556432, BD Biosciences) followed by a 2 hour incubation

with anti-mouse Cy3 secondary antibody (Jackson Labs). Nuclei were stained with Hoechst 33258 (Molecular Probes).

Microinjection and quantitation of cell survival

Our method or microinjecting sympathetic neurons with cytochrome *c* has been described previously (Wright, Linhoff et al. 2004). Immediately after injections, the number of viable cells injected was determined by counting rhodamine-positive cells that had intact phase-bright bodies. This method of assessing neuronal survival correlates well with other cell survival assays such as trypan blue exclusion and staining with calcein AM (Potts, Singh et al. 2003) At various times after injections, the number of viable injected neurons remaining was determined by using the same counting criteria and expressed as a percentage of the original number of microinjected cells.

In experiments involving microinjection of DNAs, the Apaf-1 expressing plasmid or empty vector was injected into neurons. The nucleus was injected with 200 ng/ul of the plasmid DNA along with 50 ng/ul of EGFP-expressing DNA (Clonetech laboratories, Inc.) in microinjection buffer. After 24 hours to allow for expression, GFP-expressing cells were identified by fluorescence microscopy and re-injected with cytochrome *c*. The survival of these double injected cells was assessed by morphological criteria.

Western Blot analysis

Western analysis was performed as described previously (Potts, Singh et al. 2003). The following antibodies were used; anti-Apaf-1 (13F11; Alexis Biochemicals), anti-XIAP (AF822; R&D Systems), anti-LDH (Rockland), anti-alpha-Tubulin (Sigma). Anti–

mouse/rabbit HRP-conjugated secondary antibodies were purchased from Pierce Chemical Co. For quantitation, Western blots were developed with ECL-plus reagents (Amersham Biosciences) and analyzed on a Typhoon fluorescent imager (Amersham Biosciences) using Image Quant software (Amersham Biosciences).

Quantitation of mRNA expression

Our method of quantitative RT-PCR analysis has been described previously (Potts, Singh et al. 2003). The gel was stained with Sybr Green I (Molecular Probes) and visualized on a Typhoon fluorescence imager (Amersham Biosciences) and quantitated using ImageQuant software.

Annexin V and PI staining

Sympathetic neuronal cultures were treated with etoposide for 36 hours, followed by staining for Annexin V and Propidium Iodide (PI) using the TACS Annexin V-FITC Apoptosis Detection kit (R&D Systems) according to the manufacturer's protocol. Briefly, cells were washed in PBS followed by a 15 min incubation with 2.5 ug/mL PI, and 1.25 ug/mL AnnexinV-FITC. Cells were washed twice and viewed immediately on a Leica inverted fluorescence microscope.

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.

Primers

Primers used to genotype the following knockout mice:

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

SMAC: Forward WT: 5’ GAGACAGAAAGGTAGAGGTGC 3’

Forward KO: 5’ GGTGGATGTGGAATGTGTG 3’

Reverse (common): 5’ TATAGAGCCCGAATGTCAGAA 3’

WT: 600 base pairs; SMAC KO: 750 base pairs

Primer sets used for PCR amplification of Apaf-1 and GAPDH were as follows.

Apaf-1: Forward 5′ GAG GCA CAA TGG ATG CAA AGG 3′; Reverse 5′ GGC TGC TCG TTG ATA TTG AGT GG 3′

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

Acknowledgements

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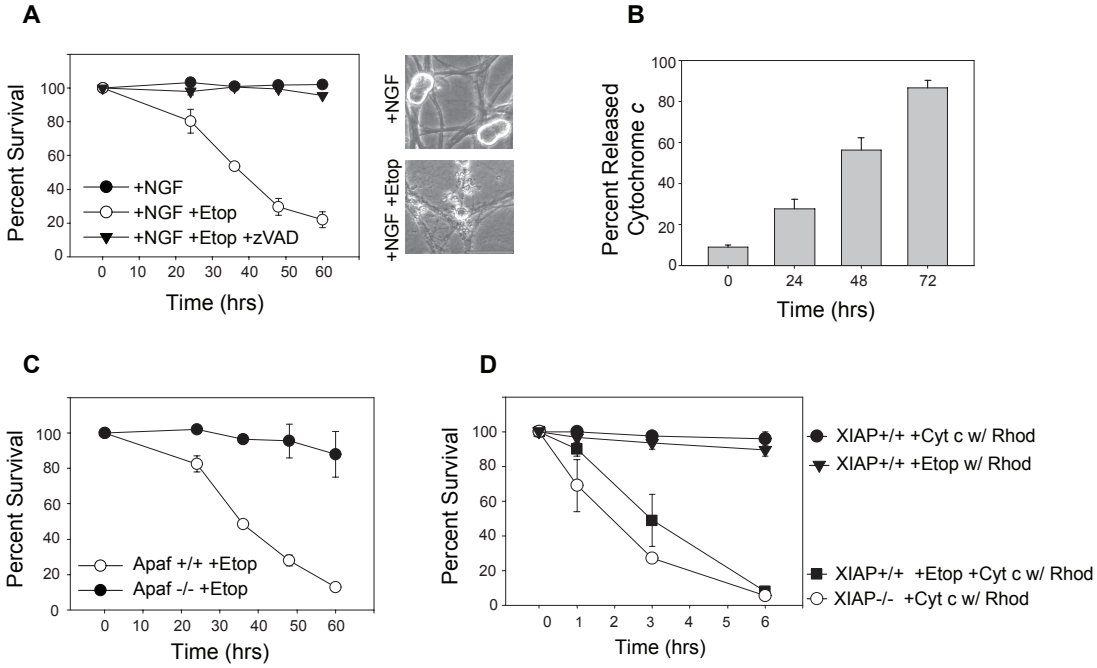
F. Figures and Legends

Figure 2.1. Etoposide induces a cytochrome *c*- and apoptosome-dependent apoptosis in sympathetic neurons. **A)** Cultures of sympathetic neurons were either left untreated, treated with 20 μM etoposide, or treated with etoposide and the caspase inhibitor zVAD-fmk (50 μM). At various time points, cell survival was assessed by morphological criteria (healthy neurons remain intact and phase bright). This method of assessing cell death correlates well with trypan blue exclusion (Fig. S1A) and calcein AM staining (Potts, Singh et al. 2003). Data shown are mean ±SEM of three experiments. Photographs show untreated or etoposide-treated sympathetic neurons at 60 hours. **B)** Cultures of sympathetic neurons were treated with 20 uM etoposide for various time points, and the status of cytochrome *c* in these neurons was examined by immunocytochemical techniques. Neurons showing a punctate staining pattern have maintained cytochrome *c* in the mitochondria, whereas neurons showing a loss of staining have released cytochrome *c* from the mitochondria. Approximately 100 neurons per condition were counted to determine the status of cytochrome *c*. **C)** Sympathetic neurons isolated from Apaf-1-deficient mice or their wildtype littermates were treated with 20 μM etoposide and cell survival was assessed as described above. **D)** Sympathetic neuronal cultures isolated from XIAP+/+ or XIAP-/- mice were injected with cytochrome *c* and rhodamine dextran dye (Rhod) to mark injected cells, or rhodamine alone. Wildtype sympathetic neurons (XIAP+/+) were also treated with etoposide (20 uM) for 24 hrs or left untreated and then injected with cytochrome *c* and rhodamine dextran dye (Rhod), or rhodamine alone. Cell survival of these neurons was then assessed at various time points following injection.

Figure 2.1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Percent Survival
Time (hrs)
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+NGF +Etop
+NGF +Etop +zVAD
+NGF
+NGF +Etop
B
Percent Released Cytochrome c
Time (hrs)
C
Percent Survival
Time (hrs)
Apaf +/+ +Etop
Apaf -/- +Etop
D
Percent Survival
Time (hrs)
XIAP+/+ +Cyt c w/ Rhod
XIAP+/+ +Etop w/ Rhod
XIAP+/+ +Etop +Cyt c w/ Rhod
XIAP-/- +Cyt c w/ Rhod

Figure 2.2. Development of competence by etoposide is independent of XIAP degradation, and requires protein synthesis. **A)** Protein levels of XIAP were examined from whole cell lysate of sympathetic neurons maintained in NGF (+NGF), deprived of NGF (-NGF) for 24 hours, or treated with 20 μM etoposide (+NGF +Etop) for 24 hours. Levels of LDH were also examined as a loading control. **B)** Quantitation of XIAP levels (mean ± SEM of three experiments) in NGF-deprived and etoposide-treated neurons at 24 and 48 hours as compared to NGF maintained neurons is shown and is normalized to LDH. **C)** Cycloheximide (CHX) (1 μg/ml) treated wildtype sympathetic neurons, or neurons isolated from Bax-deficient mice were deprived of NGF for 24 hours. Cells were then injected with 10 mg/ml cytochrome *c* along with rhodamine dextran or with rhodamine dextran dye alone. At each time point after injections, the number of microinjected cells that remained viable was determined and expressed as a percentage of the total number of microinjected cells. **D)** CHX (1 μg/ml) treated wildtype sympathetic neurons, or neurons isolated from Bax-deficient mice were treated with etoposide (20 μM) for 24 hours. Cells were then injected with 10 mg/ml cytochrome *c* along with rhodamine dextran or with rhodamine dextran dye alone. At each time point after injections, viable cells were quantified as in (C). Results shown are a mean (± SEM) for three experiments with 70-100 cells injected in each experiment.

Figure 2.2

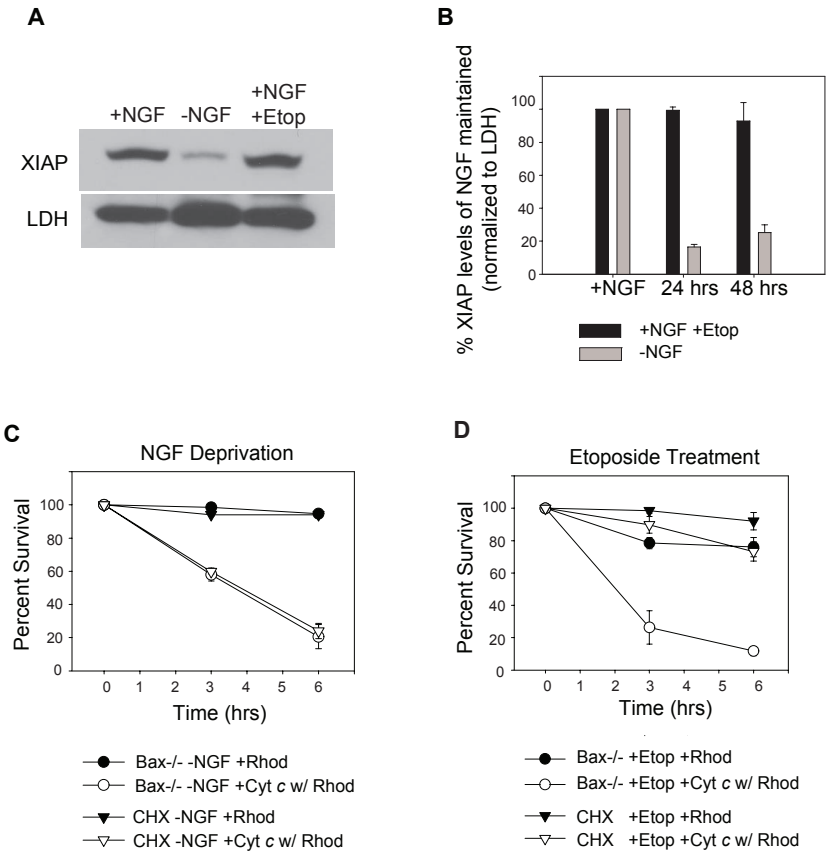

A
+NGF
-NGF
+NGF +Etop
XIAP
LDH
B
% XIAP levels of NGF maintained (normalized to LDH)
100
80
60
40
20
0
+NGF
24 hrs
48 hrs
+NGF +Etop
-NGF
C
NGF Deprivation
Percent Survival
Time (hrs)
Bax-/- -NGF +Rhod
Bax-/- -NGF +Cyt c w/ Rhod
CHX -NGF +Rhod
CHX -NGF +Cyt c w/ Rhod
D
Etoposide Treatment
Percent Survival
Time (hrs)
Bax-/- +Etop +Rhod
Bax-/- +Etop +Cyt c w/ Rhod
CHX +Etop +Rhod
CHX +Etop +Cyt c w/ Rhod

Figure 2.3. Etoposide treatment results in an increase in Apaf-1 protein and mRNA in sympathetic neurons. **A)** Sympathetic neuronal cultures were either left untreated (+NGF), deprived of NGF (-NGF) or treated with 20 μM etoposide (+NGF +Etop) for 24 hours. Cells were collected and levels of Apaf-1 protein analyzed by Western blot. LDH was used as a loading control. Quantitation of Apaf-1 protein levels normalized to LDH following etoposide treatment at 24 and 48 hours is shown. Data are mean (±SEM) for three independent experiments. **B)** Sympathetic neurons were either left untreated, or treated with 20 etoposide for 24 hours. Levels of Apaf-1 and GAPDH (loading control) mRNAs were examined by quantitative RT-PCR and expressed as a percentage of NGF maintained as normalized to GAPDH. Quantitation shown is mean (±SEM) for three independent experiments.

Figure 2.3

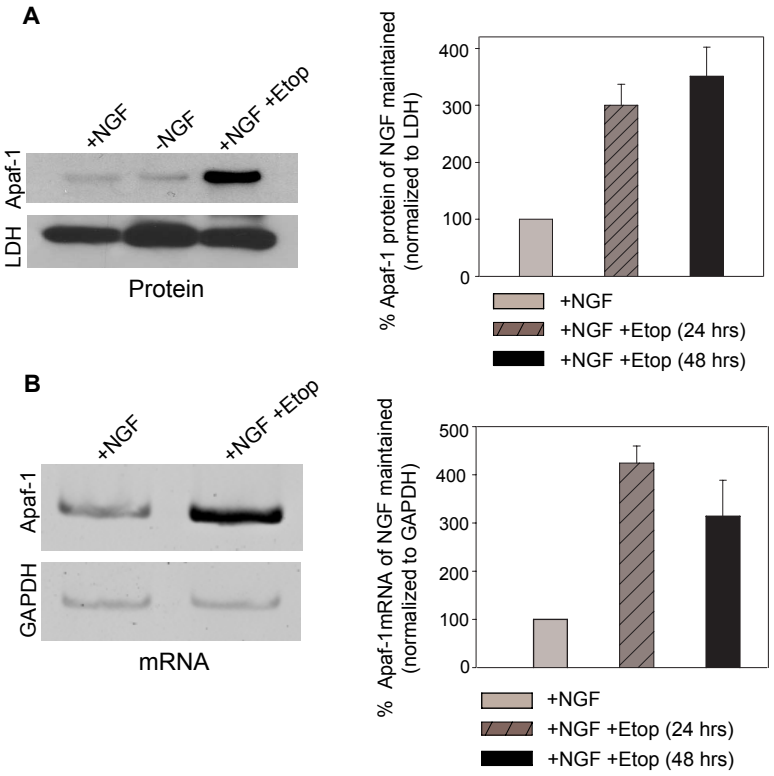

A
+NGF
-NGF
+NGF +Etop
Apaf-1
LDH
Protein
% Apaf-1 protein of NGF maintained (normalized to LDH)
400
300
200
100
0
+NGF
+NGF +Etop (24 hrs)
+NGF +Etop (48 hrs)
B
+NGF
+NGF +Etop
Apaf-1
GAPDH
mRNA
% Apaf-1mRNA of NGF maintained (normalized to GAPDH)
500
400
300
200
100
0
+NGF
+NGF +Etop (24 hrs)
+NGF +Etop (48 hrs)

Figure 2.4. p53-deficient neurons develop competence in response to NGF deprivation but not etoposide. **A)** Cultures of sympathetic neurons from p53+/+ or p53-/- mice were treated with 20 μM etoposide. At various time points, cell survival was assessed by morphological criteria (healthy neurons remain intact and phase bright). Data are a mean ±SEM for three experiments with greater than 100 cells counted in each experiment. Representative photographs at 60 hours after etoposide treatment are shown. Scale bars represent 50 μm. **B)** p53+/+ or p53-/- sympathetic neurons were treated with etoposide for 24 hours. Cells were then microinjected with cytochrome *c* (10 mg/ml) alone or cytochrome *c* and SMAC (1 mg/ml) together protein along with rhodamine dye, or rhodamine alone. Cell survival was assessed at various time points as indicated. **C)** p53+/+ or p53-/- sympathetic neurons were deprived of NGF in the presence of cycloheximide (1 μg/ml) for 24 hours. Cells were then microinjected with cytochrome *c* protein (10 mg/ml) along with rhodamine dye, or rhodamine alone. Cell survival was assessed at various time points as indicated.

Figure 2.4

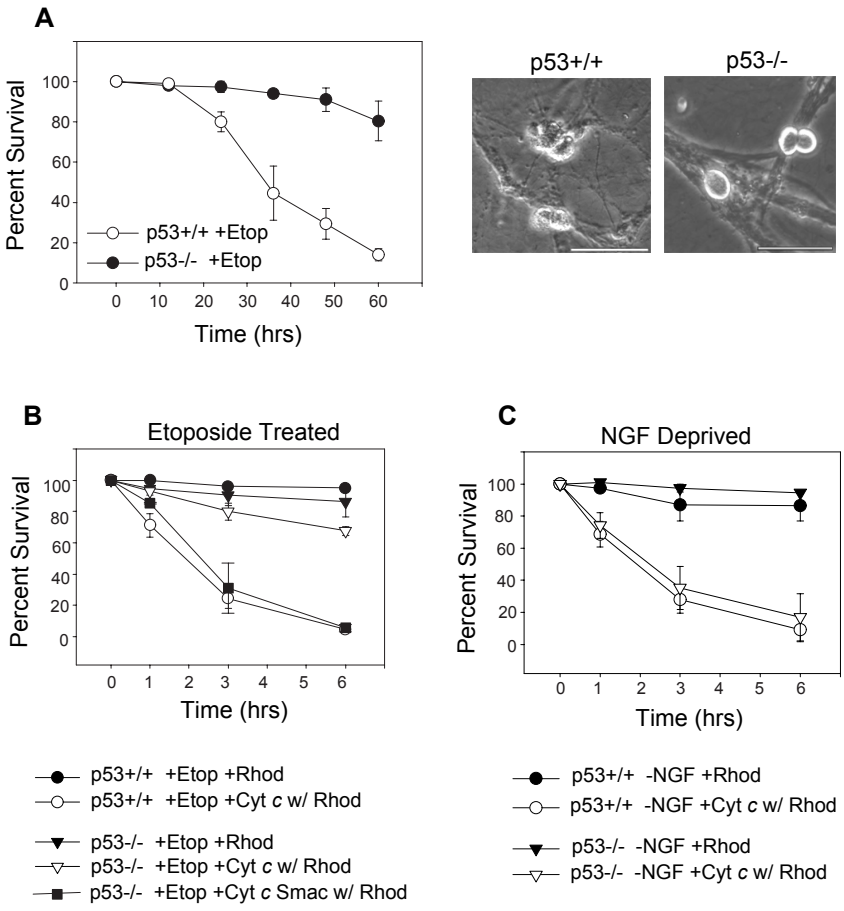

A
Percent Survival
Time (hrs)
p53+/+ +Etop
p53-/- +Etop
p53+/+
p53-/-
B
Etoposide Treated
Percent Survival
Time (hrs)
p53+/+ +Etop +Rhod
p53+/+ +Etop +Cyt c w/ Rhod
p53-/- +Etop +Rhod
p53-/- +Etop +Cyt c w/ Rhod
p53-/- +Etop +Cyt c Smac w/ Rhod
C
NGF Deprived
Percent Survival
Time (hrs)
p53+/+ -NGF +Rhod
p53+/+ -NGF +Cyt c w/ Rhod
p53-/- -NGF +Rhod
p53-/- -NGF +Cyt c w/ Rhod

Figure 2.5. p53 links Apaf-1 induction with XIAP inactivation during DNA damage-induced neuronal apoptosis. **A)** p53+/- or p53-/- sympathetic neurons were either left untreated, or treated with etoposide (20 μM) for 24 hours. Cells were then collected and Apaf-1 protein levels analyzed by Western blot. Tubulin is used as a loading control. These data are representative of multiple experiments. **B)** p53-/- sympathetic neurons were injected with either a plasmid encoding Apaf-1 (200 ng/μl) along with EGFP (50 ng/μl), or empty vector (200 ng/μl) along with EGFP (50 ng/μl) and allowed to express DNAs for 24 hours. Cells were then treated with 20 μM etoposide for 24 hours (a time at which endogenous cytochrome *c* is retained in the mitochondria; Fig. S2), after which GFP expressing cells were injected with either cytochrome *c* protein (10 mg/ml) along with rhodamine, or rhodamine alone. Cell survival of double injected cells was assessed by morphological criteria at the indicated time points. Data are a mean $\pm$ SEM of three independent experiments with 70-100 cells injected per experiment. Note that the data represented with open and filled circles are closely overlapping.

