IMMUNE MOLECULES REGULATE MEDULLOBLASTOMA AND NEURONAL APOPTOSIS

Elizabeth R. W. Knight

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Neurobiology in the School of Medicine.

Chapel Hill
2013

Approved by:

Mohanish Deshmukh

Jay Brenman

Glenn Matsushima

Jeff Rathmell

Jenny Ting

ABSTRACT

Elizabeth R. W. Knight: Immune Molecules Regulate Medulloblastoma and Neuronal Apoptosis
(Under the direction of Mohanish Deshmukh)

While the brain has long been considered an immunoprivileged region, recent research reveals that immune genes play important roles in neurons and the nervous system. Neurons not only express immune genes but these genes can serve immune or neuron-specific functions. Additionally, cytokines produced by immune cells can influence neuronal characteristics and survival. In this work, I investigated the role of an immune cytokine, interferon-gamma (IFN-γ), on neuronal apoptosis and the role of an immune gene, ASC (Apoptosis-associated speck-like protein containing a caspase recruitment domain), in cancer of the cerebellum.

While IFN-γ induces apoptosis in many cell types to limit the spread of infection, here we show that it protects sympathetic neurons from death with NGF deprivation or pan-kinase inhibition. Specifically, we determined that IFN-γ inhibited apoptosis at the point of mitochondrial permeabilization, yet did not induce expression of a number of proapoptotic genes in sympathetic neurons that are upregulated in other cell types. The ability of IFN-γ to promote sympathetic neuronal survival while inducing pro-death pathways in pathogens is likely a physiologically important mechanism which could ensure the long-term survival of these neurons during critical situations of infection.

We also examined the function of another immune gene, ASC, in the context of brain development and medulloblastoma, the most common malignant pediatric brain cancer. ASC exerts pro-death effects in several cell types, is silenced in many cancers, and acts as a tumor suppressor in colon cancer. Here, we present the unexpected findings that ASC deficiency robustly suppressed tumor incidence, delayed age of tumor onset, reduced premalignant lesion size, decreased EGL (external granule layer) proliferation, and increased TGF-β pathway expression and signaling in a mouse model of medulloblastoma. These results identify a critical function of ASC in driving proliferation and tumorigenesis in this medulloblastoma model. Therapies targeting ASC may be a promising strategy for preventing tumor progression of this challenging disease. Together, these studies illustrate the ability of immune genes and signals to exert powerful effects on neuronal apoptosis and on a cancer of the nervous system.

ACKNOWLEDGEMENTS

For the interferon-gamma project, we thank Dr. Julie Clarke Williams, Dr. Kasturi Puranam, and Dr. Anna Cliffe for thoughtful discussion. We thank Dr. Glenn Matsushima and Dr. Kasturi Puranam at UNC and Dr. Divaker Choubey at the University of Cincinnati for *ifi202b* plasmids. This work was supported by grants NS042197 and GM078366 to MD.

For the ASC project, we thank Drs. Vishva Dixit (Genentech) and James Olson (Fred Hutchinson Cancer Research Center) for generously sharing $ASC^{-/-}$ and ND2:SmoA1 mice, respectively. Thank you to Drs. Jenny Ting, Glenn Matsushima, Ryan Miller, and Drew Dudley for collaborations. We appreciate the technical assistance provided by Esita Patel, Vivian Xu, Cornelius Flowers, Michael Conlin, and Meera Patel in the Deshmukh Lab; Janice Weaver, Lily Wai, and Yongjuan Xia in the UNC Histopathology Core; Terese Camp and Ling Li in the UNC Genomics Core; Mark Vincent Olorvida, Stephanie Cohen, and Bentley Midkiff in the UNC Translational Pathology Laboratory (TPL); and Joel Parker, George Wu, Chandri Yandava, and Chris Fan at UNC for bioinformatics and biostatistics guidance. The UNC TPL is supported in part by grants from the National Cancer Institute (3P30CA016086) and the UNC University Cancer Research Fund. We would like to thank members of the Deshmukh laboratory for critical review of this manuscript. TRG is supported by NIH grant 1K08NS077978 and St. Baldrick's Foundation. This work was supported by grants NS042197 and GM078366 to MD.