Figure 2.5

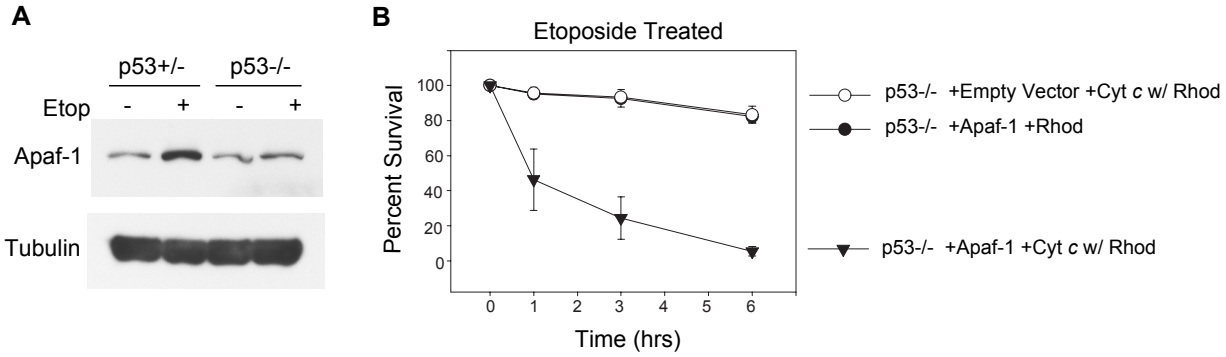
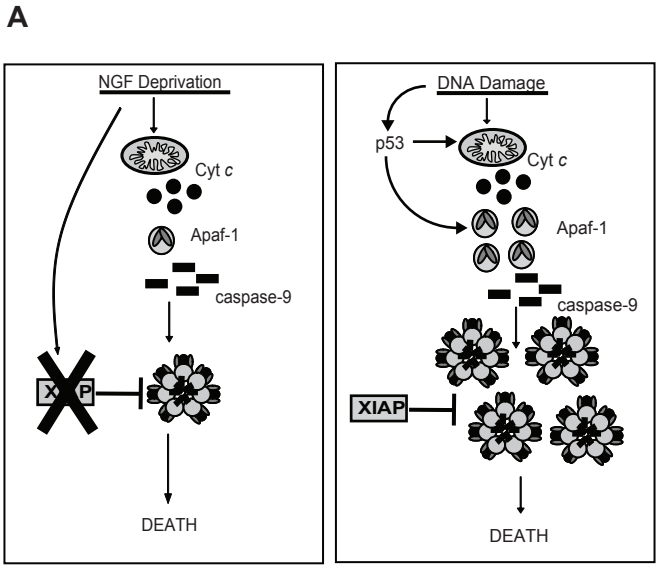
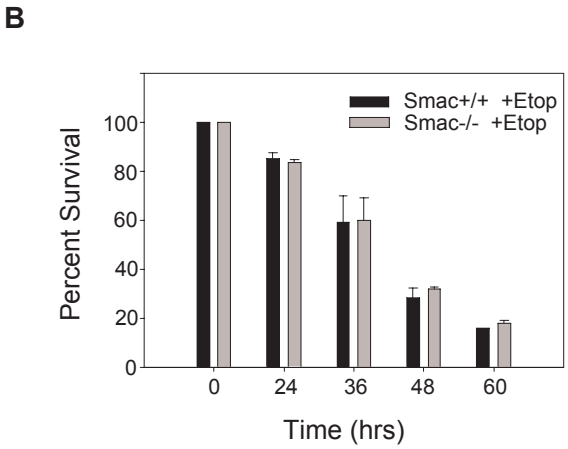

A
p53+/-
p53-/-
Etop
-
+
-
+
Apaf-1
Tubulin
B
Etoposide Treated
Percent Survival
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (hrs)
p53-/- +Empty Vector +Cyt c w/ Rhod
p53-/- +Apaf-1 +Rhod
p53-/- +Apaf-1 +Cyt c w/ Rhod

Figure 2.6. Distinct SMAC-independent mechanisms by which XIAP is relieved in NGF deprivation and DNA damage models of neuronal apoptosis. **A)** While both NGF deprivation and DNA damage activate a pathway which releases cytochrome *c* from the mitochondria, the mechanisms by which these two stimuli induce competence and overcome XIAP's inhibition of caspases are distinct. NGF Deprivation selectively degrades XIAP, while DNA damage overcomes XIAP activity via a p53-mediated induction of Apaf-1. **B)** Cultures of sympathetic neurons isolated from SMAC -/- or wildtype mice were either left untreated or treated with 20 μM etoposide. Shown is quantitation of cell survival at various time points.

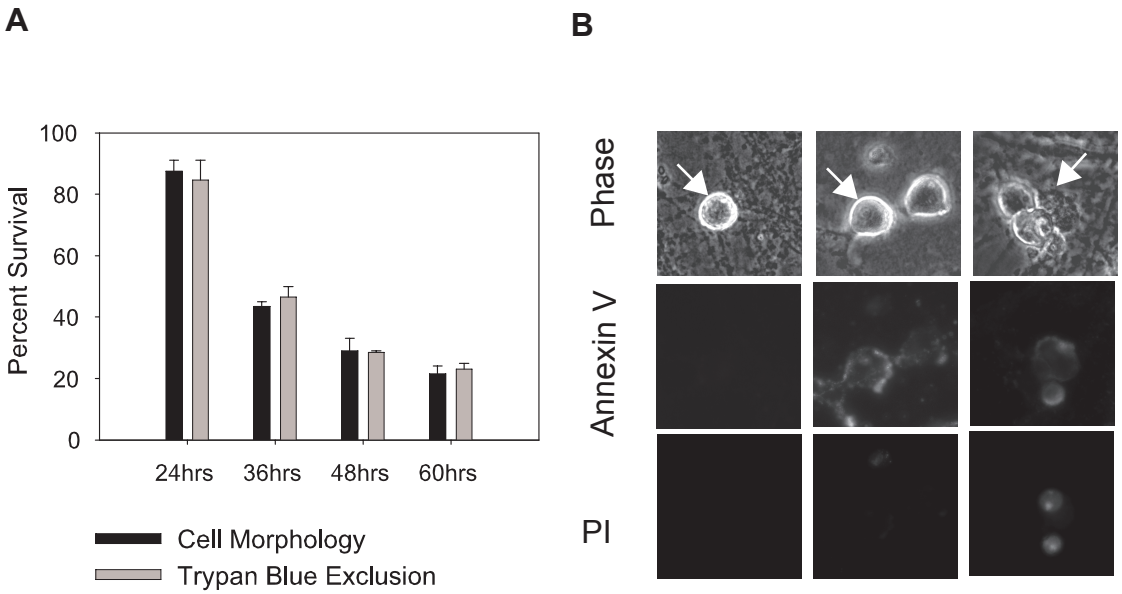
Figure 2.6


A
NGF Deprivation
Cyt c
Apaf-1
caspase-9
XIAP
DEATH
DNA Damage
p53
Cyt c
Apaf-1
caspase-9
XIAP
DEATH


B
Smac+/+ +Etop
Smac-/- +Etop
Percent Survival
100
80
60
40
20
0
0
24
36
48
60
Time (hrs)

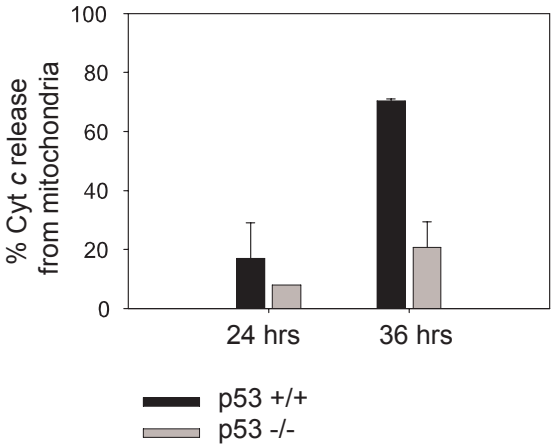
Supplemental Figure 2.1. Assessment of cell death by multiple criteria. **A)** Sympathetic neurons were treated with 20 uM etoposide for various time points. For assessment of cell death by cell morphology, cells with intact phase bright cell bodies are counted as alive. For trypan blue staining, cells were incubated in a 0.04 % trypan blue solution for 5 min and the number of cells that excluded the trypan blue dye was counted. **B)** Sympathetic neurons were treated with etoposide for 36 hours, followed by staining with Annexin V and Propidium Iodide (PI) as described in the Methods section. Photographs depict healthy Annexin V negative, PI negative cells (left hand column), Annexin V positive, PI negative cells (middle column), and Annexin V positive, PI positive cells (right column).

Supplemental Figure 2.1



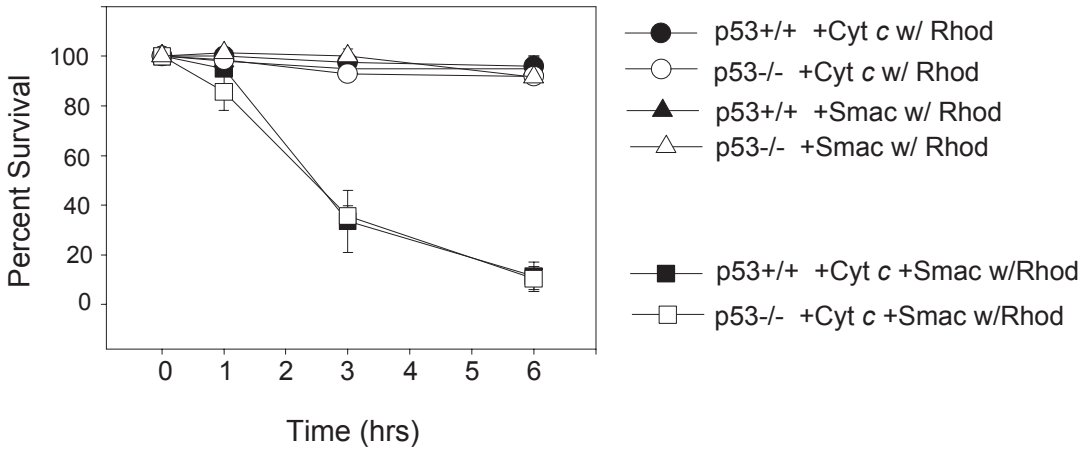
Supplemental Figure 2.2. Cytochrome *c* release by etoposide is inhibited in p53-deficient neurons. p53+/+ or p53-/- sympathetic neurons were treated with etoposide (20 uM) for 24 or 36 hours, and the status of cytochrome *c* in these neurons was examined by immunohistochemical techniques. Neurons showing a punctate staining pattern have maintained cytochrome *c* in the mitochondria, whereas neurons showing a loss of staining have released cytochrome *c* from mitochondria. Approximately 100 neurons per condition were counted to determine the status of cytochrome *c*.

Supplemental Figure 2.2



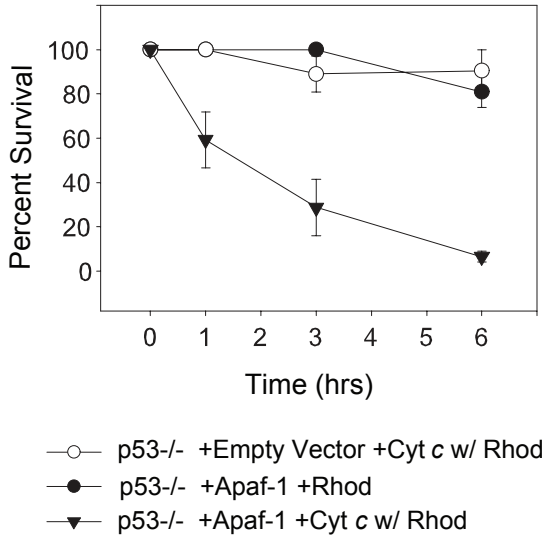
Supplemental Figure 2.3. Inactivation of XIAP is sufficient to permit p53-/- neurons to undergo cytochrome *c*-mediated apoptosis. p53+/+ or p53-/- sympathetic neurons were microinjected with cytochrome *c* protein (10 mg/ml), or cytochrome *c* along with mature SMAC protein (1 mg/ml), or SMAC alone. At various time points following injection, cells survival was assessed by morphological criteria. Data are a mean $\pm$ SEM of three independent experiments with 70-100 cells injected per experiment.

Supplemental Figure 2.3



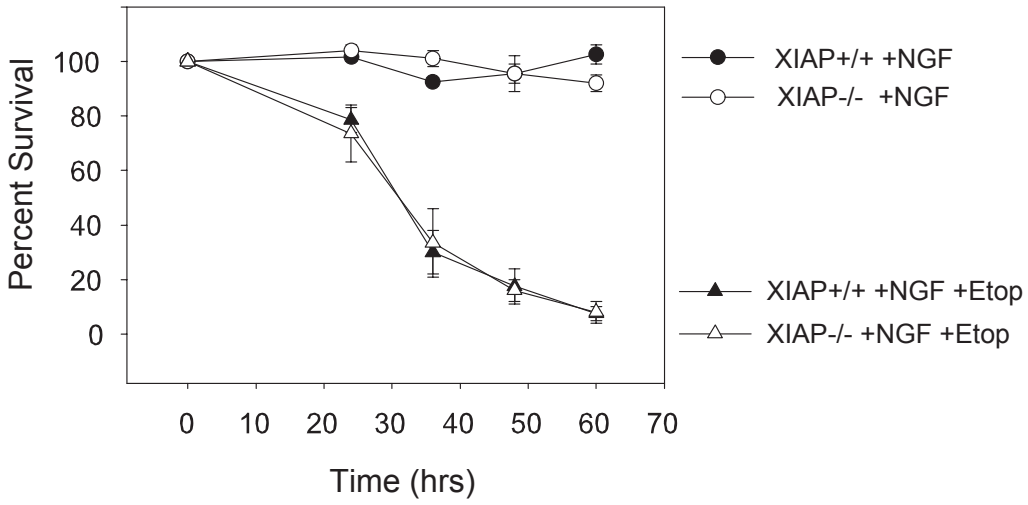
Supplemental Figure 2.4. Apaf-1 induction is the only step required by etoposide to sensitize p53-deficient neurons to cytochrome *c*. p53-/- sympathetic neurons were injected with either a plasmid encoding Apaf-1 (200 ng/µl) along with EGFP (50 ng/µl), or empty vector (200 ng/µl) along with EGFP (50 ng/µl). After 24 hours, GFP expressing cells were injected with either cytochrome *c* protein along with rhodamine, or rhodamine alone. Cell survival of double injected cells was assessed by morphological criteria at the indicated time points. Data are a mean $\pm$ SEM of three independent experiments with 70-100 cells injected per experiment.

Supplemental Figure 2.4



Supplemental Figure 2.5. Timecourse of etoposide-induced death in wildtype and XIAP-deficient neurons. Sympathetic neurons isolated from XIAP-/-, or their wildtype littermates (XIAP+/+) were treated with 20 uM etoposide (+NGF+Etop), or left untreated (+NGF). Cell survival was assessed by morphological criteria at various time points.

Supplemental Figure 2.5



III. CHAPTER THREE:

Glucose Metabolism Inhibits Apoptosis in Neurons and Cancer Cells by Redox Inactivation of Cytochrome *c*

A. Abstract

Neurons and cancer cells utilize glycolysis extensively, yet the precise advantage of this adaptation remains elusive. Here we show that these cells strictly inhibit cytochrome *c*-mediated apoptosis by a mechanism dependent on glucose metabolism. We report that the proapoptotic activity of cytochrome *c* requires its oxidation and that this is accomplished by increases in intracellular ROS following an apoptotic insult. In healthy neurons and cancer cells, cytochrome *c* is reduced and held inactive by intracellular glutathione (GSH) generated as a result of glucose metabolism by the pentose phosphate pathway. These results uncover a striking similarity in apoptosis regulation between neurons and cancer cells and provide insight into an adaptive advantage offered by the Warburg effect for cancer cell evasion of apoptosis and for long-term neuronal survival.

B. Introduction

Apoptosis is a genetically regulated process that is essential for the development of the organism, but its dysregulation can lead to neurodegenerative disorders as well as cancer (Yuan and Yankner 2000; Green and Evan 2002). A critical event in the apoptotic pathway is the release of cytochrome *c* from the mitochondria. In healthy cells, cytochrome *c* resides in the mitochondrial intermembrane space where it serves as a redox carrier for the electron transport chain. However, in response to many apoptotic stimuli, cytochrome *c* is released into the cytosol where it can initiate the formation of the apoptosome complex, leading to caspase activation and subsequent cell death (Wang 2001). Emerging evidence indicates that cells such as postmitotic neurons, which last the lifetime of the organism, as well as cancer cells, which must overcome a cell death response, both strictly inhibit the apoptotic pathway (Wright and Deshmukh 2006). Interestingly, despite the striking morphological and functional differences between neurons and cancer cells, both depend on glycolysis as their primary source of energy (Warburg 1956; Schubert 2005). Here we examined whether the mutual reliance on glycolysis was of critical importance to the increased resistance to apoptosis in neurons and cancer cells.

C. Results

We assessed the ability of endogenous cytochrome *c* to activate apoptosis in sympathetic neurons by using truncated Bid (tBid). Full length Bid is cleaved intracellullarly into tBid in response to certain apoptotic stimuli, where it then acts as a potent inducer of cytochrome *c* release from mitochondria (Esposti 2002). While expression of tBid-GFP plasmid DNA in mouse embryonic fibroblasts (MEFs) resulted in rapid and complete

apoptosis, nerve growth factor (NGF)-maintained sympathetic neurons remained remarkably resistant to expression of tBid-GFP (Fig. 3.1A). Sympathetic neurons are known to be resistant to exogenously microinjected cytochrome *c* because of the strict inhibition of caspases by XIAP. In contrast to wildtype neurons, XIAP-deficient sympathetic neurons are sensitive to microinjection of exogenous cytochrome *c* (Potts, Singh et al. 2003). However, we were surprised to find that the release of *endogenous* cytochrome *c* with tBid was incapable of inducing apoptosis even in XIAP-deficient neurons (Fig. 3.1B). This inability to induce apoptosis is not a defect in tBid itself, as expression of tBid in sympathetic neurons induced potent release of cytochrome *c* from the mitochondria (Fig. 3.1C). Thus, in NGF-maintained neurons, the release of endogenous cytochrome *c* was incapable of inducing apoptosis even in the absence of XIAP. To focus on this unexpected XIAP-independent mechanism of postcytochrome *c* regulation, we conducted all following experiments in neurons isolated from XIAP-deficient mice.

Despite the resistance of neurons to direct cytochrome *c* release by tBid, sympathetic neurons readily undergo cytochrome *c*-mediated cell death in response to multiple apoptotic stimuli (Neame, Rubin et al. 1998; Vaughn and Deshmukh 2007). Thus, apoptotic stimuli must render endogenous cytochrome *c* capable of activating apoptosis in neurons. Indeed, NGF deprivation as well as DNA damage with etoposide sensitized sympathetic neurons to apoptosis induced by tBid-mediated cytochrome *c* release (Fig. 3.1D, Supplemental Fig. 3.1A). Since tBid may cause the release of multiple factors from the mitochondria, we examined whether this observed effect was a direct result of sensitization to cytochrome *c*. Indeed, injection of cytochrome *c* in NGF-deprived or DNA-damaged neurons (at a time

point prior to endogenous cytochrome *c* release) also showed increased sensitivity (Fig. 3.1E, Supplemental Fig. 3.1B).

To be apoptotically active, cytochrome *c* must exist as a holoenzyme complete with its heme prosthetic group (Yang, Liu et al. 1997). Results from *in vitro* studies that have examined whether the redox state of cytochrome *c* affects its apoptotic activity remain controversial. While some show that oxidized cytochrome *c* is more apoptotically active, others suggest that the reduced form can also function effectively (Kluck, Martin et al. 1997; Hampton, Zhivotovsky et al. 1998; Pan, Voehringer et al. 1999; Hancock, Desikan et al. 2001; Chen, Crosby et al. 2003; Suto, Sato et al. 2005). To determine whether the intracellular redox environment affects the sensitivity of intact neurons to cytochrome *c,* we treated neurons with either low levels of hydrogen peroxide (H_2O_2) to create a more oxidized environment, or cell permeable reduced glutathione (GSH) to make the environment more reduced. Neurons injected with 2.5 ug/ul cytochrome *c* exhibit substantial death only 10-16 hrs after the injections (Fig. 3.2A, B). However, H_2O_2 greatly increased the sensitivity of neurons to injected cytochrome *c* (Fig. 3.2A) while neurons treated with GSH are resistant (Fig. 3.2B). These results show that in intact cells, the redox environment has a dramatic affect on the ability of cytochrome *c* to promote apoptosis.

As changes in the redox environment can have widespread affects, we determined whether the ability of the reduced cellular environment to inhibit cytochrome *c*-mediated apoptosis is a result of the specific inactivation of cytochrome *c*. Incubation of cytochrome *c* with cytochrome *c* reductase rendered this now reduced cytochrome *c* (Supplemental Fig. 3.2) ineffective for promoting apoptosis in injected neurons (Fig. 3.2C). In addition, we generated both wildtype cytochrome *c* protein, and a N52I mutant of cytochrome *c* which has

increased stability, making it less prone to oxidation (Doyle, Waldner et al. 1996). Injection of the N52I mutant cytochrome *c* into sympathetic neurons induced significantly less apoptosis than did wildtype cytochrome *c* (Fig. 3.2D). This mutant protein also exhibited a diminished ability to activate caspases in an *in vitro* assay (Supplemental Fig. 3.3).

The observation that cytosolic cytochrome *c* requires an oxidized cellular environment to be proapoptotic leads to two predictions. First, that the ability of tBid-induced cytochrome *c* release to promote apoptosis in MEFs but not in NGF-maintained neurons could be due to a marked difference in their redox environment. Consistent with this, we find that the average intensity of the redox-sensitive dye 5- (and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2 DCFDA) was 5 fold less in sympathetic neurons as compared to MEFs, indicating reduced levels of reactive oxygen species (ROS) in sympathetic neurons (Supplemental Fig. 3.3A). Second, that NGF deprivation, which sensitizes neurons to cytochrome *c*-induced death, may result in an increased oxidized cellular environment. Indeed, CM-H_2 DCFDA intensity was elevated 2.5 fold after NGF deprivation in neurons (Supplemental Fig. 3.3B; (Greenlund, Deckwerth et al. 1995; Kirkland and Franklin 2001)). This increase in ROS following NGF deprivation was important to permit cytochrome *c*-mediated apoptosis as addition of GSH inhibited the ability of NGF deprivation to sensitize neurons to cytochrome *c* release (Fig 3.2E). Importantly, we examined whether the levels of ROS accumulating after NGF deprivation were sufficient to oxidize cytochrome *c*. While cytochrome *c* added to NGF-maintained neuronal lysates remained in a reduced state, NGF-deprived neuronal lysate was able to oxidize cytochrome *c*, as measured by a decrease in absorbance peak at 550 nm (Fig. 3.2F).

GSH is one of the most prevalent cellular reducing agents and maintains redox homeostasis by scavenging ROS. We found that intracellular GSH is necessary for maintaining cytochrome *c* in an inactive state in NGF-maintained neurons, as inhibition of GSH with Diethyl Maleate (DEM) leads to an increase in ROS and sensitizes neurons to cytosolic cytochrome *c* (Fig. 3.3A,B). Levels of GSH are maintained in cells by reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is generated when glucose is metabolized via the pentose phosphate pathway (Kaplowitz, Aw et al. 1985). We examined whether the pentose phosphate pathway was important for regulating the redox status of neurons, and thus cytochrome *c*-mediated apoptosis. Neurons treated with the pentose phosphate pathway inhibitor, dehydroepiandrosterone (DHEA), accumulated high levels of ROS and became sensitive to endogenous cytochrome *c* release with tBid (Fig. 3.3C,D). This effect was not unique to DHEA as another inhibitor of the pentose phosphate pathway, 6-anicotinamide (6-AN) was also able to sensitize neurons to cytochrome *c* release (Fig. 3.3D). Together, these data show how glucose metabolism via the pentose phosphate pathway promotes neuronal survival by maintaining GSH levels and directly restricting cytochrome *c*-mediated apoptosis.

Like neurons, many cancer cells are known to depend predominantly on glycolysis for ATP generation, a phenomenon known as the Warburg effect (Warburg 1956). To investigate whether increased glycolysis in cancer cells directly regulates apoptosis at the point of cytochrome *c,* we first examined the sensitivity of cancer cells to cytosolic injection of cytochrome *c*. Cytochrome *c* was injected in the presence of SMAC to eliminate the activity of IAPs that are known to block caspase activation in many cancer cells (Verhagen and Vaux 2002), thus focusing on IAP-independent mechanisms of cytochrome *c* regulation.

While normal cells (MEFs, Human Dermal Fibroblasts-HDFs) readily underwent apoptosis in response to cytosolic cytochrome *c,* multiple cancer cells tested showed an increased resistance (Fig. 3.4A). Strikingly, the sensitivity to cytochrome *c* correlated with the levels of intracellular GSH in these cancer cells, with the highly resistant hepatoma cell line JM2 containing as much as 5 fold more intracellular GSH than noncancerous cells (Fig. 3.4B).

We examined whether enhanced glycolysis and the pentose phosphate pathway in HeLa and JM2 cancer cells contributes to cellular redox homeostasis, and thus resistance to cytochrome *c.* Indeed, addition of the pentose phosphate inhibitor DHEA increased ROS levels and specifically rendered these cancer cells sensitive to cytosolic cytochrome *c* (Fig 3.4. C,D). Importantly, reducing glycolysis by glucose deprivation also resulted in a similar increase in sensitivity to cytochrome *c* (Fig. 3.4E). Together, these results show that the ability of cytochrome *c* to induce apoptosis is strictly regulated by its redox state and that glycolysis flux is a critical regulator of cytochrome *c*-mediated apoptosis.

D. Discussion

By coupling the proapoptotic activity of cytochrome *c* to the pentose phosphate pathway, cells which have increased glycolytic flux, such as neurons and cancer cells, are able to effectively maintain a restrictive environment for cytochrome *c*-mediated apoptosis. Such a regulation would be of particular physiological importance for neurons that have limited regenerative potential and survive long term, and advantageous to cancer cells in evading apoptosis. Flux through the pentose phosphate pathway is likely to inhibit apoptosis in multiple scenarios. For example, upregulation of TIGAR following p53 activation by DNA damage was recently shown to engage the pentose phosphate pathway and promote cell

survival (Bensaad, Tsuruta et al. 2006). Overexpression of glucose-6-phosphate dehydrogenase is also shown to promote tumor formation by increasing GSH through pentose phosphate pathway (Kuo, Lin et al. 2000). Our results suggest that a specific mechanism by which this could inhibit apoptosis is through direct inactivation of cytochrome *c*.

Although exactly how a change in the redox status can influence the apoptotic activity of cytochrome c is unknown, it is likely that the redox status affects cytochrome c binding to its downstream apoptotic effector, Apaf-1. While the overall structural differences between oxidized and reduced cytochrome is not substantial, the oxidation state does have a more pronounced affect on the orientation specific residues, such as Lysine 72 (Kar, Sherman et al. 1994). Interestingly, modification of Lysine 72 is an important factor in determining whether cytochrome c from different species will be proapoptotic (Kluck, Ellerby et al. 2000).

Although maintenance of redox homeostasis is a primary function of the pentose phosphate pathway, flux through this pathway may also inhibit apoptosis at other points. For example, NADPH production through the pentose phosphate pathway maintains caspase-2 in an inactive state in *Xenopus* egg extracts by a mechanism independent of GSH synthesis (Nutt, Margolis et al. 2005).

Neurons undergoing cytochrome *c*-mediated apoptosis must overcome multiple blocks in order to undergo cell death. We have shown previously that endogenous XIAP is a potent inhibitor of cytochrome *c*-mediated apoptosis in neurons (Potts, Singh et al. 2003), and here, that endogenous cytochrome *c* itself is incapable of inducing apoptosis in NGF-maintained neurons. In response to developmental apoptosis after trophic factor deprivation, these neurons not only exhibit degradation of XIAP to allow for caspase activation, but also a

marked decrease in glucose uptake and increase in ROS (Supplemental Fig. 3.3B) (Deckwerth and Johnson 1993; Greenlund, Deckwerth et al. 1995; Kirkland and Franklin 2001; Potts, Singh et al. 2003) which can activate cytochrome *c* and permit apoptosis.

Increases in intracellular ROS as well as mitochondrial damage are commonly seen during aging and associated with apoptotic cell death in many neurodegenerative diseases, including Parkinson's Disease (Carney, Smith et al. 1994; Squier 2001; Rego and Oliveira 2003). Our results provide mechanistic insight into how even a modest increase in ROS would prime neurons to undergo apoptosis in response to otherwise potentially non-lethal events of mitochondrial damage and cytochrome *c* release (Deshmukh, Kuida et al. 2000).

Neurons and cancer cells are indeed strikingly distinct by most criteria. Different cancer cells themselves appear to inhibit the apoptotic pathway at different points. However, these results bring into focus the possibility that the multiple mechanisms evolved by neurons to restrict apoptosis would be the same ones adapted by some mitotic cells during their progression to becoming cancerous.

E. Materials and Methods:

Reagents.

All reagents were purchased from Sigma (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). Recombinant SMAC protein was purified from bacteria as previously described (Potts, Singh et al. 2003) and used at a concentration of 1.5 mg/ml. MEFs were a kind gift provided by Dr. Douglas Green (St. Jude's Children's Research Hospital, Memphis, TN). HDFs and HeLa cells were obtained

from UNC Tissue Culture Facility, and JM2 cells were a kind gift of Dr. Ekhson Holmuhamedov (UNC, Chapel Hill, NC). XIAP-/- mice were a kind gift from Dr. Craig Thompson (University of Pennsylvania, Philadelphia, PA).

Sympathetic neuronal cultures.

Primary sympathetic neurons were dissected from the superior cervical ganglia of postnatal day 0-1 mice from wildtype ICR or XIAP-deficient C57BL/6 mice and maintained in culture as described previously (Deshmukh, Kuida et al. 2000). Cells were plated on collagen coated dishes at a density of 10,000 cells per well. Neurons were grown for 4-5 days in NGF-containing media before treating them with experimental conditions. For NGF deprivation, cultures were rinsed three times with medium lacking NGF, followed by the addition of goat anti-NGF neutralizing antibody to this media. Etoposide was used at a concentration of 20 μM.

*Immunofluorescence analysis***.**

The status of cytochrome *c* following expression of tBid-GFP (whether intact in the mitochondria or released) was examined by immunofluorescence. Sympathetic neurons exhibit a punctate mitochondrial staining with anti-cytochrome *c* antibody, which is lost as cytochrome *c* is released from the mitochondria. Briefly, cultured sympathetic neurons that had been expressing tBid for 24 hours were fixed in 4% paraformaldehyde and incubated overnight with anti-cytochrome *c* (556432, BD Biosciences), or anti-GFP (Cell Signaling) primary antibody followed by a 2 hour incubation with anti-mouse Cy3 and anti-Chicken

Alexa 488 secondary antibody (Jackson Labs). Nuclei were stained with Hoechst 33258 (Molecular Probes).

Microinjection and quantitation of cell survival.

Cells were injected with horse cytochrome *c* protein at a concentration of 2.5 ug/ul. Horse cytochrome *c* (Sigma) is predominantly in an oxidized state (Supplemental Fig. 3.2). Immediately after injections, the number of rhodamine-positive cells were counted. At various times after injections, the number of viable injected cells remaining was determined using the same counting criteria and expressed as a percentage of the original number of microinjected cells. This method of assessing survival has correlated well with other cell survival assays such as trypan blue exclusion and staining with calcein AM (Potts, Singh et al. 2003).

In experiments involving microinjection of DNAs, 100 ng/ul of the tBid expressing plasmid, or EGFP vector in microinjection buffer was injected into the nucleus of neurons or MEFs. To quantitate survival after injection, GFP-positive cells were counted 5 hours after injection as well as 24 hours following injection. Cell survival is expressed as a ratio of viable GFP-positive cells at 24 hours post-injection over 5 hours post injection. In experiments involving assessment of cell survival following tBid expression in the absence of NGF or presence of etoposide, neuronal cultures were deprived of NGF or treated with etoposide for 12 hrs followed by microinjection with tBid-GFP or EGFP alone. Cell survival was expressed as a ratio of viable GFP-positive cells at 16 hours post-injection over 5 hours post-injection.

ROS measurement.

The redox-sensitive dye CM-H_2 DCFDA (Molecular Probes) was used to measure ROS levels in sympathetic neuronal cultures using the protocol described previously (Kirkland and Franklin 2001). CM-H_2 DCFDA is non-fluorescent when reduced and following permeabilizing of the cell is cleaved by esterases and is trapped in the cell where oxidation converts it to a fluorescent form. After treatment of cells with various experimental conditions, cells were incubated with 10 μM CM-H_2 DCFDA for 20 minutes followed rinsing 3 times in PBS. Fluorescent intensity of cells was represented as an image, and or quantitated by measuring pixel intensity in a 50 μm^2 area within individual soma using Metamorph software.

*Cytochrome c redox status***.**

The redox status of cytochrome *c* was assessed by monitoring the absorbance of horse cytochrome *c* (40 μM) at 550 nm on Nanodrop spectrophotometer. For experiments involving analysis of redox activity of cytochrome *c* following NGF deprivation, sympathetic neurons were plated at a density of 100,000 cells, and maintained in NGF, or deprived of NGF for 12 hours. Neuronal lysate was collected and incubated with reduced cytochrome *c* (reduced with 0.5 mM DTT) prior to analysis as described above.

*Reduced glutathione measurements***.**

Measurements of intracellular GSH were carried out according to a modification of the method of Tietze (Tietze 1969). Briefly, cultured cells were washed three times in PBS followed by lysis in 3% perchloric acid for 15 minutes at 4°C. After centrifugation,

supernatants were immediately measured for GSH content by assessing the colorimetric change of 600 μM 5,5'-dithiobis (nitrobenzoic acid) (DTNB) at 412 nm. GSH standards were prepared fresh for each experiment in 0.01N HCl. The perchloric acid pellet was resuspended in 1N NaOH, and the solubilized protein measured by Biorad Protein Assay with BSA as a standard. GSH was normalized to total cell protein.

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.

Acknowledgements

We thank Dr. Jeffery Rathmell, Dr. Eugene Johnson, and members of the Deshmukh Lab for helpful discussions and critical review of this manuscript. This work was supported by NIH grants NS42197 (to MD) and NS055486 (to AEV).

F. Figures and Legends

Figure 3.1. Endogenous cytochrome *c* release is incapable of inducing apoptosis in NGF-maintained sympathetic neurons. **A)** Cultures of MEFs or sympathetic neurons were injected with tBid-GFP, or GFP plasmid. Cell survival was quantified by cell morphology and expressed as a percentage of alive and healthy green cells at 24 hrs compared to 5 hrs post-injection. * represents no MEF survival. **B)** XIAP -/- sympathetic neurons or wildtype littermates were injected with EGFP or tBid-GFP, and cell survival was quantified as in (A). **C)** tBid-GFP was injected into sympathetic neurons, and allowed to express for 24 hrs. Cells were fixed, and the status of cytochrome *c* analyzed by immunofluorescence. **D)** XIAP-/- sympathetic neurons were either maintained in NGF-containing media, or deprived of NGF for 12 hrs, followed by injection with EGFP or tBid-GFP. Cell survival was quantified by cell morphology and expressed as a percentage of alive and healthy green cells at 16 hrs compared to 5 hrs post-injection. **E)** XIAP-/- sympathetic neurons were deprived of NGF for 12 hrs followed by injection with rhodamine alone, or cytochrome *c* protein along with rhodamine to mark injected cells. Cell survival was assessed at various time points following injection. Error bars represent ±SEM of three independent experiments.

Figure 3.1

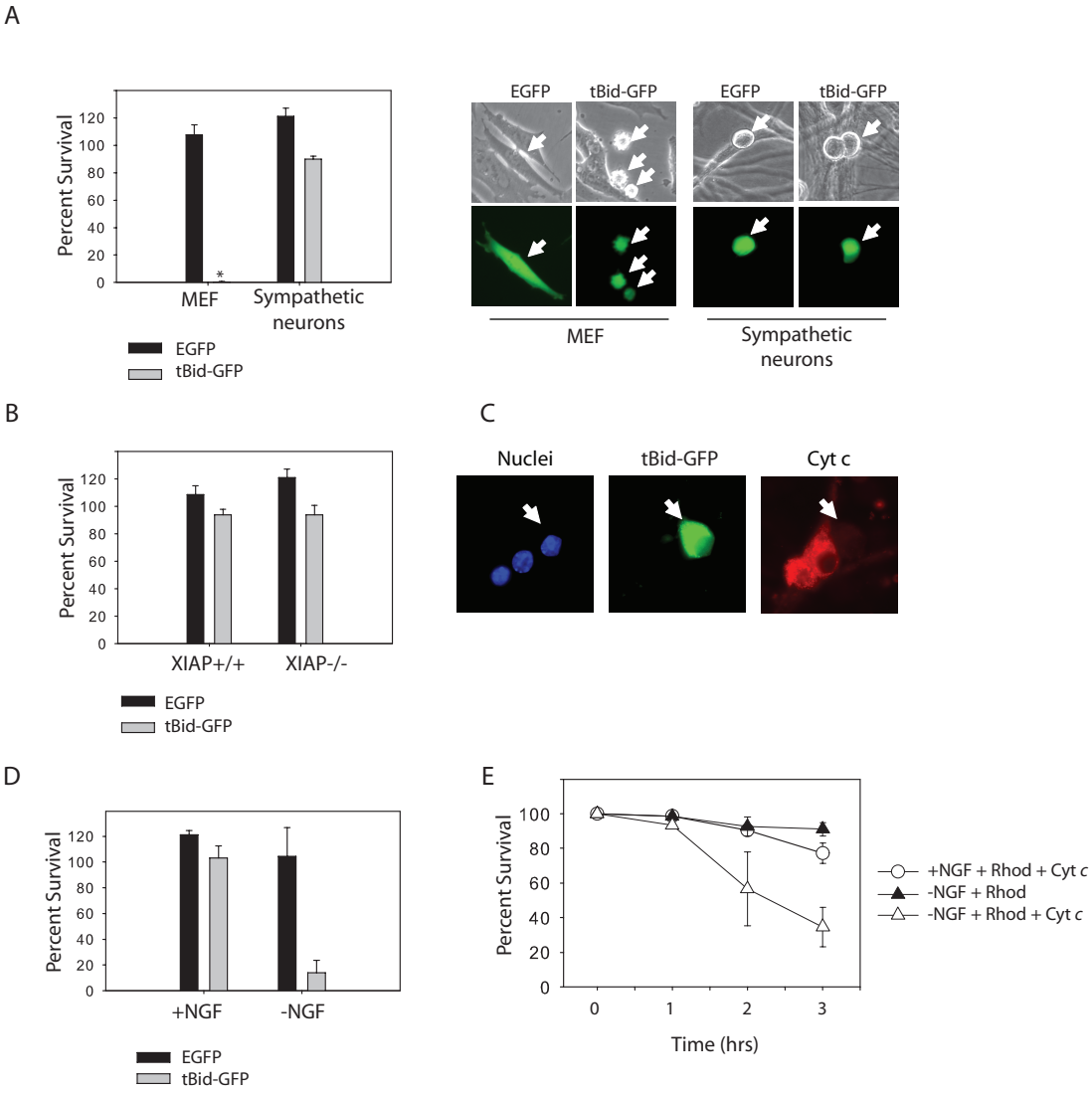

A
Percent Survival
120
100
80
60
40
20
0
*
MEF
Sympathetic neurons
EGFP
tBid-GFP
EGFP
tBid-GFP
EGFP
tBid-GFP
MEF
Sympathetic neurons
B
Percent Survival
120
100
80
60
40
20
0
XIAP+/+
XIAP-/-
EGFP
tBid-GFP
C
Nuclei
tBid-GFP
Cyt c
D
Percent Survival
120
100
80
60
40
20
0
+NGF
-NGF
EGFP
tBid-GFP
E
Percent Survival
100
80
60
40
20
0
0
1
2
3
Time (hrs)
+NGF + Rhod + Cyt c
-NGF + Rhod
-NGF + Rhod + Cyt c

Figure 3.2. Oxidation of cytochrome *c* increases its apoptotic activity. **A)** XIAP-/- sympathetic neurons were treated with 20-50 μM of H_2O_2 for 20 minutes, followed by injection with cytochrome *c* protein and rhodamine, or rhodamine dye alone. Cell survival was assessed at various time points following injection. **B)** XIAP-/- sympathetic neurons were treated with 10 mM GSH ethyl ester for 12 hrs, followed by injection of cytochrome *c* along with rhodamine, or rhodamine alone. Cell survival was assessed as in (A). **C)** Cytochrome *c* was incubated with 10 Units/ml of cytochrome *c* reductase (NADPH), followed by injection into sympathetic neurons (XIAP-/-). Cell survival was assessed at various time points following injection. **D)** XIAP-/- sympathetic neurons were injected with recombinant wildtype (WT) or N52I cytochrome *c* (4 μg/ul) and assessed for survival after 4 hrs. **E)** XIAP-/- sympathetic neurons were deprived of NGF for 12 hrs in the presence of 10mM GSH, followed by injection with EGFP or tBid-GFP constructs. Cell survival was quantified by cell morphology and expressed as a percentage of alive and healthy green cells at 16 hrs compared to 5 hrs post-injection. **F)** Reduced cytochrome *c* was added to neuronal extracts, and A_{550} was measured. Results are mean (±SEM) of three independent experiments.