Thank you to my advisor, Mohanish, for his guidance and dedication, for teaching thinking, writing, and speaking skills, and for fostering a positive lab environment. Thank you to my committee members and Dr. Tim Gershon for guidance, thoughtful input, and collaborations. To all of my lab mates, thank you for being positive, cooperative, and available for scientific or nonscientific discussion. I especially appreciate Drs. Vivian Gama and Allyson Evans for providing guidance, support, and scientific know-how. Thank you to the undergraduates who cheerfully supported the lab's foundation. Esita and Vivian, I have thoroughly enjoyed working with you and seeing you both grow in the lab.

I would never have been exposed to research in college without Dr. Elena Mendez of Converse College who introduced me to REU research opportunities. Thank you to all the professors and teachers who have selflessly taught, challenged, and encouraged me throughout my education. I appreciate the Neurobiology and IBMS programs and my classmates for their friendships and community.

Thank you to my dear friends who have supported and encouraged me throughout, and who patiently and generously waited for me to have free time. Thank you to my family, especially my mother who built up my confidence and kept telling me I could do great things.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIF	Apoptosis initiating factor
ALS	Amyotrophic lateral sclerosis
APAF-1	Apoptotic protease-activating factor-1
ASC	Apoptosis-associated speck-like protein containing a CARD
BCL	B-cell lymphoma
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
BIR	Baculoviral IAP repeat
C. elegans	Caenorhabditis elegans
CARD	Caspase activation and recruitment domain
CD2	Cyclin D2
CGNPs	Cerebellar granule neuron progenitors
CGNs	Cerebellar granule neurons
dATP	Deoxyadenosine triphosphate
DED	Death effector domain
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
E	Embryonic-day
EGL	External granule layer
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal regulated kinases

GAPDH	Glyceraldehyde phosphate dehydrogenase
GAS	Gamma interferon-activated sequence
GFP	Green fluorescent protein
IAP	Inhibitor of apoptosis protein
ICE	Interleukin-1β converting enzyme
ifi202b	Interferon-inducible protein 202b
IFN	Interferon
IFNAR	IFN-α receptor
IFNGR	IFN-γ receptor
IGF-1	Insulin-like growth factor-1
IGL	Internal granule layer
IL	Interleukin
Irf	Interferon-regulatory factor
IRFs	IFN regulatory factors
ISGs	Interferon-stimulated genes
ISRE	IFN-stimulated response element sequence in promoter
JAK	Janus tyrosine kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MB	Medulloblastoma
MEF	Mouse embryonic fibroblast
Mig	Monokine induced by interferon-gamma

miR-29	MicroRNA-29
MLK	Mixed lineage kinase
mRNA	Messenger ribonucleic acid
NAIP	Neuronal apoptosis inhibitory protein
ND2	Neuro D2
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NGF	Nerve growth factor
NK	Natural killer
P	Postnatal-day
pH3	Phospho-histone H3
PKR	Protein kinase interferon-inducible double stranded RNA dependent
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RIP1	Receptor interacting protein kinase-1
RLR	RIG-I-like receptors
SCG	Superior cervical ganglia
Shh	Sonic hedgehog
SMAC	Second mitochondrial activator of caspases
Smo	Smoothened
SOD	Superoxide dismutase
STAT	Signal transducers and activators of transcription
STS	Staurosporine
tBid	Truncated Bid

Tgfb1i1	Transforming growth factor beta-1-induced transcript 1
TGF-β	Transforming growth factor-β
TLR	Toll-like receptors
TMS-1	Target of methylation-induced silencing-1
TNFα	Tumor necrosis factor alpha
XAF-1	XIAP associated factor 1
XIAP	X-lined inhibitor of apoptosis protein

CHAPTER I: INTRODUCTION

1.1 Apoptosis Overview

Introduction

Cells can die by one of several mechanisms: apoptosis, pyroptosis, autophagy, or necrosis. In apoptosis, the cell degrades its contents via cysteine proteases and compartmentalizes its fragments into apoptotic bodies to be engulfed by phagocytic cells. Thus apoptosis is an orderly way for a cell to die, leaving no trace behind. Apoptosis is required for normal development, maintaining homeostasis, and preventing tumors and autoimmune diseases (Fink and Cookson, 2005; Taylor et al., 2008; Duprez et al., 2009).

Pyroptosis, the newly discovered inflammatory cell death in immune cells is caused by the inflammasome complex activation of cysteine protease caspase-1, resulting in the cell lysing, spilling its contents into the milieu. Released cellular cytokines activate the immune system to respond to the alarm, recruiting immune cells to the site of damage, where they can either promote healing or further destruction. Pyroptosis is important for limiting infection and evoking immune response to pathogen and danger signals (Fink and Cookson, 2005; Duprez et al., 2009).