Figure 3.2

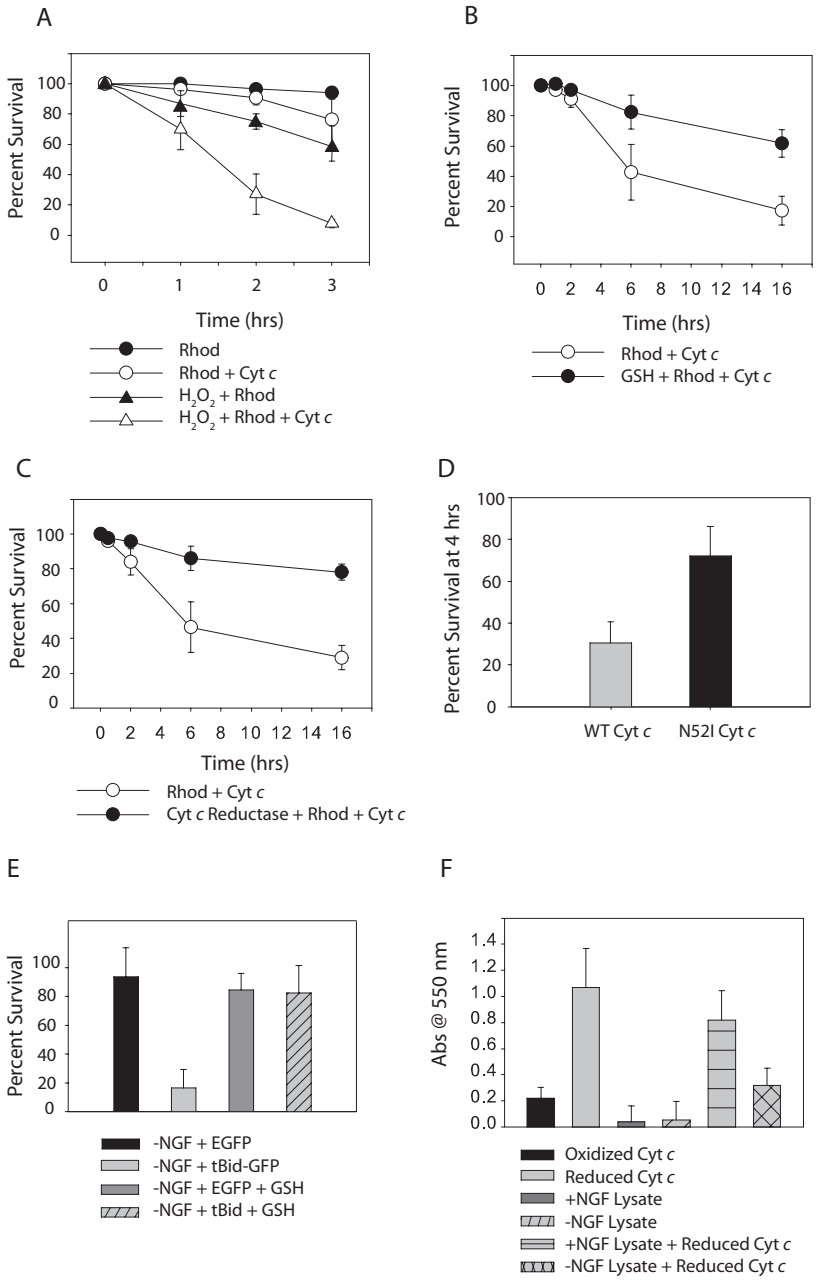

A
Percent Survival
Time (hrs)
Rhod
Rhod + Cyt c
H2O2 + Rhod
H2O2 + Rhod + Cyt c
B
Percent Survival
Time (hrs)
Rhod + Cyt c
GSH + Rhod + Cyt c
C
Percent Survival
Time (hrs)
Rhod + Cyt c
Cyt c Reductase + Rhod + Cyt c
D
Percent Survival at 4 hrs
WT Cyt c
N52I Cyt c
E
Percent Survival
-NGF + EGFP
-NGF + tBid-GFP
-NGF + EGFP + GSH
-NGF + tBid + GSH
F
Abs @ 550 nm
Oxidized Cyt c
Reduced Cyt c
+NGF Lysate
-NGF Lysate
+NGF Lysate + Reduced Cyt c
-NGF Lysate + Reduced Cyt c

Figure 3.3. Role of the glycolytic pentose phosphate shunt in cytochrome *c*-mediated apoptosis. **A)** Average ROS levels in sympathetic neurons were observed by fluorescence intensity of the redox-sensitive dye CM-H_2DCFDA following 30 min of GSH depletion with 0.1 mM DEM. **B)** XIAP-/- sympathetic neurons were treated with 0.1 mM DEM for 30 min followed by injection of cytochrome *c* and rhodamine, or rhodamine dye alone. **C)** Average ROS levels were observed as in (A) following inhibition of the Pentose Phosphate Pathway with 200 μM DHEA for 24 hrs. **D)** XIAP -/- sympathetic neurons were treated with 200 μM DHEA. After 6 hrs, neurons were injected with tBid-GFP or EGFP constructs alone. Cell survival was expressed as a percentage of healthy green cells at 16 hrs compared to 5 hrs post-injection. Error bars represent ±SEM.

Figure 3.3

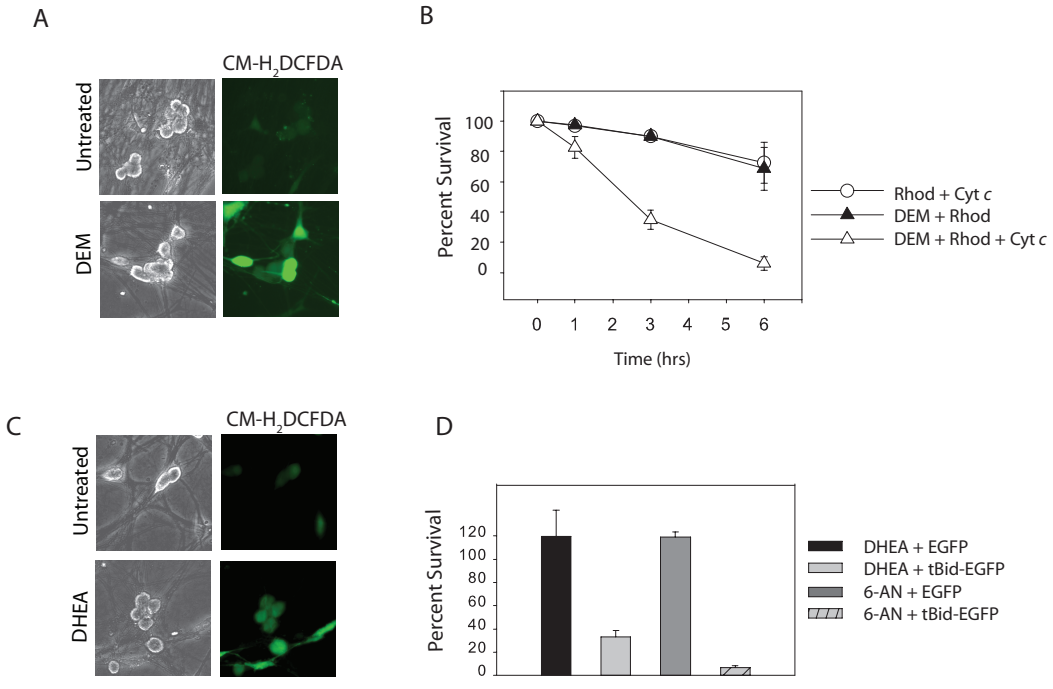
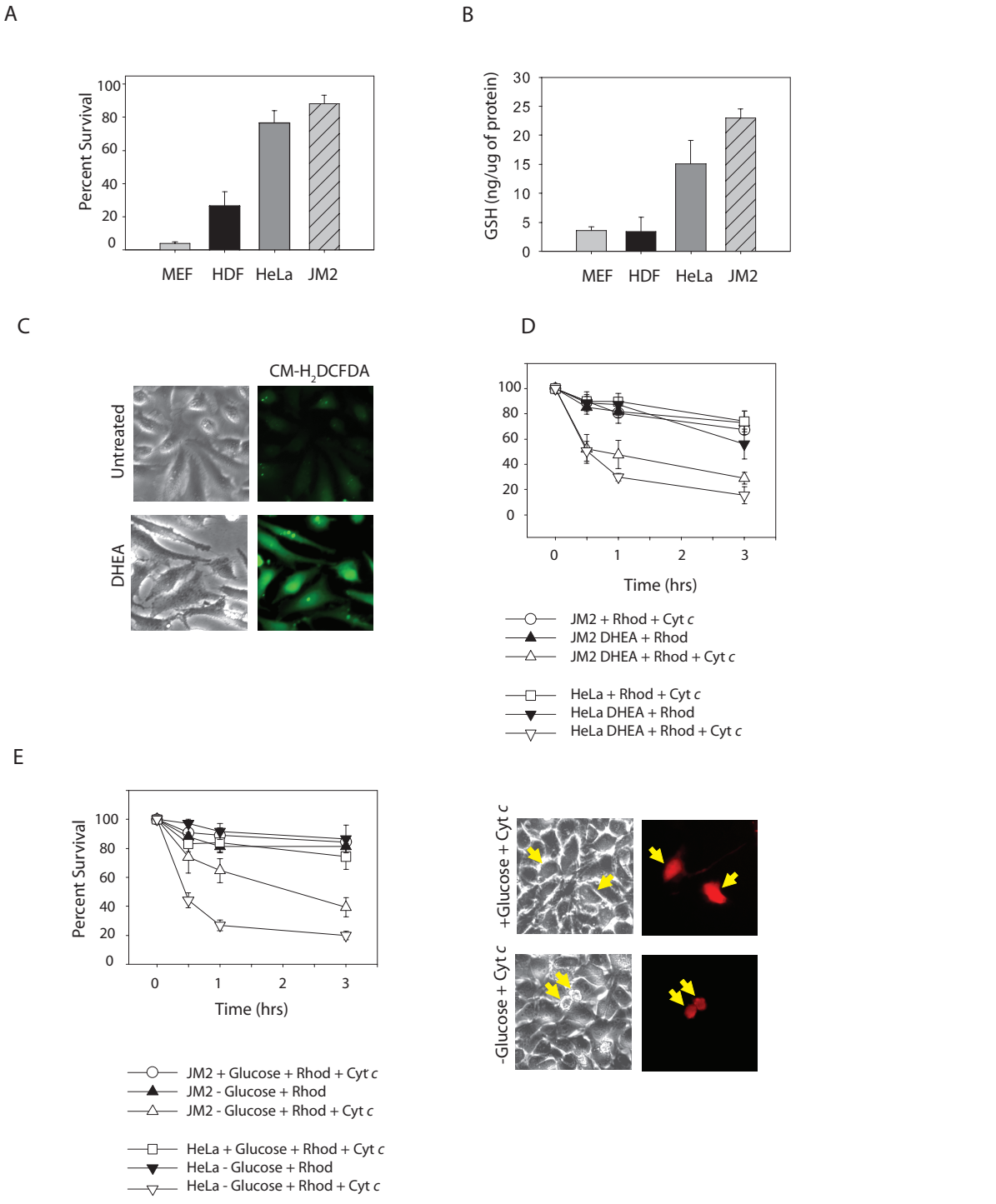

A
CM-H2DCFDA
Untreated
DEM
B
Percent Survival
100
80
60
40
20
0
0 1 2 3 4 5 6
Time (hrs)
Rhod + Cyt c
DEM + Rhod
DEM + Rhod + Cyt c
C
CM-H2DCFDA
Untreated
DHEA
D
Percent Survival
120
100
80
60
40
20
0
DHEA + EGFP
DHEA + tBid-EGFP
6-AN + EGFP
6-AN + tBid-EGFP

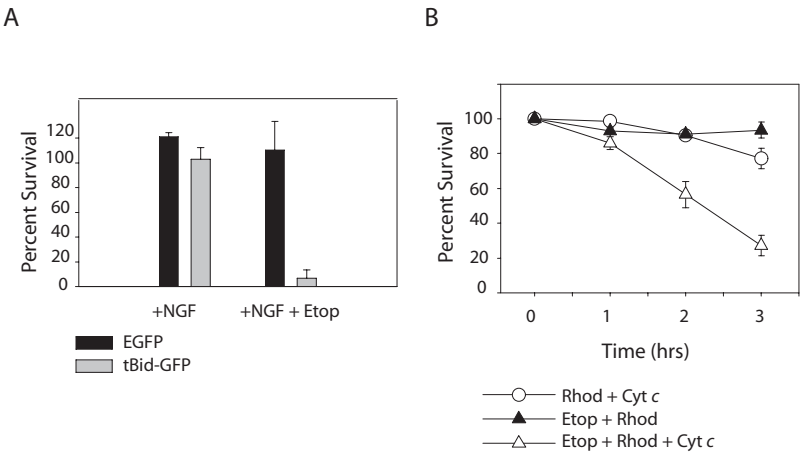
Figure 3.4. Cancer cells exhibit glycolysis-meditated protection from cytochrome *c*. **A)** MEFs, HDFs or various cancer cell lines were injected with cytochrome *c* and SMAC protein and cell survival was assessed after 30 minutes. **B)** GSH content was measured in normal mitotic cells (MEFs, HDFs) as well as cancer cell lines. GSH content is expressed as a ratio of GSH to total cellular protein. **C)** Average ROS levels in HeLa cells measured by fluorescence of CM-H_2DCFDA in the absence or presence of the pentose phosphate pathway inhibitor, DHEA (200 μM) for 6 hrs. **D)** The pentose phosphate pathway was inhibited with 200 μM DHEA in JM2 as well as HeLa cells for 6 hrs by addition of 200 μM DHEA, followed by injection of cytochrome *c* and SMAC protein. Cell survival was assessed at various time points. **E)** JM2 and HeLa cells were deprived of glucose for 16 hrs followed by injection with cytochrome *c* and SMAC, and assessed for survival at various time points. Images are representative of JM2 cells at 3 hrs following cytochrome *c* injection. Error bars represent ±SEM.

Figure 3.4


A
Percent Survival
100
80
60
40
20
0
MEF
HDF
HeLa
JM2
B
GSH (ng/ug of protein)
30
25
20
15
10
5
0
MEF
HDF
HeLa
JM2
C
CM-H2DCFDA
Untreated
DHEA
D
100
80
60
40
20
0
0
1
2
3
Time (hrs)
JM2 + Rhod + Cyt c
JM2 DHEA + Rhod
JM2 DHEA + Rhod + Cyt c
HeLa + Rhod + Cyt c
HeLa DHEA + Rhod
HeLa DHEA + Rhod + Cyt c
E
Percent Survival
100
80
60
40
20
0
0
1
2
3
Time (hrs)
JM2 + Glucose + Rhod + Cyt c
JM2 - Glucose + Rhod
JM2 - Glucose + Rhod + Cyt c
HeLa + Glucose + Rhod + Cyt c
HeLa - Glucose + Rhod
HeLa - Glucose + Rhod + Cyt c
+Glucose + Cyt c
-Glucose + Cyt c

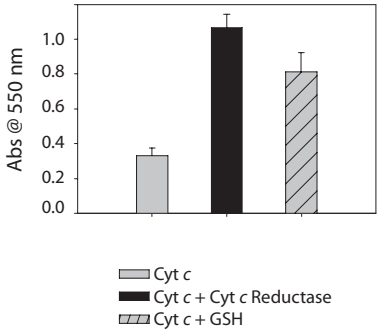
Supplemental Figure 3.1. DNA damage sensitizes neurons to cytochrome *c*-mediated apoptosis. **A)** XIAP-/- sympathetic neurons were treated with 20 μM etoposide for 12 hrs, followed by injection of tBid-GFP or EGFP plasmids. Cell survival was assessed by morphology by quantifying the number of viable GFP positive cells at 16 hrs compared to 5 hrs post-injection. **B)** XIAP-/- sympathetic neurons were treated with 20 μM etoposide for 12 hrs followed by injection with cytochrome *c* and rhodamine, or rhodamine alone. Cell survival was assessed at multiple time points.

Supplemental Figure 3.1



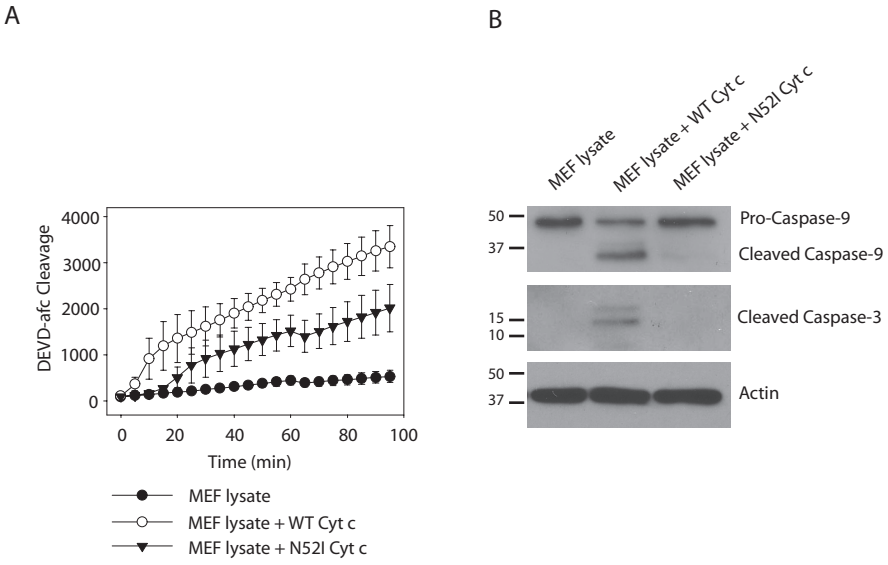
Supplemental Figure 3.2. GSH and cytochrome *c* reductase convert oxidized cytochrome *c* to reduced cytochrome *c*. 40 μM bovine cytochrome *c* was incubated with either 10 mM glutathione ethyl ester or 10 units/ml cytochrome *c* reductase (NADPH) for 15 minutes, followed by measurement at Abs_{550}.

Supplemental Figure 3.2



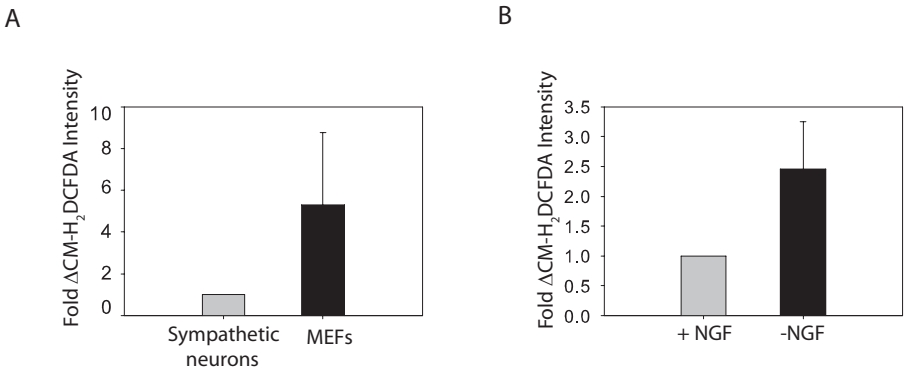
Supplemental Figure 3.3. N52I Cytochrome *c* has diminished ability to activate caspases *in vitro*. **A)** Cytosolic extracts from MEFs were assessed for caspase activation after the addition of Wildtype or N52I mutant cytochrome *c* (2.5 μM) by measuring the cleavage of the fluorogenic substrate, DEVD-afc. **B)** Western blot showing the status of caspase-9 and caspase-3 cleavage in cytosolic lysate after the addition of cytochrome *c* as described in (A). Actin is shown as loading control.

Supplemental Figure 3.3



Supplemental Figure 3.4. ROS levels in MEFs, and sympathetic neurons after NGF deprivation. **A)** MEFs were co-cultured with sympathetic neurons, followed by incubation with 10 μM CM-H_2DCFDA redox-sensitive dye. Fluorescent intensity was measured in a 50 μm^2 area within individual soma and quantified using Metamorph software. **B)** XIAP-/- sympathetic neurons were maintained in NGF, or deprived of NGF for 24 hrs, followed by incubation with CM-H_2DCFDA. Fluorescent intensity was quantified as in (A).

Supplemental Figure 3.4



IV. CHAPTER FOUR:

Age Dependent Changes in the Regulation of the Apoptosome in Neurons

A. Abstract

Our data in PC12 cells and primary sympathetic neurons suggests that as mitotic cells become postmitotically differentiated, they more stringently regulate caspase activation and apoptosis. To examine whether this phenomenon extends to neurons of the developing brain, we examined whether the cerebellum exhibits age-dependent changes in the regulation of cytochrome *c* induced apoptosis. Here, we show that cytochrome *c* was sufficient to activate caspases in extracts from postnatal day five (P5) but not P13 and P19 cerebellum. The resistance of mature P19 cerebellar neurons to cytochrome *c* correlated with a dramatic reduction in the levels of all three apoptotic mediators, Apaf-1, caspase-9 and caspase-3. In contrast, P13 cerebellar neurons exhibited remarkable resistance to cytochrome *c* despite maintaining high levels of these proapoptotic proteins. Complementation experiments identified a specific and marked defect in Apaf-1 activity in P13 extracts. Interestingly, the activity of Apaf-1 in the developing cerebellum correlated with its phosphorylation, while inactive Apaf-1 in P13 brain remained unphosphorylated.

A stricter control of apoptosis in neurons as they mature is arguably beneficial for these postmitotic cells to survive the lifetime of the organism. Our data define three specific stages in which developing neurons exhibit an age-dependent change in the regulation of apoptosis and identify a potentially novel mechanism by which Apaf-1 function is regulated in maturing neurons.

B. Introduction

A clear understanding of how the apoptotic pathway is regulated is of particular importance in the nervous system. During development, neuronal elimination is crucial to ensure the proper setup of the nervous system, however, aberrant loss of neurons can result in neurodegenerative disease.

A critical trigger of the initrinsic pathway of apoptosis is the release of cytochrome *c* from the mitochondria. Once in the cytosol, cytochrome *c* binds to and induces a conformational change in the protein, Apaf-1, causing it to oligimerize. Oligimerized Apaf-1 then recruits and activates caspase-9, which can then go on to activate the executioner caspases of apoptosis such as caspase-3 and caspase-7.

These major components of the apoptotic pathway have been well characterized in mitotic cells and biochemical assays. However, recent work by our lab has suggested that sympathetic neurons more strictly regulate the apoptotic pathway than do mitotic cells. For example, addition of cytochrome *c* (by either microinjection or addition to cytosolic extracts) to the cytosol of mitotic cells such as fibroblasts results in rapid caspase activation and apoptosis (Liu, Kim et al. 1996; Li, Srinivasan et al. 1997; Brustugun, Fladmark et al. 1998; Juin, Hueber et al. 1999; Wright, Linhoff et al. 2004). Whereas, microinjection of cytochrome *c* into the cytosol of differentiated sympathetic neurons is unable to initiate cell death (Deshmukh and Johnson 1998; Wright, Linhoff et al. 2004). Here, we examine whether other postmitotic neurons, particularly those of the central nervous system (CNS), acquire similar brakes on the cytochrome *c*-mediated apoptotic pathway, and if so, by what mechanisms.

Cerebellar granule neurons (CGNs) are the most abundant neurons in the brain, comprising greater than 90% of cells in the cerebellum (Contestabile 2002). CGN neurogenesis occurs largely postnatally. In the mouse, CGNs lie in the external granule layer, and proliferate there during the first week following birth. By the second postnatal week, they stop dividing, and migrate towards the inner graule layer of the cerebellum and differentiate (Hatten 1999).

Apoptosis of cerebellar granule neurons occurs during the development of the cerebellum. Although the exact stimulus that triggers this cell death is not well understood, these cells die in a caspase-dependent manner (Contestabile 2002). In addition, caspase-dependent apoptosis of CGNs is also seen as a result of various pathological stimuli (Ferrer 1999; Tanaka, Momoi et al. 2000; Puig, Tortosa et al. 2001). Here, we describe a novel mechanism by which neurons of the developing cerebellum becoming increasingly resistant to cytochrome *c*-mediated caspase activation as they differentiate.

C. Results

1. Cytochrome *c* is sufficient to induce caspase activation in P5 (mitotic), but not in P13 (postmitotic) cerebellar extracts

To determine whether neurons of the CNS become resistant to cytochrome *c*-mediated apoptosis as they differentiate, cytosolic extracts were made from the cerebellum of mice at different stages of development and their ability to activate caspases as measured by the cleavage of the fluorogenic caspase substrate, DEVD-afc, with the addition of exogenous cytochrome *c* was assessed. Indeed, we found that postnatal day five (P5) lysates readily activated caspases, while lysates made in a similar manner from the cerebellums of postnatal

day 13 (P13) and postnatal day 19 (P19) animals were unable to activate caspases upon the addition of cytochrome *c* (Fig. 4.1A). Western analysis confirms this finding. P5 lysates show potent caspase-9 as well as caspase-3 processing, while P13 and P19 lysates exhibit neither processed caspase-9 or caspase-3 after cytochrome *c* addition (Fig. 4.1B). While these extracts were made from whole cerebellum, it is likely that these results most accurately represent the properties of CGNs, which make up over ninety percent of the neurons in the cerebellum. To examine this directly, cytosolic lysates were made from CGNs isolated from P4 animals and left in culture for one day (equivalent of P5), or from CGNs cultured for nine days (P13 equivalent). CGNs cultured for one day were competent to activate caspases upon the addition of cytochrome *c,* however CGNs equivalent of P13 were markedly resistant to cytochrome *c* (Fig. 4.1C). Together, this data suggests that as cerebellar granule neurons differentiate and become postmitotic, they acquire a resistance to cytochrome *c*-mediated apoptosis.

2. The inability of cytochrome *c* to induce apoptosis in P13 cerebellar extracts is not due to regulation by IAPs

Previously, our lab has shown that postmitotic cells such as sympathetic neurons and cardiomocytes are resistant to cytochrome *c*-mediated caspase activation due to strict inhibition of caspases by the X-linked inhibitor of apoptosis protein (XIAP) (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). To examine whether IAPs play a role in mediating the resistance of P13 cerebellum to cytochrome *c,* P13 cerebellar extracts were incubated with cytochrome *c* along with the IAP inhibitor SMAC. Surprisingly, even in the presence of SMAC, where IAPs are inhibited, cytochrome *c* was unable to induce caspase activation in

P13 lysates (Fig 4.2A). To confirm this, these extracts were analyzed by Western blot for caspase activation. Even with the addition of excess SMAC, neither caspase-9 or caspase-3 was processed in P13 cerebellar extracts treated with cytochrome *c* (Fig 4.2B). In addition, lysates made from P19 animals are also unable to activate caspases with cytochrome *c* and SMAC. Although these results suggest that XIAP is not important for the restriction of apoptosis in cerebellar neurons, cerebellar lysates made from XIAP deficient mice from a slightly earlier age (P10) exhibited an increase in caspase activation compared to wildtype littermates (Fig. 4.2C). These data show that while XIAP mediates resistance in P10 cerebellar neurons, as these neurons mature (P13 and older), they develop an IAP-independent mechanism of resistance to cytochrome *c*.

3. P13 cerebellar neurons exhibit a specific defect in Apaf-1 activity, despite maintaining high levels of Apaf-1 protein

We then asked whether the inability of the differentiating cerebellum (P13) to undergo apoptosis in response to cytochrome *c* was due to a defect in expression of one of the components of the apoptosome. Despite the difference in apoptotic activity, we found that all components necessary for apoptosome activity (Apaf-1, caspase-9, and caspase-3) were present to similar levels in both P5 and P13 cerebellar lysates (Fig 4.3A).

Since the IAP family of caspase inhibitors was not responsible for resistance of P13 lysates to cytochrome *c*, we asked whether this resistance was mediated by an as yet unidentified inhibitor of apoptosome formation of caspase activation. If this were the case, we would expect that mixing activateable P5 lysates with lysate from P13 would inhibit the overall activity of the P5 lysate. Surprisingly, a mixture of P5 and P13 cerebellar lysate

activated quite readily, arguing against the presence of a potent inhibitor of caspase activation in P13 lysates (Fig 4.3B).

In contrast, by P19, the levels of Apaf-1 and caspase-3 were markedly reduced (Fig. 4.3A). This suggests that the resistance of P19 extracts to cytochrome *c* was likely due to the absence of Apaf-1 and caspase-3.

We next examined whether the resistance of P13 cerebellar extracts to cytochrome *c* was due to a functional defect in Apaf-1, caspase-9, or caspase-3. S-100 Cytosolic lysates were prepared from mouse embryonic fibroblasts (MEFs) isolated from wildtype mice or mice deficient in Apaf-1, caspase-9, or caspase-3, and assessed for their ability to complement the defect in P13 cerebellar extracts. Addition of caspase-9-/- or caspase-3-/- MEF extracts (which have no independent ability to cleave DEVD-afc upon the addition of cytochrome c) fully complemented the P13 cerebellar extracts and allowed cytochrome *c* to activate caspases, indicating that there was no defect in caspase-9 or caspase-3 in P13 cerebellar neurons (Fig. 4.3C). In contrast, Apaf-1-/- MEF extracts failed to complement the P13 cerebellar extracts. These results indicate that while P13 cerebellar extracts maintain levels of Apaf-1 protein, this protein is nonfunctional. Indeed, we found that the simple addition of recombinant Apaf-1 protein (human Apaf-1; kind gift of Dr. Xiaodong Wang, UT Southwestern) fully restored cytochrome *c*-mediated caspase activation to P13 cerebellar extracts (Fig. 4.3D,E).

Addition of Apaf-1 protein alone to P19 cerebellar extracts was not sufficient to induce activation of caspases upon the addition of cytochrome *c* (Fig. 4.3D,E), presumably since these extracts still lack caspase-3.

Together, these data show that cerebellar neurons go though multiple, distinct events in which cytochrome *c*-mediated caspase activation becomes increasingly restricted with maturation (Fig. 4.6). In particular, our data point to a novel, posttranslational mechanism by which Apaf-1 activity is inhibited in P13 cerebellar neurons

4. Apaf-1 phosphorylation occurs in P5 but not P13 cerebellar neurons

The inability of Apaf-1 to function in P13 cerebellar lysates could be due to an inability of cytochrome *c* to bind Apaf-1, or a defect in the recruitment of caspase-9 to Apaf-1. To examine this more closely, either His-tagged cytochrome *c* protein, or GST tagged CARD domain of caspase-9 was incubated with P5 or P13 lysates. Cytochrome c binding to Apaf-1 occurs via Apaf-1's C-terminal WD-40 repeat domain, whereas caspase-9 and Apaf-1 interact via a CARD/CARD interaction (Fig 4.4A). The ability of endogenous Apaf-1 to bind either His-Cyt c or GST-Casp-9-CARD was then analyzed by Western blot. While cytochrome *c* appeared to bind Apaf-1 equally in both P5 and P13 lysates, the ability of caspase-9 to interact with Apaf-1 was diminished in P13 lysates (Fig. 4.5A).

Although no role for phosphorylated Apaf-1, nor a specific phsophorylation site on this protein has been identified, recent data suggests that Apaf-1 can become phosphorylated in BCR-Abl overexpressing cells, or by overexpression of PKA (Deming, Schafer et al. 2004; Martin, Allan et al. 2005). To examine whether phosphorylation of Apaf-1 affects its activity in the developing cerebellum, we examined the phosphorylation status of Apaf-1 in P5 and P13 lysates. Due to the large size of Apaf-1 and the difficulty in immunoprecipitating this protein, we constructed a GST tagged domain of Apaf-1 deleted for the WD-40 repeat region. This GST-Apaf (aa 1-543) retains the caspase-9 binding CARD domain, as well as

the Nucleotide Binding Domain (Fig 4.4B). Purified Apaf-1 GST (aa 1-543) protein was then incubated with either P5 or P13 cerebellar lysate in the presence of [γ-^{32}P]ATP and analyzed by autoradiography. Interestingly, P5, but not P13 was able to phosphorylate GST-Apaf-1 (aa 1-543) (Fig 4.5B). Although only correlative, this data suggests that phosphorylation of Apaf-1 may be necessary for its activity, and that as cerebellar neurons differentiate, phosphorylation of Apaf-1 is lost to ensure a stricter regulation of apoptosis.

D. Discussion

In this study, we have examined whether differentiating neurons of the CNS acquire an increased regulation of the apoptotic pathway. Indeed, we find that just as postmitotic sympathetic neurons are resistant to cytochrome *c*, differentiating granule neurons develop an inability to undergo cytochrome *c*-mediated apoptosis. In neurons of the developing cerebellum, we identify four distinct stages of apoptotic regulation (Fig 4.6). 1) At an early developmental age (P5), apoptosome components are maintained at high levels, Apaf-1 is in a phosphorylated state, and cerebellar neurons are completely sensitive to cytochrome *c*. 2) At P10, apoptosome components are still highly expressed, but neurons have a reduced sensitivity to cytochrome *c* that is mediated, in part, by XIAP's inhibition of caspases. 3) As neurons differentiate and begin to migrate toward the inner granule layer (P13) they lose the ability to undergo apoptosis induced by cytochrome *c*. Expression of apoptosome components are still high, however, Apaf-1 is no longer functional, perhaps due to its dephosphorylation. 4) Mature neurons of the cerebellum (P19) are completely resistant to cytochrome *c* and have severely reduced Apaf-1 and caspase-3 levels.

We were intrigued to find that the inhibition of cytochrome *c*-mediated apoptosis in cerebellar neurons was a result of posttranslational inactivation of Apaf-1. Our lab has extensively studied the regulation of the apoptotic pathway in postmitotic sympathetic neurons as well as cardiomyocytes. In these cells, cytochrome *c*-mediated apoptosis is inhibited by the coordinated suppression of caspase activation by XIAP and low Apaf-1 levels. In mitotic cells and immature sympathetic neurons, Apaf-1 levels are high, overwhelming XIAP's ability to inhibit caspase activation and apoptosis. In postmitotic sympathetic neurons as well as cardiomyoctyes, however, the levels of Apaf-1 are extremely low, and XIAP becomes a critical inhibitor of caspase activation and cell death (Fig 1.2), (Wright and Deshmukh 2006; Vaughn and Deshmukh 2007). The data from this study, however, suggests that not all postmitotic neurons regulate cytochrome *c*-mediated caspase activation by similar mechanisms. In CGNs, the transient role that XIAP plays to inhibit caspase activation (P10 cerebellum) (Fig. 4.2), occurs ar a stage when Apaf-1 levels are quite high. It is likely that the ability of XIAP to affectively inhibit caspase activation at this stage is because Apaf-1 begins to be inactivated by dephosphorylation.

Interestingly, by P13, cerebellar lysates were unable to be activated by cytochrome *c* even when XIAP activity was inhibited. Despite this, Apaf-1 levels remain high in P13 cerebellar neurons. Our data suggests that cerebellar neurons can regulate apoptosis by a posttranslational modification in Apaf-1 that inhibits the ability of Apaf-1 to interact with caspase-9. Although the exact mechanism by which Apaf-1 is inhibited in P13 cerebellum is still unclear, the phosphorylation status of Apaf-1 directly correlates with its activity in the developing cerebellum.

At later ages in the mature cerebellum, the apoptotic pathway becomes further restricted by multiple means. By almost three weeks after birth, the addition of recombinant Apaf-1 protein is no longer able to restore activity to these lysates, and levels of Apaf-1 and caspase-3 are virtually undetectable (Fig 4.3D,E). While our lab and others have shown that levels of apoptosome components are reduced with neuronal maturation (Yakovlev, Ota et al. 2001; Donovan and Cotter 2002; Wright, Linhoff et al. 2004), here, we uncover multiple and distinct stages by which developing neurons confer resistance to apoptosis.

These multiple stages of increasing inhibition on the apoptotic pathway could be advantageous to ensure the proper developoment of the cerebellum. During the first postnatal week, mitotic neuronal precursors have no need to strictly inhibit the apoptotic pathway as they are plentiful and have regenerative potential. As these neurons begin to differentiate, however, they become more cautious about undergoing apoptosis. Posttranslational inhibition of Apaf-1 is an efficient mechanism by which neurons can quickly restrict the apoptotic pathway, or easily reverse this inhibition in order to remove a damaged or misdirected cell. Once a neuron has matured, it downregulates the expression of multiple apoptotic components. It's abilty to strictly inhibit apoptosis is crucial at this age, as these neurons have now established elaborate neural connections, and cannot be readily replaced.

E. Materials and Methods

Reagents

All reagents were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise stated. [γ-^{32}P]ATP was purchased from Perkin Elmer. Recombinant

SMAC protein was purified from bacteria as described previously (Potts 2003). Apaf-1 protein was a kind gift of Dr. Xiaodong Wang (UT Southwestern, Dallas). ICR outbreed mice (Harlan Sprague Dawley) were used for all experiments except those involving Apaf-1, caspase-3, XIAP, and caspase-9 deficient cells. For these the background is C57BL/6. Apaf-1 deficient mice were generated by Dr.Joachim Herz (UT Southwestern) and were kindly provided by the laboratory of Dr. Susan Ackerman (Jackson Laboratories).

Preparation of Cytosolic Extracts

For extracts from whole cerebellum, cerebellum from various aged ICR mice were dissected, minced, trypsinized, and dissociated. Cells were passed through a cell filter, washed in PBS and resuspended in hypotonic lysis buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 0.5 mM EDTA, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT) and rotated at 4 degrees for 20 minutes, followed by passage through a p200 pipette tip to lyse cells. S100 lysates were prepared by centrifugation at 16,000 x g to remove nuclei and heavy membrane fractions. The supernatant was then centrifuged for 30 min at 100,000 x g to remove the light membrane fraction. For cultured cells, cells were washed 3 x in PBS, and cells collected by scraping. The cell pellet was lysed as above only passaged through a 25-gauge needle. Centrifugation was done as described above, and the resulting supernate was used for caspase activation assays.

In vitro caspase activation assays were performed by incubating equal protein (50 ug) of cytosolic lysate with 1mM dATP and 10 uM cytochrome *c* in oligimerization buffer (25 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM KCl, 1.5 mM MgCl2, 10% glycerol, 1mM DTT) for 15 minutes at 37 degrees. The caspase substrate, DEVD-afc was then added at a

concentration of 50 uM to bring the total volume up to 100 ul. Fluorescent intensity was measured over time on a Fluoroskan fluorescent plate reader.

Granule Neuron Cultures

Primary cultures of Cerebellar Granule Neurons were obtained as described previously (Miller and Johnson 1996). Briefly, postnatal day 5 (P5) cerebellar were dissected triturated, and plated into 0.1 mg/ml poly-L-lysine coated 60 mm dishes containin K25 + S medium at density of four million cells per plate. After 24 hours, neurons were treated with 100 nM AraC to reduce the accumulation of non-neuronal cells. These cultures were maintained for 1 day or 12 days in culture before preparation of cytosolic extracts.

Western Blot Analysis

To assess the endogenous levels of proteins throughout cerebellum development, Western analysis was performed on untreated cerebellar. To assess caspase processing, cytochrome *c* alone or along with SMAC or Apaf-1 protein was incubated with lysates. The following antibodies were used; anti-Apaf-1 (13F11; Alexis Biochemicals), anti-LDH (Rockland), anti-active caspase-3 (Cell Signaling), anti-active caspase-9 (Cell signaling). Anti-rabbit/rat HRP-conjugated secondary antibodies were purchased from Pierce Chemical Co. Western blots were developed with ECL-plus reagents (Amersham Biosciences).

Phosphorylation Assay

GST-Apaf-1 (aa 1-543) attached to glutathione sepharose was incubated with 0.5 mg cytosolic lysate from either P5 or P13 cerebellum in the presence of 15 mM MgCl2, 10 mM

cold ATP and 0.01 mCi [γ-^{32}P]ATP in a volume of 100 ul for one hour at 30°C. Beads were washed 5 x in RIPA + 500 mM NaCl. Beads were then boiled in SDS sample buffer, and supernate was run on an 8% SDS PAGE gel and analyzed by scanning onto the Typoon phosphoimager.

Acknowledgements

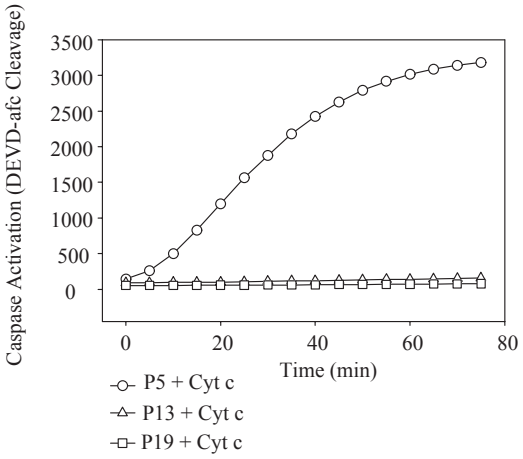
We kindly thank Dr. Xiaodong Wang for providing the Apaf-1 recombinant protein, and Dr. Sally Kornbluth's lab for their help with the initial phosphorylation assays and for providing the GST-Apaf (aa 1-543) plasmid.

F. Figures and Legends

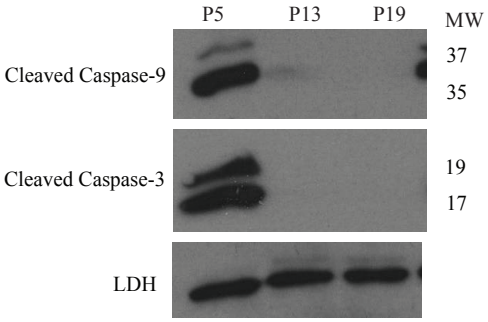
Figure 4.1. Cytochrome *c* is sufficient to induce caspase activation in P5 (mitotic), but not in P13 (postmitotic) cerebellar extracts. **A)** S100 extracts were made from the cerebellum of P5, P13, or P19 widltype mice. The ability of activated caspases to cleave DEVD-afc was measured over time following the addition of 10 uM bovine cytochrome *c*. **B)** Lysates activated in (A) were analyzed by Western analysis with antibodies detecting activated caspase-9, activated caspase-3, and LDH as a loading control. **C)** Cerebellar granule neurons were cultured from P4 mice for either 24 hours (P5 equivalent), or for nine days (P13 equivalent). Neurons were then collected and made into S100 extract. The ability of these extracts to activate caspases was measured by DEVD-afc cleavage after the addition of 10 uM cytochrome *c*.