Autophagy, an intracellular catabolic mechanism that recycles cellular components inside autophagic vacuoles, can promote cell survival but can also induce cell death.

Autophagy-induced cell death is implicated in the demise of salivary gland cells during *Drosophila* metamorphosis (Fink and Cookson, 2005; Duprez et al., 2009).

Necrosis has been the catch-all phrase for uncontrolled, passive cell death involving caspase-1-independent cell lysis. Necrosis is considered a back-up cell death mechanism when other types of cell death are blocked. For instance, when death receptors are activated but apoptosis is blocked by caspases inhibition, a necrosis pathway involving RIP1 (receptor interacting protein kinase -1) called necroptosis ensues. Necrosis also results following TLR (toll-like receptors) or RLR (RIG-I-like receptors) activation by pathogen- or danger-associated signals and occurs in pathological conditions such as myocardial infarction, stroke, or traumatic brain injury. Thus, cells can die by one of several mechanisms, characterized by environmental signal, biochemical pathways, and morphology (Fink and Cookson, 2005; Duprez et al., 2009).

History of apoptosis

Apoptosis was first described in 1842 in the notochord of tadpoles by Carl Vogt, followed by extensive discoveries in chondrocytes, neurons, and other cell types (Clarke and Clarke, 1996). Apoptosis was coined in 1972 by Kerr, Wyllie and Currie for the Greek meaning "falling off" of leaves from trees and was characterized in several human and rat tissues as an active, controlled cell deletion, with cells breaking apart into membrane-bound fragments which are then phagocytosed (Kerr et al., 1972). The absence of cellular debris following cell death is a hallmark of apoptosis which allows a quiet death that does not elicit an immune response (Danial and Korsmeyer, 2004). In 1976, John E. Sulston published his

findings on the programmed death of cells in *C. elegans*, demonstrating that cells reproducibly die during development. Ellis and Horvitz then published in 1986 their findings in *C. elegans* of the first known genes required for apoptosis, *ced-3* and *ced-4* (Ellis and Horvitz, 1986). Since these seminal discoveries, much progress has been made to elucidate the apoptotic machinery and mechanisms in varied organisms and cell types.

Physiological functions of apoptosis

Apoptosis was first discovered as a developmental process, and is required for normal organism development including organogenesis and vestigial organ removal, tissue remodeling including interdigital web removal (Milligan et al., 1995; Sharma et al., 2009), and death of neurons in the immature nervous system (Davies, 2003) (Figure 1.1). Apoptosis is also instrumental in maintaining homeostasis, for example by executing faulty or self-reactive immune cells (Marsden and Strasser, 2003), eliminating cells with a high turnover rate (e.g. red blood cells which die at a rate of 3000 cells per second) (Erwig and Henson, 2007), and post-development tissue remodeling, such as during mammary gland involution which restores mammary epithelial cells to pre-pregnancy number (Erwig and Henson, 2007). Through the elimination of cells in the adult organism, apoptosis is able to balance the number of cells created to maintain homeostasis (Vaux and Korsmeyer, 1999).

In addition to regulating development and homeostasis, apoptosis can either prevent or exacerbate various diseases (Fadeel and Orrenius, 2005). Human embryonic stem cells exposed to DNA damage undergo rapid apoptosis, thereby eliminating chance of propagating mutated cells during embryogenesis (Dumitru et al., 2012). Likewise, epithelial

cells undergo apoptosis following DNA damage (Green and Evan, 2002). Apoptosis of cells infected with virus or bacteria limits the spread of infection (Dafny and Yang, 2005; Maher et al., 2007). Finally, immune cells induce apoptosis in tumor cells to block cancer development (Wang and El-Deiry, 2003). Thus, apoptosis eliminates infected, dysfunctional, or damaged cells to promote the health of the organism (Carson and Ribeiro, 1993).

When apoptosis does not occur normally, cells that have accumulated mutations may proliferate uncontrollably, becoming tumor cells. If these tumor cells escape apoptosis by evading immune surveillance, they may develop into cancers. Furthermore, some mutations can suppress the apoptotic pathway or enhance survival pathways, thereby rendering the tumor cell resistant to external apoptotic signals (Martin, 2003; Yu and Zhang, 2003; Wright and Deshmukh, 2006). Inadequate apoptosis of immune cells can also cause systemic autoimmunity (Siegel et al., 2000).