Figure 4.1

A



B



C

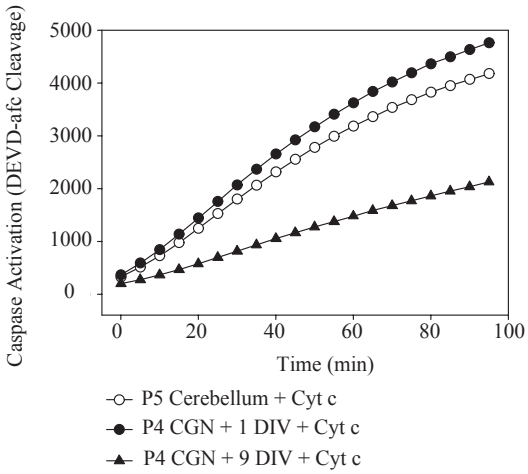


Figure 4.2. The inability of cytochrome *c* to induce apoptosis in P13 cerebellar extracts is not due to regulation by IAPs. **A)** Cerebellar extracts from P5 and P13 mice were examined for their ability to activate caspases (cleave DEVD-afc) in the presence of cytochrome *c* (10 uM) and the IAP inhibitor, SMAC (4 mg/ml). **B)** Lysates from (A) were analyzed or Caspase-9 and Caspase-3 cleavage by Western blot. LDH serves as a loading control. **C)** Extracts were made from the cerebellum of either XIAP-/- mice, or their wildtype littermates at P10. Their ability to activate caspases was measured following addition of cytochrome *c*.

Figure 4.2

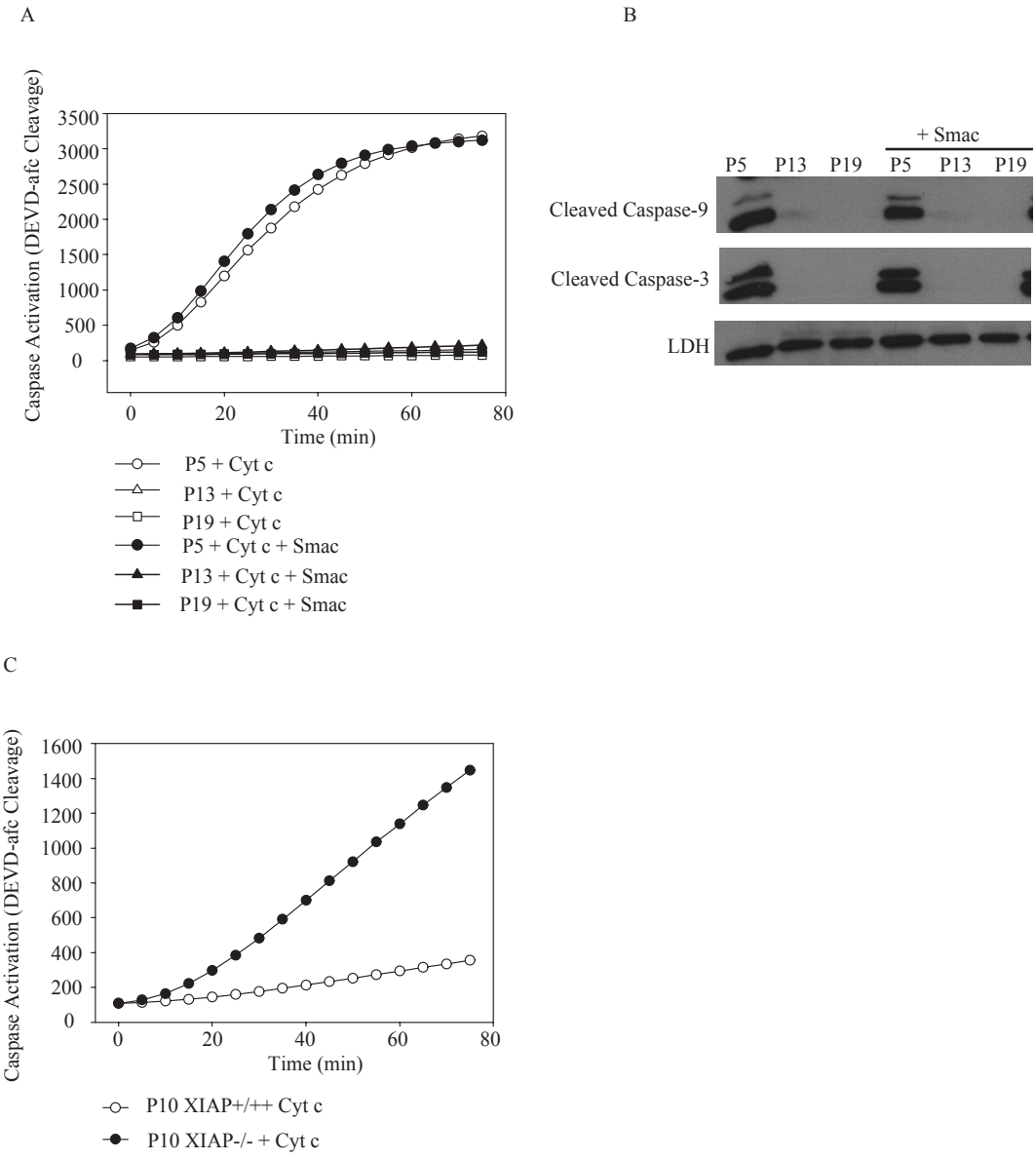

A
Caspase Activation (DEVD-afc Cleavage)
3500
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
Time (min)
P5 + Cyt c
P13 + Cyt c
P19 + Cyt c
P5 + Cyt c + Smac
P13 + Cyt c + Smac
P19 + Cyt c + Smac
B
+ Smac
P5
P13
P19
P5
P13
P19
Cleaved Caspase-9
Cleaved Caspase-3
LDH
C
Caspase Activation (DEVD-afc Cleavage)
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
Time (min)
P10 XIAP+/++ Cyt c
P10 XIAP-/- + Cyt c

Figure 4.3. P13 Cerebellar Neurons Exhibit a Specific Defect in Apaf-1 Activity, Despite maintaining High Levels of Apaf-1 Protein. **A)** Cerebellar extracts were made from P5, P13 and P19 cerebellum, and the levels of the apoptosome components were examined by Western analysis. LDH is shown as a loading control. **B)** Cerebellar extracts from P5 and P13 were mixed, and the ability of cytochrome *c* to activate caspases was measured by caspase activation assay. **C)** S100 cytosolic extracts were made from cultures of wildtype, caspase-3-/-, caspase-9-/- and Apaf-/- MEFs. The ability of these extracts to complement the defect in P13 cerebellar extracts was examined by incubating MEF lysate (50 ug) with P13 lysate (50ug) in the presence of cytochrome *c.* Caspase activation was measured as described for the caspase activation assay. **D)** Recombinant Apaf-1 (20 nM) was incubated with P13 or P19 cerebellar lysate in the presence of cytochrome *c,* followed by quantitation of caspase activation. **E)** Lysates from (C) were analyzed for caspase activation by Western blot.

Figure 4.3

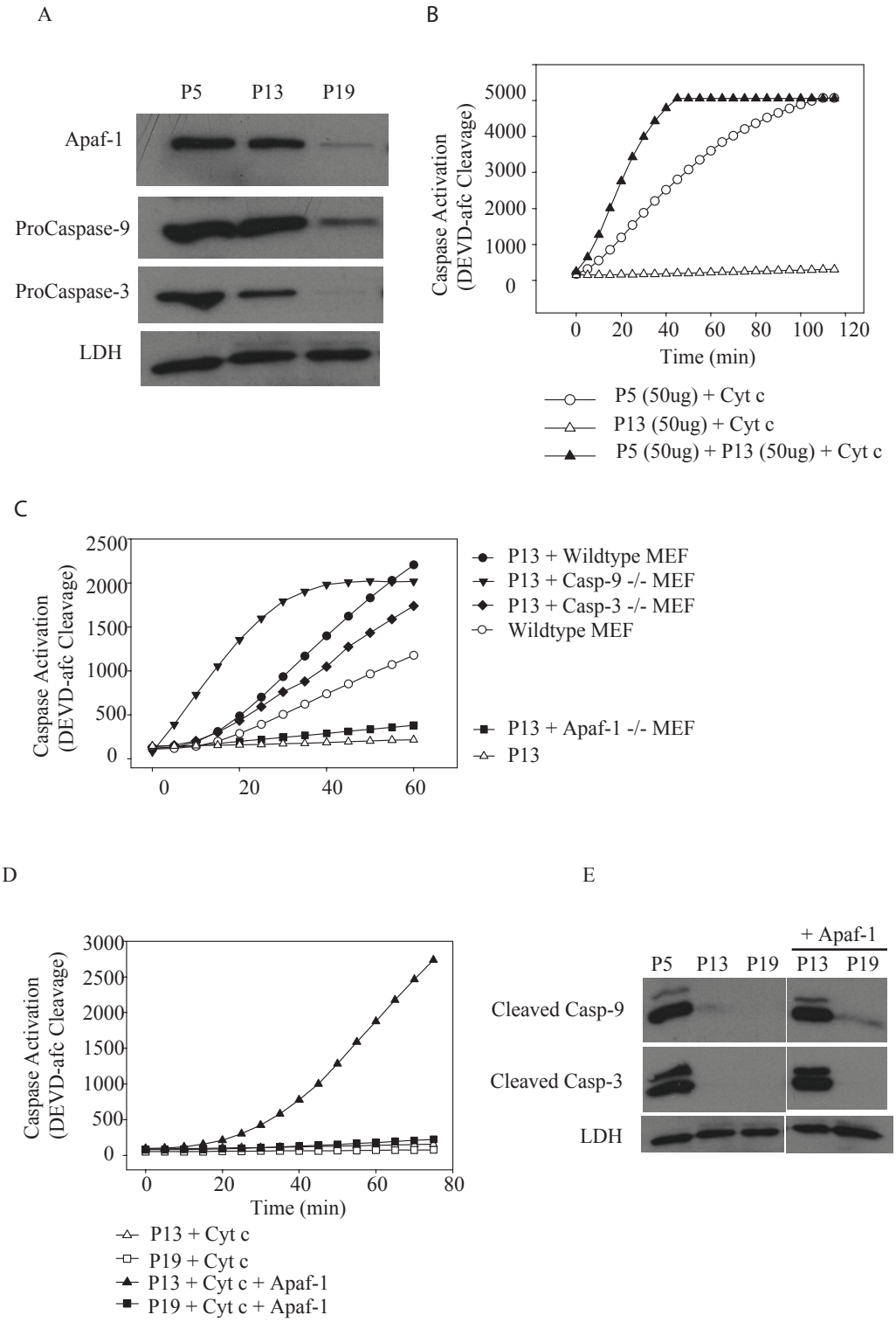

A
P5
P13
P19
Apaf-1
ProCaspase-9
ProCaspase-3
LDH
B
Caspase Activation (DEVD-afc Cleavage)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
120
Time (min)
P5 (50ug) + Cyt c
P13 (50ug) + Cyt c
P5 (50ug) + P13 (50ug) + Cyt c
C
Caspase Activation (DEVD-afc Cleavage)
2500
2000
1500
1000
500
0
0
20
40
60
P13 + Wildtype MEF
P13 + Casp-9 -/- MEF
P13 + Casp-3 -/- MEF
Wildtype MEF
P13 + Apaf-1 -/- MEF
P13
D
Caspase Activation (DEVD-afc Cleavage)
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
Time (min)
P13 + Cyt c
P19 + Cyt c
P13 + Cyt c + Apaf-1
P19 + Cyt c + Apaf-1
E
+ Apaf-1
P5
P13
P19
P13
P19
Cleaved Casp-9
Cleaved Casp-3
LDH

Figure 4.4 Domain structure of Apaf-1. **A)** Apaf-1 contains an N-terminal Caspase Recruitment Domain (CARD), a Nucleotide Binding Domain (NBD), and 13 C-terminal WD-40 repeats. **B)** Apaf-1 lacking the WD-40 repeats was fused to a GST tag to make GST-Apaf-1 (aa 1-543).

Figure 4.4

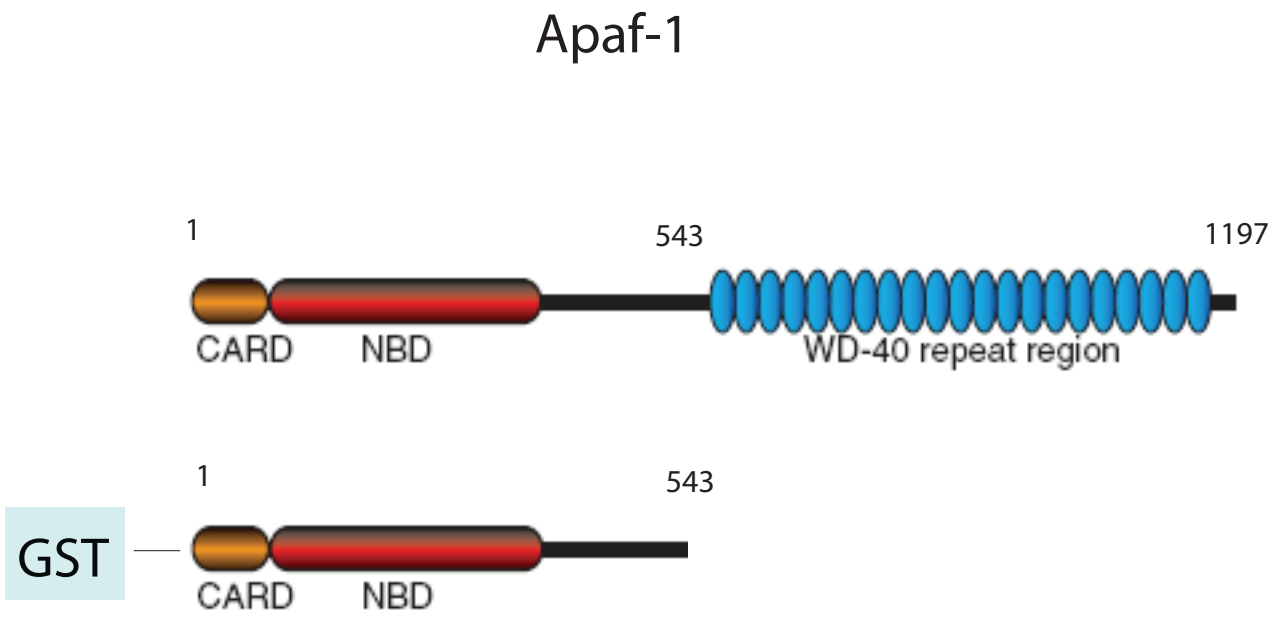

Apaf-1
1
543
1197
CARD
NBD
WD-40 repeat region
GST
1
543
CARD
NBD

Figure 4.5 Apaf-1 phosphorylation occurs in P5 but not P13 cerebellar neurons. A) The ability of His-Cyt *c* or GST-Casp-9-CARD was assed for its ability to bind Apaf-1 in P5 and P13 lysates. Recombinant protein was incubated with cerebellar extracts, followed by removal of His-Cyt *c* and GST-Casp-9-CARD with nickel beads, or Glutathione sepharose, respectively. Beads were washed in 500 mM NaCl, and binding partners examined by Western analysis. **B)** Recombinant protein of GST-Apaf-1 (aa 1-543) was incubated with lysates in the presence of [γ-^{32}P]-ATP. GST-Apaf-1 (aa 1-543) was removed from the lysates with Glutathione Sepharose, and phosphorylation of GST-Apaf-1 (aa 1-543) analyzed by autoradiography.

Figure 4.5

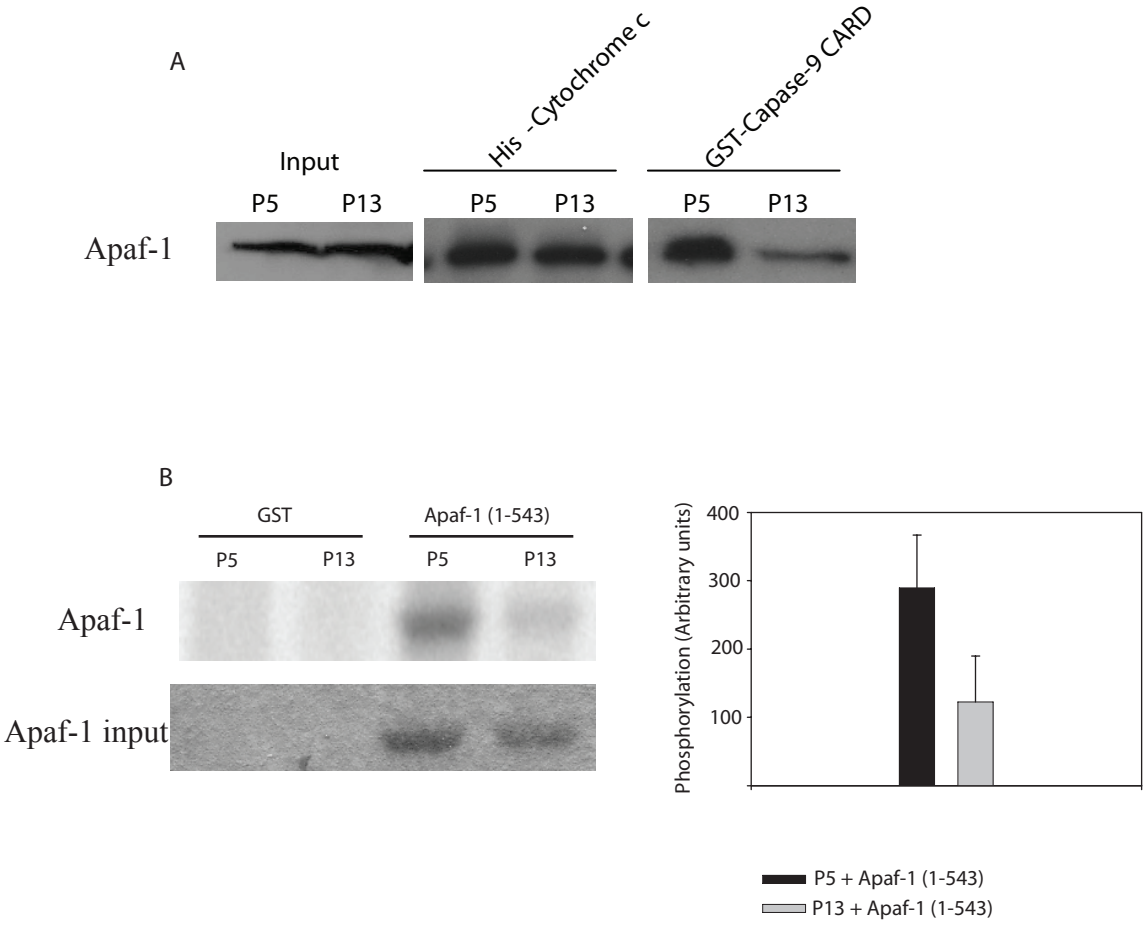
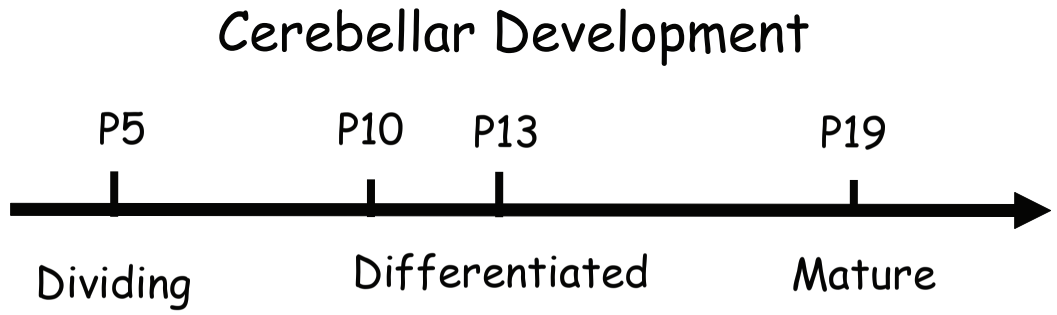

A
Input
His -Cytochrome c
GST-Capase-9 CARD
P5
P13
P5
P13
P5
P13
Apaf-1
B
GST
Apaf-1 (1-543)
P5
P13
P5
P13
Apaf-1
Apaf-1 input
Phosphorylation (Arbitrary units)
400
300
200
100
P5 + Apaf-1 (1-543)
P13 + Apaf-1 (1-543)

Figure 4.6 In neurons of the developing cerebellum, we identify four distinct stages of apoptotic regulation. 1) At an early developmental age (P5), apoptosome components are maintained at a high level, Apaf-1 is in a phosphorylated state, and cerebellar neurons are completely sensitive to cytochrome *c*. 2) At P10, apoptosome components are still highly expressed, but neurons have a reduced sensitivity to cytochrome *c* that is mediated in part by XIAP. 3) As neurons differentiate and begin to migrate toward the inner granule layer (P13) they lose the ability to undergo apoptosis induced by cytochrome *c*. Expression of apoptosome components are still high, however, Apaf-1 is no longer functional, perhaps due to a reduction in its phosphorylation. 4) Mature neurons of the cerebellum (P19) are completely resistant to cytochrome *c* and have severely reduced Apaf-1 and caspase-3 levels.