While insufficient apoptosis may lead to pathology, increased apoptosis can also occur in disease states. Reperfusion following an ischemic event induces apoptosis in cardiac or brain tissue (Honda and Ping, 2006; Jung et al., 2010). Also, many neurodegenerative diseases are characterized by apoptosis of mature neurons, which normally live for the lifetime of the organism (Honig and Rosenberg, 2000; Gorman, 2008; Nakamura et al., 2012). Neurotoxins released in neurodegenerative disease states induce apoptosis in neurons (Gorman, 2008). Blocking apoptosis can reduce the deleterious effects of diseases. Repressing apoptosis in models of traumatic brain injury, stroke, or ALS (amyotrophic lateral sclerosis) reduces cell death and improves clinical outcome (Hara et al.,

1997; Hara et al., 1997; Kostic et al., 1997; Yakovlev et al., 1997). Furthermore, inhibiting apoptosis in telencephalic neurons exposed to fibrillar amyloid beta, a hallmark of Alzheimer disease, prevented neuron death (Selznick et al., 2000). Thus, apoptosis can be both a cause and a consequence in many pathologies.

Apoptotic mechanisms

In 1986, Ellis and Horvitz demonstrated a requirement for *ced-3* and *ced-4* genes in normal developmental apoptosis in *C. elegans* (Ellis and Horvitz, 1986). The Horvitz lab later discovered the requirement of *ced-9* "to protect cells that normally survive from undergoing programmed cell death" (Hengartner and Horvitz, 1994). Ced-9 prevents Ced-4 from activating Ced-3 to subsequently cause apoptosis (Hengartner et al., 1992; Danial and Korsmeyer, 2004). The mammalian homologs of the nematode genes were determined to be *BCL-2* (*B-cell lymphoma 2*; *Ced-9*), *Caspase-3* (*Ced-3*), and *Apaf-1* (*Ced-4*) (Danial and Korsmeyer, 2004). Sequence homology analysis to *Ced-3* led to the discovery of an entire family of caspases, inactive zymogens that, upon activation, become cysteine proteases that cleave aspartic acid motifs. Apoptotic caspases, including caspases-8, -9, and -3, degrade a multitude of cellular substrates to cause the apoptotic morphology characterized by cell shrinkage, membrane blebbing, DNA fragmentation, and chromatin condensation (Danial and Korsmeyer, 2004).

After several decades of research since the discovery of the nematode genes, the major players in the apoptotic pathway have been elucidated. Apoptosis can occur due to extrinsic or intrinsic signals. Extrinsic signals such as Fas, TRAIL, or TNFα bind to their

respective receptors, inducing activation of the extrinsic apoptotic pathway. Ligand-receptor binding activates caspase-8, which cleaves Bid into tBid, which translocates to the mitochondria to cause mitochondrial permeabilization and cytochrome *c* release (Ozoren and El-Deiry, 2003; Riedl and Shi, 2004). Since our lab studies intrinsic apoptosis, this section will focus on the intrinsic, or mitochondrial, apoptotic pathway.

In intrinsic apoptosis, signals from DNA damage, trophic factor deprivation, ER stress, or other insults trigger the activation of pro-apoptotic BH3-only proteins through upregulation or phosphorylation (Vaux and Korsmeyer, 1999). Pro-apoptotic BH3-only proteins are in the Bcl-2 family and contain a single BH3 (Bcl-2 homology -3) domain. Examples of BH3-only proteins are Bim, Bad, Bid, Puma, Noxa, Bmf, and DP5 (Chao and Korsmeyer, 1998). Anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-XL, and Bcl-w contain BH domains 1-4 and are localized at the mitochondrial membrane where they prevent mitochondrial permeabilization by associating with pro-apoptotic proteins Bax and Bak (Cory and Adams, 2002). Bax and Bak are Bcl-2 family members that contain BH domains 1-3 (Danial and Korsmeyer, 2004). BH-3 only proteins, such as Bim and Bid, can be “activators" by binding and activating Bax/Bak directly through induction of an allosteric conformational change (Figure 1.2). Other BH-3 only proteins, including Bad and BMF, are “sensitizers” that compete for binding to anti-apoptotic Bcl-2 and Bcl-XL, preventing their suppression of Bax/Bak (Letai et al., 2002; Deng et al., 2007; Ren et al., 2010). When released from repression by Bcl-2 and Bcl-XL, Bax and Bak undergo a conformational change, inserting and oligomerizing in the mitochondrial membrane, forming a pore in the

outer membrane that then releases cytochrome *c*, Smac, AIF, and other mitochondrial proteins (Danial and Korsmeyer, 2004; Chipuk and Green, 2008).

In the mitochondria, cytochrome *c* transfers electrons as an essential part of the electron transport chain. When cytochrome *c* is released from the mitochondria into the cytosol, it binds to the WD40 repeat domain of Apaf-1 (Figure 1.3). Binding of cytochrome *c* to Apaf-1 in the presence of dATP releases the autoinhibition of WD40 and induces a conformational change which exposes the CARD (caspase activation and recruitment domain) of Apaf-1. The CARD domain recruits procaspase-9 via homotypic CARD binding. This tripartite structure composed of cytochrome *c*, Apaf-1, and procaspase-9 oligomerizes as a heptameric apoptosome, a ~700 kDa structure that activates procaspase-9 through autocatalytic cleavage (Srinivasula et al., 1998; Acehan et al., 2002; Cain et al., 2002; Riedl et al., 2005).

Caspase-9, once activated by the apoptosome, induces a cascade of caspase activation. Caspase-9 activates caspase-3/7 and indirectly activates caspase-6. Caspase 3 then can activate caspase-2/6, which then activate caspase-8/10. Caspase 3 also can activate caspase-8/9, escalating a positive feedback loop of caspase activating amplification (Creagh et al., 2003; Riedl and Shi, 2004).

Caspases are cysteine proteases that exist as zymogens until activated. While individual caspases can have many substrates, each caspase cleaves a specific substrate sequence of four residues amino-terminal to an aspartic acid motif (Hengartner, 2000). Initiator caspases include caspase-9, -8, -2 and -10, which are activated directly by the apoptotic machinery to initiate the caspase cascade (Figure 1.4). Initiator caspases have a

CARD or DED (death effector domain) N-terminal prodomain that allows for homotypic protein interactions (Riedl and Shi, 2004; Boucher and Denault, 2012). Initiator caspases activate effector caspases, such as caspase-3, -6 and -7, which cleave an array of substrates to produce the morphology of apoptosis- membrane blebbing, DNA fragmentation, cell shrinkage, and chromatin condensation (Thornberry and Lazebnik, 1998; Riedl and Shi, 2004).

Interestingly, caspases can have nonapoptotic functions, for instance caspase-3 plays a role in differentiation of erythroblasts and inhibition of B-cell proliferation (Nhan et al., 2006; Lamkanfi et al., 2007). Furthermore, additional caspases exist that do not play a role in apoptosis. Caspase-1, originally known as ICE (interleukin-1β converting enzyme) was first discovered as the homolog to the *C. elegans* gene *ced-3*, which promoted apoptosis in nematodes, yet caspase-1 has been determined to have a role not in apoptosis, but in inflammation (Riedl and Shi, 2004). Likewise, caspase-4, -5, (human) and -11 (mouse) are also activated primarily in inflammatory responses (Creagh et al., 2003). Inflammatory caspases have an N-terminal CARD or DED prodomain for homotypic protein binding (Figure 1.4) (Creagh et al., 2003; Martinon and Tschopp, 2007).

Following apoptosome formation and initiation of the caspase activation cascade, apoptotic caspases can be blocked by inhibitors of apoptosis proteins (IAPs). Eight mammalian IAPs have been discovered thus far and include XIAP (X-linked IAP), NAIP (neuronal apoptosis inhibitory protein), c-IAP1, c-IAP2, ML-IAP (melanoma IAP)/Livin, Survivin, ILP2 (IAP-like protein-2), and Bruce. IAPs contain a baculoviral IAP repeat (BIR) domain and can block caspases by binding directly or targeting them to the ubiquitin-

proteasome pathway for degradation. XIAP is the only mammalian IAP that has been shown to bind caspases directly. XIAP mediates caspase inhibition by binding a tetrapeptide motif on active caspases directly through one of its BIR domains and sterically blocking substrates from binding with the active caspases. Research also suggests that XIAP may ubiquitinate caspases through its C-terminal RING finger domain, which has E3 ubiquitin ligase activity (Riedl and Shi, 2004; Eckelman et al., 2006).