Figure 4.6



Sensitivity to Cytochrome c	Yes	No		No
Expression of apoptosome components	High	High		Low
Regulation By XIAP	N/A	Yes	No	No
Apaf-1 Activity/ Phosphorylation	Yes	No		N/A

V. CHAPTER FIVE:
Discussion

A. Summary of Findings

Unlike normal mitotic cells which are easily replaceable, postmitotic neurons have limited regenerative potential and must last the lifetime of the organism. The work from this dissertation provides evidence for two main hypotheses about neuronal apoptosis. *First*, it proposes that unlike normal mitotic cells, neurons have developed multiple mechanisms by which they strictly regulate the apoptotic pathway to ensure their longterm survival. In support of this hypothesis, I have identified two novel mechanisms (described in Chapter Three and Four) by which neurons inhibit the apoptotic pathway. *Second*, this dissertation proposes that the mechanisms by which neurons strictly inhibit the apoptotic pathway are reversible in order to cell death in appropriate situations. In Chapter Two and Three, I describe novel mechanisms by which blocks on the apoptotic pathway are removed in response to DNA damage and developmental and pathological situations of increasing ROS, respectively. In addition, work from this dissertation uncovers a specific mechanism utilized by neurons and cancer cells to restrict apoptosis. This discovery opens the possibility that the multiple mechanisms present in neurons to restrict apoptosis and ensure their longterm survival may also be used by cancer cells for the evasion of apoptosis. Chapter Three addresses similarities between how neurons and cancer cells regulate apoptosis via the redox state of cytochrome *c*.

CHAPTER TWO: Essential Postmitochondrial Role of p53 Uncovered in DNA Damage-Induced Apoptosis in Neurons

Summary of Findings

1. XIAP is degraded following NGF deprivation, but remains stable after DNA damage

2. Unlike NGF deprivation, etoposide-induced death requires translation to overcome XIAP

3. Apaf-1 is markedly induced in etoposide treated sympathetic neurons

4. p53 deficient neurons are unable to overcome XIAP's inhibition of caspases in response to DNA damage

5. p53 induces the expression of Apaf-1 induction in response to DNA damage; an event necessary to overcome XIAP's inhibition of caspases and promote apoptosis in neurons

Previous work from this lab and others has shown that while cytosolic cytochrome *c* is sufficient to induce apoptosis in mitotic cells, it is unable to do so in postmitotic sympathetic neurons (Deshmukh 1998; Neame, Rubin et al. 1998). Work that followed, demonstrated that the resistance of sympathetic neurons to cytosolic cytochrome *c* was due to the inhibition of caspase activation by XIAP (Potts, Singh et al. 2003). This finding was: 1) unique because a function for endogenous XIAP had not been desmonstrated for other cell types (Harlin, Reffey et al. 2001), and 2) curious because cytochrome *c* was found to be capable of inducing apoptosis in mitotic cells which express ample amounts of XIAP.

The removal of XIAP is required for cytosolic cytochrome *c* to induce apoptosis in neurons. Therefore, we wanted to examine the mechanism by which XIAP is overcome in pathological situations of DNA damage-induced apoptosis. Interestingly, we found that, unlike NGF deprivation which inhibits XIAP by selectively degrading it, DNA damage did not alter the levels of XIAP. Instead, XIAP was overcome by a p53-mediated upregulation of Apaf-1. These data are significant because they demonstrate that the mechanisms utilized

by neurons to overcome XIAP to permit cytochrome *c*-mediated apoptosis are stimulus specific. These results are also the first to identify a postmitochondrial mechanism by which p53 can regulate the apoptotic pathway in mammalian cells (discussed in more detail in Chapter Two). It is likely that other postmitotic cells which exhibit increased regulation of apoptosis due to XIAP activity (e.g. Cardiomyocytes; (Potts, Vaughn et al. 2005)) would also require p53-mediated induction of Apaf-1 in order to undergo apoptosis by DNA damage.

CHAPTER THREE: Glucose Metabolism Inhibits Apoptosis in Neurons and Cancer Cells by Redox Inactivation of Cytochrome *c*

Summary of Findings

1. Endogenous cytochrome *c* release is capable of inducing apoptosis in mitotic fibroblasts, but incapable of inducing apoptosis in sympathetic neurons.

2. Endogenous cytochrome *c* release is incapable of inducing apoptosis even in XIAP deficient sympathetic neurons.

3. NGF deprivation makes endogenous cytochrome *c* capable of inducing apoptosis in sympathetic neurons

4. An increase in the oxidative environment enables cytochrome *c* to induce apoptosis cells, whereas a reduced cellular environment inhibits the proapoptotic activity of cytochrome *c*. Whereas cytochrome c is active in an oxidized state, reduced cytochrome is less able to induce apoptosis.

5. Healthy neurons maintain a highly reduced environment via glucose metabolism through the pentose phosphate pathway and production of GSH.

6. Inhibition of GSH synthesis through the pentose phosphate pathway sensitizes neurons to cytochrome *c*.

7. NGF deprivation creates a cellular environment capable of oxidizing cytochrome *c*, thereby permitting apoptosis.

8. Like neurons, cancer cells have elevated GSH and are resistant to cytochrome *c*.

9. Glucose deprivation or inhibition of the pentose phosphate pathway increases ROS and sensitizes cancer cells to cytochrome *c* induced apoptosis.

Because postmitotic neurons have an increased need to regulate apoptosis, it is likely that multiple mechanisms exist in neurons that would inhibit apoptosis. In Chapter Three, I identified a novel mechanism by which neurons inhibit the apoptotic pathway. In brief, I report that the ability of cytochrome *c* to activate the apoptotic pathway is dependent on its redox status. The endogenous release of cytochrome *c* is not capable of inducing apoptosis in neurons because it is held in a reduced and inactive state.

It is important to note that this observation could not have been made using the same techniques which identified XIAP as an inhibitor of apoptosis in sympathetic neurons. In those experiments, an excess amount of exogenous cytochrome *c* protein (which is mostly oxidized, and therefore capable of activating apoptosis in the absence of XIAP) was introduced by microinjection into the cytosol of cells. Only by directly inducing endogenous release of cytochrome *c* from mitochondria in intact cells (via tBid) did it become apparent that even in the absence of XIAP, cytochrome *c* was still incapable of promoting apoptosis in neurons.

Here we show that in healthy neurons, glucose flux through the pentose phosphate pathway produces reduced glutathione which helps to maintain cytochrome *c* in a reduced and inactive state. Just as we see with XIAP inhibition in Chapter Two, the redox inactivation of cytochrome *c* in neurons is reversible. During apoptosis induced by NGF deprivation or DNA damage, glycolytic pathways are compromised resulting in an increase in ROS. As cytochrome *c* is released into the cytosol, it becomes oxidized and apoptotically active.

This work is significant because it provides a direct link between cellular metabolism, the production of ROS, and the activation of key players in the apoptotic pathway. It also 1) proposes that cancer cells and neurons have adapted similar methods by which they can evade apoptosis, and 2) provides a mechanism by which the Warburg effect (and enhanced use of glycolysis in cancer cells) could mediate protection or cancer cells from apoptosis.

CHAPTER FOUR: Age-Dependent Changes in the Regulation of Apoptosis in Neurons

Summary of findings

1. Cytochrome *c* is sufficient to induce caspase activation in P5 (mitotic), but not in P13 (postmitotic) cerebellar neurons.

2. The inability of cytochrome *c* to induce apoptosis in p13 cerebellar neurons is not due to inhibition of caspases by IAPs.

3. Differentiating cerebellar neurons exhibit a specific defect in Apaf-1 *activity*, despite maintaining high levels of Apaf-1 protein.

4. P13 neurons exhibit a defect in the ability of Apaf-1 to bind to caspase-9.

5. P5 and P13 neurons exhibit differential phosphorylation of Apaf-1.

6. With subsequent maturation (P19), cerebellar neurons exhibit a marked reduction in expression of apoptosome components.

While our lab and others have extensively studied the mechanisms by which sympathetic neurons regulate the apoptotic pathway in response to cytochrome *c,* whether this is a phenomenon shared by other neurons, particularly those of the CNS, is less known. In Chapter Four, I have used cell-free assays to determine that, like sympathetic neurons, granule neurons in the early stages of cerebellar development remain sensitive to cytochrome *c,* but become completely resistant as the cerebellum continues to develop and mature. While a reduction in Apaf-1 levels contributes to the resistance of sympathetic neurons to

cytochrome *c*, cerebellar neurons lose Apaf-1 activity despite maintaining high levels of Apaf-1 protein. While this data is still preliminary, we find that the phosphorylation status of Apaf-1 positively correlates to the sensitivity of neuronal extracts to cytochrome *c*. This work is significant as it is the first to identify a posttranslational regulation of Apaf-1 in a physiological context such as cerebellar development.

B. Discussion

Although all three chapters in this dissertation address novel mechanisms by which the apoptotic pathway is inhibited in neurons, Chapter Three specifically examines similarities between the regulation of apoptosis in neurons and cancer cells. In this section, I will discuss the possibility that the novel mechanisms regulating apoptosis described in both Chapters Two and Four may also be adapted by cancer cells to evade apoptosis. Finally, I will examine the implications that these findings may have on our understanding of how neuronal loss contributes to aging and neurodegenerative disease.

1. Cancer Cells and Neurons: a fight for survival

Postmitotic cell such as neurons have adapted to strictly regulate the apoptotic pathway to ensure their longterm survival. However, easily replaceable mitotic cells tend to lack these brakes, and more readily undergo apoptosis in the event of stress. A heightened susceptibility to apoptosis is especially important for mitotic cells to ensure that damaged DNA is not passed down to daughter cells. Unfortunately, rare events that inhibit the apoptotic pathway can cause a normal cell to proliferate through these checks, leading to malignancy. Therefore, despite the morphological and functional differences between

postmitotic neurons and cancer cells, a characteristic common to both cell types is their increased ability to inhibit apoptosis.

One theory that has developed from this dissertation proposes that some of the mechanisms by which neurons strictly regulate apoptosis could be the same ones adopted by cancer cells to evade apoptosis. In support of this, Chapter Three demonstrates that both neurons and cancer cells restrict apoptosis by maintaining released endogenous cytochrome *c* in a reduced and thus apoptotically inactive state. Although this is the only chapter which examines such similarities between neurons and cancer cells, I would hypothesize that cancer cells may have also commandeered the methods described in Chapters Two and Four used by neurons to inhibit apoptosis. I discuss evidence for these possibilities, and their implications below.

Do both cancer cells and neurons regulate apoptosis via XIAP activity?

In postmitotic cells such as sympathetic neurons and cardiomyocytes, XIAP plays a crucial role in mediating sensitivity of cells to cytochrome *c* (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005). Unlike normal mitotic cells where cytochrome *c* is capable of activating caspases even in the presence of XIAP, postmitotic neurons and cardiomyocytes are resistant to cytochrome *c* unless XIAP is removed. Interestingly, many cancers may have also adapted to use IAPs in the regulation of apoptosis. Levels of ML-IAP are overexpressed in the majority of melanomas, and other IAPs, including XIAP, are upregulated in nonsmall cell lung cancer, prostate cancer, and a variety of lymphomas and leukemias (Vucic, Stennicke et al. 2000; Hofmann, Simm et al. 2002; Altieri 2004; Nachmias, Ashhab et al. 2004; Zangemeister-Wittke and Simon 2004; Wright and Duckett 2005). Consistent with the

expectation that increased expression of IAPs inhibit apoptosis in cancer cells, small peptide mimics of the endogenous IAP inhibitor, SMAC, are able to sensitize a variety of tumor derived cell lines and mouse models of tumors to apoptosis (Fulda, Wick et al. 2002; Li, Thomas et al. 2004; Schimmer, Welsh et al. 2004; Bockbrader, Tan et al. 2005). If IAPs are indeed important for cancer cell survival, one prediction is that animals deficient in XIAPs may have a reduced susceptibility to certain types of tumors.

Do both cancer cells and neurons regulate cytochrome c-mediated apoptosis by modulation of Apaf-1 expression?

Although the inhibition of XIAP activity is only required for postmitotic cells, and not normal mitotic cells, to undergo cytochrome *c*-mediated apoptosis, there is no difference in the levels of XIAP between these two cell types. Instead, levels of the proapoptotic protein, Apaf-1 are dramatically reduced in postmitotic cells as compared to normal mitotic cells (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005). It is this low level of Apaf-1 in neurons and cardiomyocytes which allows the XIAP brake to be engaged, making these cells insensitive to cytochrome *c*. Interestingly, certain cancer cells have also hijacked this technique of inhibiting apoptosis, as a variety of cell lines including melanoma and leukemia have decreased expression of Apaf-1, and thus a higher threshold for apoptosis (Jia, Srinivasula et al. 2001; Soengas, Capodieci et al. 2001).

Does p53 regulate apoptosis downstream of mitochondria in neurons and cancer cells?

In Chapter Two of this dissertation I show that in order for neurons to overcome XIAP inhibition of caspases and undergo apoptosis following DNA damage, they must re-express

sufficient levels of Apaf-1. From these studies in neurons, one could predict that cancer cells exhibiting reduced Apaf-1 levels or increased expression of XIAP may also upregulate Apaf-1 to undergo apoptosis mediated by DNA damage. In neurons, we show that the transcription factor p53 mediates the increase in Apaf-1 in response to DNA damage. While p53 has a well known role in apoptosis at the mitochondria (e.g. upregulation of apoptotic Bcl-2 proteins), our results could provide an additional explanation to why cancers deficient in p53 are often resistant to DNA damaging therapeutics. It would be interesting to examine whether cancer cells with intact p53 respond better than p53 deficient tumors to DNA damaging therapeutics in part due to their ability to re-express Apaf-1. In addition, this would suggest that in order to treat cancers carrying mutations in p53 as well as defects in Apaf-1 expression, therapeutics must not only induce the release of cytochrome *c* from the mitochondria, but also target XIAP activity.

Do both neurons and cancer cells regulate Apaf-1 postranslationally to inhibit apoptosis?

A reduction in Apaf-1 levels in sympathetic neurons and cardiomyocytes engages XIAP activity and protects these cells from cytochrome *c*-mediated apoptosis. However, as described in Chapter Four of this dissertation, postmitotic neurons at mid stages in cerebellar development regulate apoptosis not by modulating levels of Apaf-1, but by redering it inactive posttranslationally. Interestingly, certain cancers seem regulate the apoptosome in a similar manner. Work from Dr. Doug Green's laboratory shows that a variety of ovarian cancer cell lines are defective for Apaf-1 activity despite adequate expression of this protein (Wolf, Schuler et al. 2001). Consistent with our observations in the developing cerebellum, this inactivation does not seem to be a result of a dominant Apaf-1 inhibitor, or altered

expression of Apaf-1 isoforms. Future experiments would need to be done to determine whether the posttranslational inhibition of Apaf-1 is accomplished by similar means in the developing cerebellum as in ovarian tumors.

Although correlative, our data in the developing cerebellum suggests that phosphorylation could be the posttranslational motif regulating the apoptotic activity of Apaf-1. Work in Dr. Sally Kornbluth's lab at Duke have shown that phosphorylation of Apaf-1 occurs in cells overexpressing the oncogene, BCR-Abl (Deming, Schafer et al. 2004), To date, however, the sites of Apaf-1 phosphorylation have not been determined. It would be interesting to ask in the future whether phosphorylation of Apaf-1 is a key regulator of Apaf-1 function in cerebellar neurons as well as cancer cells.

2. Relevance to Neurodegeneration and Aging

This dissertation examines three mechanisms which neurons have evolved to inhibit the apoptotic pathway. Yet, despite these multiple protective mechanisms, neuronal cell death is a key feature of many neurodegenerative diseases as well as aging. We find that the mechanisms utilized by neurons to inhibit apoptosis are reversible. Therefore, I would propose that that the failure to acquire these molecular events, or their aberrant disruption could be features of neurodegenerative disease and aging, and would sensitize neurons to stress and eventual cell death.

Is deregulation of XIAP activity or Apaf-1 expression relevant to neurodegeneration and aging?

Postmitotic neurons are unable to undergo cytochrome *c*-mediated cell death due to a brake on caspases by XIAP. Chapter Two shows that this inhibition of caspases is reversible, being overcome in developmental apoptosis by XIAP degradation, or in phathological apoptosis by a p53-mediated increase in Apaf-1. With this information, we could predict that XIAP activity would be negatively affected in the context of neurodegenerative disorders. In support of this, studies have shown that in transgenic mouse models of ALS, XIAP levels are decreased in contrast with wildtype littermates. We could also speculate that a reduction of XIAP or increase in Apaf-1 may be a risk factor for the development of neurodegenerative disorders. Yolanda Huang in our lab is in the process of examining this question using a mouse model of ALS, as well as by examining DNA from human ALS patients to look for mutations in XIAP.

In addition to the disregulation of XIAP, an inability to inhibit expression of Apaf-1 in adult neurons could be a hallmark of cell death in neuronal disease, be it cause or effect. For example, in response to traumatic brain injury, Apaf-1 is upregulated in mice (Yakovlev, Ota et al. 2001). In Chapter Two we show that p53 is required for the induced expression of Apaf-1 in response to DNA damage by etoposide. Interestingly, an induction of p53 activity has been reported in numerous neurodegenerative disorders including in Alzheimer's Disease, Parkinson's Disease, and ischemic injury. This would suggest that inhibition of p53 may be a viable target to reduce apoptosis in aging and neurodegeneration (Culmsee and Mattson 2005). Although it is clear that disruption of Apaf-1 or inhibition of XIAP activity can sensitize neurons to apoptosis, it is not clear whether disregulation of these proteins is a cause or an affect of ailments such as neurodegeneration. It would be interesting to ask whether patients susceptible to neurodegenerative disease inactivate XIAP,or fail to

downregulate Apaf-1 during development, thus sensitizing them to cellular stress that accumulates with age.

Is cytochrome c activation via ROS important for neurodegeneration and aging?

We have shown that in healthy postmitotic neurons, a reduced cellular environment holds the proapoptotic activity of cytochrome *c* in check. Such a checkpoint would be critical for a postmitotic cell in particular, in the event of accidental release of cytochrome into the cytosol. For example, in a mitotic cell, if mitochondria become damaged and leak cytochrome *c,* it may be more efficient for the organism to remove this cell by apoptosis than to repair the damage. However, for an irreplaceable neuron, the risk of losing this neuron most likely outweighs the price of repairing it.

Despite the necessity for neurons to strictly inhibit the apoptotic pathway, our work shows that the inactivation of cytochrome *c* can be reversed by a disruption in the redox balance of the cell. Interestingly, this balance is disrupted in a myriad of neurodegenerative diseases and with age. Many studies, including the free radical hypothesis of aging (Harman 1992), suggest that an imbalance between generation of oxygen radicals and antioxidants may be involved the pathogenesis of many neurodegenerative diseases (Eckert, Keil et al. 2003; Eckert, Marques et al. 2003). An increase in ROS and oxidative damage have been described as a very early event in patients with Alzhemier's disease and Parkinson's Disease (Castellani, Hirai et al. 2002; Eckert, Keil et al. 2003; Eckert, Marques et al. 2003; Schubert 2005; Nunomura, Castellani et al. 2006). Despite this multitude of research, it is still unknown whether oxidative damage constitutes a cause or effect of the disease. Data from this thesis suggests, that even if the cellular damage caused by ROS accumulation is not great

enough to cause death of the cell on its own, ROS can directly activate a key player (i.e. cytochrome *c*) in the apoptotic pathway, making neurons more susceptible to apoptosis in the event of any mitochondrial damage resulting in release of cytochrome *c* into the cytosol.

Not only does my work implicate ROS as a regulator of cytochrome *c* activity and apoptosis, but it identifies glucose metabolism as an important factor in maintaining redox homeostasis and thus restriction of apoptosis in neurons. From this we could predict that disruption of glucose metabolism would also sensitize neurons to apoptosis by cytochrome *c*. Indeed, a general disruption in glucose metabolism such as glucose deprivation can lead to accumulation of ROS (Gibson 2002). Such a disruption in glucose metabolism has been implicated in the pathology of neurodegenerative disorders such as Alzheimer's disease (Gibson 2002; Schubert 2005). For example, a reduction in glucose metabolism can predict cognitive decline in adults, which can often lead to the progression of Alzheimer's Disease (Small, Ercoli et al. 2000; Arnaiz, Jelic et al. 2001; de Leon, Convit et al. 2001; De Santi, de Leon et al. 2001). Whether such abnormalities in glucose regulation and ROS accumulation sensitize neurons to cytochrome *c*-induced apoptosis in patients or mouse models of neurodegeneration and aging remain to be examined.

VI. APPENDICES

A. APPENDIX A

Role of Apaf-1 in Neurite Outgrowth

With the completion of the human genome project, it was discovered that our DNA encodes far fewer genes than had previously been anticipated, suggesting that individual proteins may have multiple functions within a cell. The different functions of a particular protein could depend on its location, either within a certain cell type or localization within the cell itself. Alternatively, temporal or even expression level differences of a protein could dictate its distinct functions. For example, only a decade ago was it discovered that cytochrome *c,* known classically for its role in the electron transport chain, can also initiate apoptosis depending upon its localization within the cell. In addition, some proteins originally thought to be involved only in the cell cycle, take on cell cycle independent roles in postmitotic neurons. Both Pin1 and the Anaphase Promoting Complex (APC) are cell cycle genes recently implicated in axonal patterning and outgrowth, and cell death, respectively, in postmitotic neurons (Konishi, Stegmuller et al. 2004; Becker and Bonni 2006).

Interestingly, components of the apoptotic pathway are constituitively expressed in cells, despite the fact that the cell (i.e. neurons) may never undergo apoptosis. Therefore, it is conceivable that cells may utilize some of these components for other cellular tasks. Some of my preliminary findings suggest that Apaf-1 may have a function in neurite extension in addition to its well established role in the apoptotic pathway.

When sympathetic neurons from Apaf-1 deficient mice are cultured in vitro, their ability grow axonal projections seems retarded in comparison with their wildtype littermates. To examine this more closely, neuronal explants of either wildtype or Apaf-1-/- neurons were

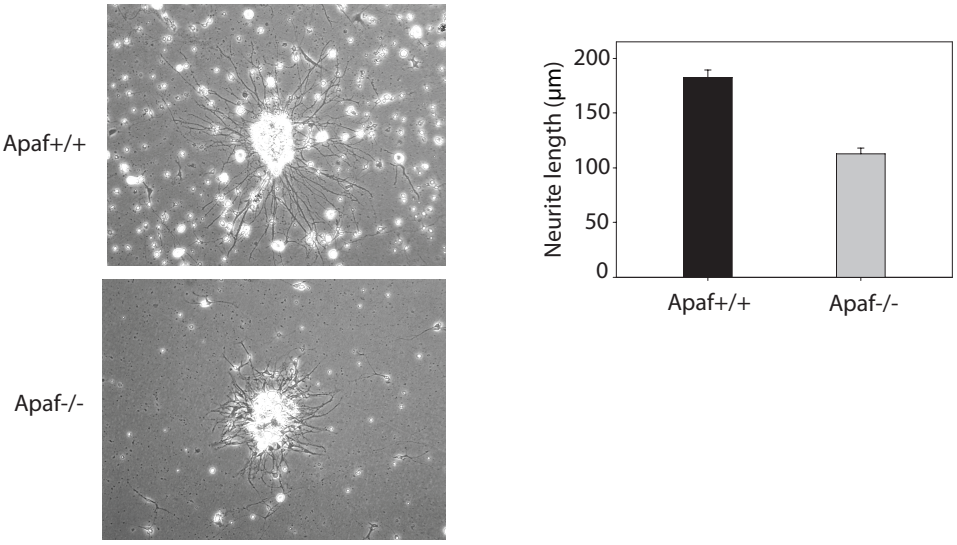
cultured in NGF containing media, followed by quantitation of neurite outgrowth. While wildtype sympathetic neurons had an average axonal length of 182 um by 16 hrs, Apaf-1-/- neurons showed an average projection length of 113 um (Fig A1). Although preliminary, this data suggests that in addition to its crucial role in the apoptotic pathway in sympathetic neurons, Apaf-1 may also function to promote neurite outgrowth.

Apaf-1 is a very large (130 kD) multidomain protein that consists of two protein interaction domains (a CARD and WD-40 domain) as well as a nucleotide binding domain. Cytochrome *c* and caspase-9 both interact with Apaf-1 to form the apoptosome during apoptosis, therefore it is possible that this same complex could be necessary for neurite outgrowth. Alternatively, Apaf-1 could interact with unknown partners to induce axon growth. To address the first hypothesis, the lab plans to ask whether neurons isolated from the caspase-9 deficient mice exhibit a similar defect in axon extension. If the second hypothesis is correct, Apaf-1 may be expected to bind to alternative partners in the cytosol of an extending neurite.

Figures and Legends

Figure A. Apaf-1 deficient neurons have a defect in neurite extension. Sympathetic neurons were cultured from Apaf-1-/- mice or their wildtype littermates. Before plating, ganglia were incompletely triturated. Populations of neurons containing roughly equal numbers were photographed 16 hrs after plating. Neurite length was measured and represented as an average lenth after 16 hrs of plating in NGF.

Figure A



B. APPENDIX B

Role of Cytochrome *c* Degradation in Neuronal Apoptosis

Data in the body of this dissertation introduce three novel ways in by which neurons tightly regulate the apoptotic pathway in order to ensure their longterm survival. In Chapter Three in particular, I describe how the apoptotic activity of cytochrome *c* itself can be modified by its redox activity. In this section, which consists of work not yet included in a publication or in the main body of this thesis, I describe an additional mode of regulation of the apoptotic pathway in neurons at the point of cytochrome *c*.

In healthy cells, including potmitotic neurons, cytochrome *c* is localized to the mitochondria where it participates in cellular respiration through the electron transport chain. However, in mitotic cells, once cytochrome *c* is released into the cytosol, it accumulates, interacts with Apaf-1, inducing its oligimerization, formation of the apoptosome and subsequent caspase activation. In postmitotic sympathetic neurons, the cytochrome *c* that is released from the mitochondria is subjected to rapid degradation which can be visualized if the subsequent steps of caspase activation are blocked. By immunofluorescence, the cytochrome *c* staining pattern goes from a punctate mitochondrial localization in NGF-maintained neurons to being virtually undetectable when cytochrome *c* is released from the mitochondria (Fig B1). Not only does this occur in response to cytochrome *c* release induced by tbid, but also occurs after cytochrome *c* release with NGF deprivation, or even etoposide (Fig. B1; data not shown). This degradation of cytochrome *c* is mediated by the proteasome, as inhibition of the 26S subunit of the proteasome with lactacystin inhibits the degradation as shown both by Western analysis and immunofluorescence (Fig B2).

The experiments described in Chapter Three of this dissertation show that the direct release of cytochrome *c* from the mitochondria of sympathetic neurons does not induce apoptosis even if XIAP is first removed (Fig 3.1). However, when cytochrome *c* release occurs in the context of NGF deprivation, cytochrome *c* is made sufficient to induce apoptosis via its oxidation. It is also possible, however, that the degradation of cytochrome in the cytosol could in part explain the inability of released cytochrome *c* to initiate apoptosis. To address this, cultures of XIAP deficient sympathetic neurons were treated with lactacystin or MG132 to inhibit the proteasome, followed by injection of tBid-GFP DNA. While untreated neurons did not undergo apoptosis in response to tBid, those treated with proteasome inhibitor died by rapid and complete apoptosis (Fig B3). Although these data are preliminary, they suggest that; 1) neurons possess a unique ability to target cytochrome *c* for degradation once it is in the cytosol, and 2) that the degradation of cytosolic cytochrome *c* may be a protective mechanism to inhibit apoptosis in postmitotic neurons.

As seen in Chapter Three of this thesis, cancer cells can also adapt mechanisms similar to those used by neurons to avoid the induction of apoptosis. While there is no concrete evidence that cancer cells inhibit the apoptotic pathway by degrading cytosolic cytochrome *c,* it is interesting to note that the prostate cancer cell line, DU145, have been found to rapidly degrade cytochrome *c* once it is released from the mitochondria (Dr. Yuri Lazebnik, personal communication).

In the future, the lab hopes to identify the ubiquitin E3 ligase that targets cytochrome *c* for proteasome-mediated degradation in sympathetic neurons. To address this, we plan to; 1) use immunoprecipitation experiments followed by mass spectrometry analysis to identify proteins which differentially bind cytosolic cytochrome *c* in cell types that degrade

cytochrome *c*, versus those cell types where cytochrome *c* remains intact; 2) take a biochemical approach to isolate factors from the cytosol that have the capability of ubiquitinating cytochrome *c* in vitro; 3) ask whether the SCF (Skp1-Cul1-F-box) complex plays a role in the degradation of cytochrome *c*. SCF is a good candidate for regulating the degradation of cytochrome *c* as SCF recognizes substrates with a WD-40 similar to the WD-40 repeat by which cytochrome *c* and Apaf-1 interact (Sun 2003).

Figures and Legends

Figure B1: Cytochrome *c* is degraded upon release from the mitochondria. Cultures of sympathetic neurons were injected with tBid-GFP DNA, and allowed to express for 24 hours. Cells were fixed with 4% paraformaldehyde, and cytochrome *c* and tbid-GFP visualized with anti-cytochrome *c* and anti-GFP antibodies, respectively.

Figure B1

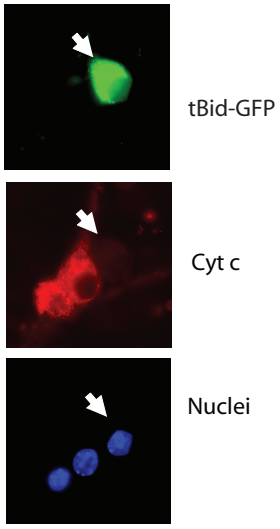
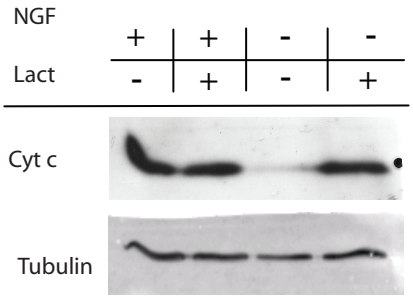


Figure B2: Cytosolic cytochrome *c* is degraded by the proteasome in sympathetic neurons. **i)** Neurons were maintained in NGF, or deprived of NGF in the presence or absence of lactacystin (10 uM) for 24 hrs. Cytochrome *c* levels were analyzed by Western blot. **ii)** Sympathetic neurons were injected with tBid-GFP in the presence of lactacystin. After 6 hours, cytochrome *c* levels were analyzed by immunofluorescence.

Figure B2

i



ii

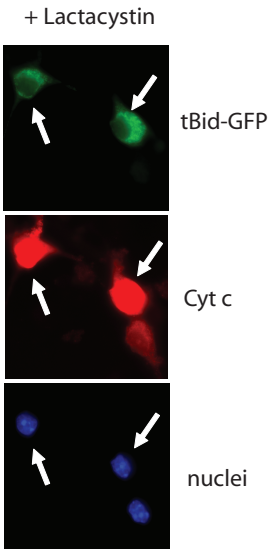
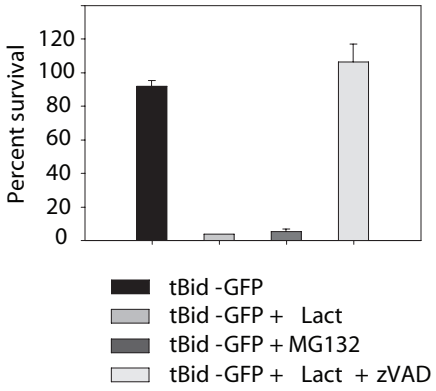


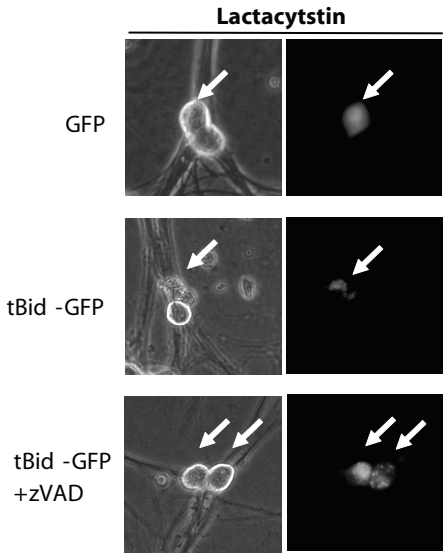
Figure B3: Inhibition of cytochrome *c* degradation allows apoptosis in sympathetic neurons. **i)** Cultures XIAP-/- sympathetic neurons were injected with tBid-GFP or GFP alone followed by treatment with lactacystin (Lact:10 uM) or MG132 (20 uM) with or without zVAD (50 uM). Cells were assessed for survival and **ii)** quantified as percentage of healthy cells at 24 hrs versus 5 hrs post-injection.

Figure B3

i



ii



C. APPENDIX C

Role of the XIAP RING Finger in Cytochrome *c*-Mediated Apoptosis

In neurons, we find that XIAP is a critical inhibitor of cytochrome *c*-mediated caspase activation and apoptosis. Although the most well documented activity of XIAP is to bind to and inhibit caspase activity, whether XIAP uses other means to regulate apoptosis is less understood.

As described in the Introduction, XIAP consists of three BIR domains, as well as a RING finger domain. The BIR3 domain of XIAP can bind directly to cleaved and activated caspase-9 (Sun, Cai et al. 2000; Srinivasula, Hegde et al. 2001), while the inhibition of caspase-3 and caspase-7 occurs through direct binding to the linker region between the BIR1 and BIR2 domain (Chai, Shiozaki et al. 2001; Huang, Park et al. 2001; Riedl, Renatus et al. 2001). At its C-terminus, XIAP contains a RING finger domain with E3 ubiquitin ligase activity (Yang, Fang et al. 2000). While it is not known whether this domain has a critical function within cells, there have been several substrates identified for the RING finger domain. For example, it has been suggested that the RING finger of XIAP can autoubiquitinate, and mutants in which the RING domain have been removed become more resistant to apoptotic stimuli (Hay, Wassarman et al. 1995; Yang, Fang et al. 2000). In addition, the RING finger has been described as anti-apoptotic as XIAP has been shown to ubiquitinate and target caspase-9, caspase-3 as well as SMAC for degradation (Suzuki, Nakabayashi et al. 2001; MacFarlane, Merrison et al. 2002; Morizane, Honda et al. 2005).

Sympathetic neurons are one of only two cell types where endogenous XIAP has been described as a critical inhibitor of apoptosis. I therefore used these cell types to ask what role, if any, the RING finger domain of XIAP plays in cytochrome *c*-mediated

apoptosis. To address this, point mutations were made in XIAP to abrogate its caspase inhibitory function, but leave the RING domain intact. The D148A mutation at the linker N-terminal of BIR2 is unable to bind processed caspase-3, and its ability to inhibit caspase-3 in vitro is severely reduced (Silke, Ekert et al. 2001). The E314S mutation is located in the caspase-9 binding groove of the BIR3 domain, and therefore is unable to inhibit caspase-9 activity (Sun, Cai et al. 2000). Unlike the wildtype XIAP, expression of the double mutant (DM) at low levels in XIAP-/- sympathetic neurons was unable to protect from cytochrome *c* injection (data not shown). These results indicate that the ability of XIAP to inhibit caspase-9 and caspase-3 is critical for it to inhibit apoptsis in neurons. Suprisingly, however, at higher expression levels, XIAP-DM protected XIAP-/- sympathetic neurons from cytochrome *c* as efficiently as the wildtype (Fig C1).

To address whether the ability of XIAP-DM to inhibit apoptosis is due to a function of E3 ubiquitin ligase activity, an H467A mutation in the Ring domain (XIAP-RM) abrogating this activity (Yang, Fang et al. 2000) was introduced separately as well as in the background of the XIAP-DM, to make the XIAP triple mutant (XIAP-TM). As expected, overexpression of the XIAP-RM, which retains the ability to inhibit caspases, was completely able to protect neurons from cytochrome *c*-induced apoptosis. However, XIAP-TM completely lost its ability to inhibit cytochrome *c*-mediated apoptosis even under the overexpression condition, indicating that the RING finger of XIAP is involved in inhibition of apoptosis (Fig. C1).

To ask whether the protection afforded by the RING finger in the XIAP-DM is a result of proteasome mediated degradation, XIAP-/- sympathetic neurons expressing XIAP-DM in the presence or absence of the proteasome inhibitor, Lactacystin, were injected with

cytochrome *c*. While XIAP-DM was able to protect neurons from cytochrome *c*-induced apoptosis, Lactacystin treated neurons became completely sensitive to cytochrome *c* (Fig. C2).

Together, these data suggest that in neurons, the RING finger domain of XIAP may have a significant antiapoptotic function. However, to determine whether the RING finger has significant antiapoptotic activity at physiological levels, it would be interesting to ask whether there is any difference in the ability of wildtype XIAP and XIAP-RM to inhibit cytochrome *c*-mediated apoptosis when expressed at more physiologic levels. Indeed, expression of wildtype XIAP at 250 pg/ul afforded significantly more protection against cytochrome *c* than equivalent expression of XIAP-RM. Although the exact targets of XIAP-mediated degradation are not known, we anticipate that caspase-9 or caspase-3 is a likely target, as these have been shown to be ubiquitinated by XIAP in vitro.

Figures and Legends

Figure C1: Diagram of XIAP motifs. Mutations at D148A abrogate the ability of XIAP to inhibit caspase-3. The E314S mutation abrogates the ability of XIAP to inhibit caspase-9. Mutation at H467A in the RING domain inhibits XIAP's E3 ligase activity.

Figure C1

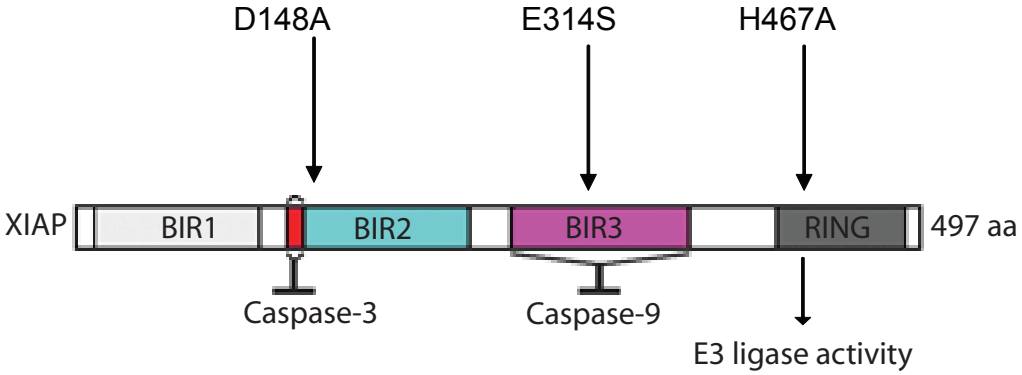
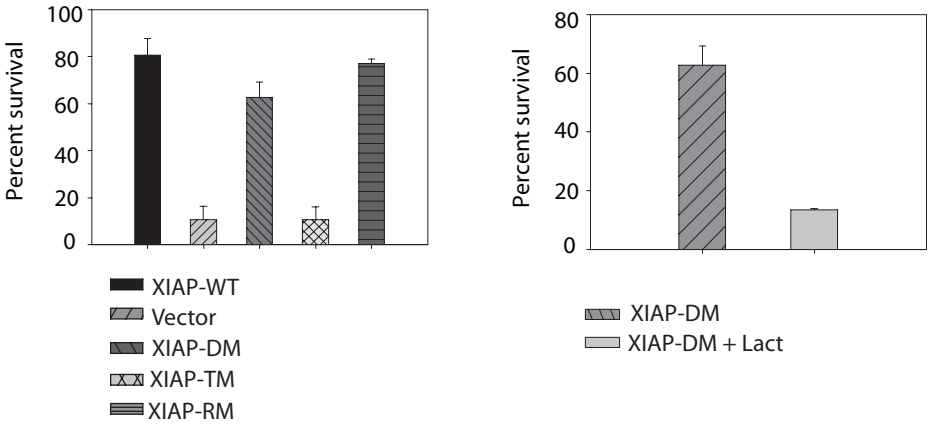

D148A
E314S
H467A
XIAP
BIR1
BIR2
BIR3
RING
497 aa
Caspase-3
Caspase-9
E3 ligase activity

Figure C2: The antiapoptotic activity of XIAP is dependent on its caspase inhibitory domains, as well as the RING finger. (**i)** XIAP-/- sympathetic neurons were injected with plasmids encoding WT XIAP, Vector alone, XIAP-DM, XIAP-TM, or XIAP-RM (200 ng/ul) along with EGFP to mark injected cells. After 24 hours to allow for expression, GFP-positive neurons were injected with 10 mg/ml cytochrome *c*. Cell survival was assessed after 8 hrs by morphological criteria. (**ii)** XIAP-/- neurons were injected with plasmids encoding XIAP-DM along with EGFP, and either left untreated, or treated with 10 uM Lactacystin. After 24 hrs, to allow for expression GFP-positive neurons were injected with 10 mg/ml cytochrome *c*. Cell survival was assessed after 8 hrs by morphological criteria.

Figure C2


Percent survival
100
80
60
40
20
0
XIAP-WT
Vector
XIAP-DM
XIAP-TM
XIAP-RM
Percent survival
80
60
40
20
0
XIAP-DM
XIAP-DM + Lact

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