Inhibitors of apoptosis proteins themselves have an inhibitor: Smac/DIABLO. Smac is a mitochondrial protein that is released with cytochrome *c* into the cytosol following mitochondrial permeabilization. The IAP-binding tetrapeptide motif in Smac competes with caspases for binding to the BIR domain of IAPs, allowing both initiator and effector caspases to remain unimpeded (Hengartner, 2000; Riedl and Shi, 2004).

1.2 Neuronal Apoptosis

Introduction

Sensory neurons were first discovered to undergo elimination in 1889, followed by a discovery of spinal ganglion cell death in chick embryos in 1906 (Clarke and Clarke, 1996). Since then, scientists have discovered that apoptosis occurs throughout the nervous system- in spinal cord, the retina, cerebellum, cortex, brain stem, and sensory and autonomic ganglia (Buss et al., 2006). The importance of apoptosis for normal nervous system development is revealed upon removal of any single gene required for apoptosis. Apaf-1, caspase-9, or caspase-3 knockout animals have enlarged brains and a high incidence of embryonic lethality (Kuida et al., 1996; Cecconi et al., 1998; Kuida et al., 1998; Buss et al., 2006). Between 20 and 80% of all neurons that are born undergo cell death during development (Oppenheim, 1991). Deletion of neurons may correct errors in cell migration, axonal pathfinding, or target innervation, and thus refine functional nervous system circuitry (Buss et al., 2006). Furthermore, apoptosis ensures that neurons that are outcompeted for limited trophic factors via target innervation are eliminated (Buss et al., 2006). In summary, neuronal apoptosis is required for proper wiring and development of the nervous system.

Sympathetic neurons

In the sympathetic nervous system, superior cervical ganglia (SCG) innervate the eye, and salivary and tear glands (Vinken and Bruyn, 1999). Sympathetic neurons of the SCG extend axons to their targets which secrete neurotrophic growth factor (NGF). NGF

was the first neurotrophic factor to be discovered and is required by sympathetic neurons for survival (Thoenen and Edgar, 1985). When neutralizing antibodies block this single growth factor *in vivo*, 99 % of the sympathetic neurons in young mouse SCGs are ablated (Levi-Montalcini and Booker, 1960).

Sympathetic neurons can be studied *in vitro* as well since primary sympathetic neurons from rat or mouse SCG can be dissociated and maintained in very pure cultures. NGF deprivation causes virtually all neurons to undergo apoptosis within 48 hours, with the classic features of DNA fragmentation, chromatin condensation, neurite degeneration, and membrane blebbing. Because these cells are able to be maintained in pure cultures and also undergo apoptosis in response to one signal, sympathetic neurons are an ideal model with which to study the apoptotic pathway (Deshmukh and Johnson, 1997; Rubin, 1997).

During development, immature sympathetic neurons extend their axons to their NGF-secreting targets. Sympathetic neurons either fail to reach their target, thus undergoing NGF-deprivation-induced apoptosis, or the neurons innervate their targets and mature, losing sensitivity to NGF deprivation with maturation around P11 (Glebova and Ginty, 2005). The sympathetic neuron experiments presented in this dissertation were conducted in young (P5) neurons, which undergo apoptosis in response to NGF deprivation.

The presence of NGF stimulates activation of pro-survival pathways, while the absence of NGF triggers an active pro-apoptotic response (Figure 1.5). NGF phosphorylates the tyrosine kinase receptor, TrkA on sympathetic neuron axon terminals, activating the PI-3-kinase/Akt pathway which promotes cell survival (Brunet et al., 2001). In the absence of NGF, an active signaling pathway requiring transcription and translation executes apoptosis

(Martin et al., 1988; Deshmukh and Johnson, 1997). NGF deprivation triggers activation of Rho GTPase Cdc42, which leads to activation of the MLK (mixed lineage kinase) and MAPK (mitogen-activated protein kinase) pathways. MKK4/MKK7 activate JNKs including JNK3, which phosphorylate c-Jun at serine residue 63 (Xia, Dickens et al. 1995; Eilers, Whitfield et al. 1998; Maroney, Finn et al. 1999; Ham, Eilers et al. 2000; Bruckner, Tammariello et al. 2001). Ser63 phosphorylation activates transcription factor activity of c-Jun, which induces transcription of c-Jun and BH3-only genes including Bim, DP5, Puma, and Bmf (Putcha, Moulder et al. 2001; Imaizumi, Benito et al. 2004; Kole, Swahari et al. 2011; Kristiansen, Menghi et al. 2011). Upregulation of the BH3-only proteins is required to block Bcl-2 and Bcl-XL and promote Bax activation. Active Bax oligomerizes and forms a pore in the mitochondrial outer membrane, releasing cytochrome c into the cytosol, as described above (see previous section). Cytochrome c binds to Apaf-1, initiating apoptosome formation and subsequent caspase activation. Blocking any step of this pathway inhibits apoptosis in NGF-deprived sympathetic neurons. Thus, NGF deprivation executes mitochondrial permeabilization and exploits the intrinsic apoptotic pathway in sympathetic neurons.

Apoptosis inhibition in sympathetic neurons

Sympathetic neurons have several mechanisms by which they can suppress key players of the apoptotic pathway. Firstly, neurons have a truncated form of Bak, N-Bak, which lacks BH3 domains necessary for pro-apoptotic activity. Thus, neurons rely solely on Bax for mitochondrial permeabilization (Deckwerth et al., 1998) and loss of Bak expression

does not alter neuronal death with NGF deprivation (Putcha et al., 2002; Sun et al., 2003; Uo et al., 2005). Furthermore, mature neurons upregulate miR-29, which blocks translation of BH3-only genes in these cells (Kole et al., 2011). MiR-29-mediated restriction of BH3-only gene expression prevents Bax activation, thus providing another obstacle to apoptosis (Kole et al., 2011).

Sympathetic neurons also restrict apoptosis at the point of cytochrome *c* release. In healthy young neurons, cytochrome *c* is inactivated by the reduced environment. Thus tBid microinjection induces apoptosis in MEFs (mouse embryonic fibroblasts) but no death in sympathetic neurons (Vaughn and Deshmukh, 2008). NGF deprivation in neurons generates reactive oxygen species which oxidize the cytosol, allowing cytochrome *c* to become activated and cause programmed cell death (Vaughn and Deshmukh, 2008). Autophagy-independent degradation may be another mechanism to suppress cytochrome *c* (Davidescu et al., 2012).

Once functional cytochrome *c* is released, Apaf-1 binding is required for apoptosome formation. Apaf-1 protein levels have been shown to decrease with development (P3 vs E16) and are further reduced with maturation (Wright et al., 2004) in sympathetic neurons *via* chromatin repression (Wright et al., 2007). Apaf-1 levels are also decreased in PC12 cells with neuronal-like differentiation (Wright et al., 2004). Apaf-1 expression is required for apoptosis in sympathetic neurons (Wright et al., 2007) and some types of apoptotic insults, such as DNA damage, upregulate Apaf-1 mRNA and protein to promote apoptosis (Vaughn and Deshmukh, 2007).

Following apoptosome formation, XIAP serves as a formidable opponent to caspase activation in sympathetic neurons. Caspase-9 and -3 are required for sympathetic neuron apoptosis with NGF deprivation (Wright et al., 2007) and are rendered incompetent by XIAP (Vaughn and Deshmukh, 2008). Endogenous Smac released from the mitochondria is insufficient to block XIAP in sympathetic neurons (Vaughn and Deshmukh, 2008), yet microinjection of excess Smac along with cytochrome *c* enables XIAP neutralization and death (Potts et al., 2003). Thus, endogenous Smac does not serve as a brake to XIAP inhibition of caspases in sympathetic neurons. To remove XIAP, neurons degrade XIAP mRNA and protein in response to NGF deprivation, enabling these cells to undergo apoptosis (Potts et al., 2003; Vaughn and Deshmukh, 2007). Thus, even at the level of caspase activation, sympathetic neurons have developed an important guardian of cell survival, XIAP, to constrain apoptosis.

By utilizing these hurdles to inhibit apoptosis at every step of the pathway, sympathetic neurons ensure survival in instances of slight damage, cytochrome *c* release, or caspase activation. These brakes prevent accidental initiation of apoptosis yet enable cell death warranted by significant insult. This remarkable resistance to apoptosis ensures the survival of these cells which must persist for the lifetime of the organism.

1.3 Medulloblastoma

Medulloblastoma (MB), a tumor of cerebellar progenitors, is the most common malignant brain cancer in children (Hatten and Roussel, 2011), with 20% of pediatric central nervous system tumors, or 540 cases (0.6 per 100,000 children 0-19 years old) in the U.S. diagnosed each year (Polkinghorn and Tarbell, 2007; Lau et al., 2012). Symptoms of medulloblastoma include morning vomiting, ataxia, and headaches. 30% of childhood cases show cerebrospinal fluid metastasis at diagnosis (Polkinghorn and Tarbell, 2007). 5 year overall survival rates are approximately 60%, yet side effects from radiation treatment include cognitive impairment (reduction of up to 30 IQ points), psychiatric illness, bone growth retardation, hearing loss, and endocrine disruption (Polkinghorn and Tarbell, 2007; Ellison, 2010; Roussel and Hatten, 2011).

In the past, MBs have been classified into histological categories including classic, desmoplastic, large cell/anaplastic (LC/A), and medulloblastoma with extensive nodularity (MBEN) (Gilbertson and Ellison, 2008; Roussel and Hatten, 2011). Recent research, however, has used molecular characteristics (mutations, deletions, amplifications, etc.) to categorize tumors based on the specific pathway altered, including SHH (Sonic Hedgehog), WNT/β-catenin, MYC, Notch, BMP, and/or TGF-β signaling pathways (Marino, 2005; Polkinghorn and Tarbell, 2007; Ellison, 2010; Gibson et al., 2010; Cho et al., 2011; Hatten and Roussel, 2011; Roussel and Hatten, 2011; Northcott et al., 2012). Amplification of the SHH pathway is the best characterized of these subgroups, with 25% of MBs having mutations in *Patched*, *Sufu* (*Suppressor of Fused Homolog*), *Smoothened*, or other genes in the pathway (Marino, 2005; Gibson et al., 2010). During normal development, activation of

the SHH pathway stimulates proliferation of cerebellar granule neuron progenitors (CGNPs), which undergo rapid division in the external granule layer (EGL), then differentiate and migrate to the internal granule layer (IGL) during the early postnatal period (Hatten and Heintz, 1995). In medulloblastoma, overactive SHH signaling induces CyclinD1 and CyclinD2 expression which promote the hyperproliferation of CGNPs. Mutations that activate SHH signaling cause predisposition to medulloblastoma in humans with Gorlin Syndrome and in genetically engineered mouse models (Polkinghorn and Tarbell, 2007; Hatten and Roussel, 2011). These models, which operate through either *Patched* deletion or insertion of constitutively active alleles of *Smoothened*, consistently implicate CGNPs as the cells of origin for SHH-driven medulloblastoma (Hallahan, Pritchard et al. 2004; Yang, Ellis et al. 2008).

In conclusion, while advances in treatment have increased the survival of medulloblastoma patients, mortality remains significantly high (30%) and debilitating side effects result from current treatment regimens (Polkinghorn and Tarbell, 2007; Jones et al., 2012). Thus, advancing the understanding of the genetic components of medulloblastoma tumorigenesis is needed to develop improved targeted therapies.

1.4 Figures and Legends

Figure 1.1. **Syndactylyl is caused by insufficient apoptosis**

Apoptosis is required for normal development, including cell death of interdigital webbing.

Figure 1.1

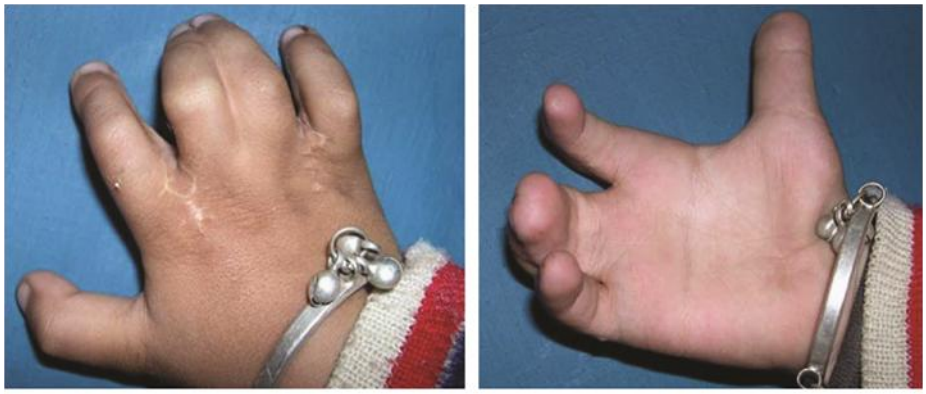


Figure adapted from Sharma et al., *Hand* (2009)

Figure 1.2. **The Bcl-2 family**

The Bcl-2 family is comprised of pro-apoptotic Bax and Bak, anti-apoptotic Bcl-2 and Bcl-XL, and sensitizer or activator BH3-only proteins.

Figure 1.2

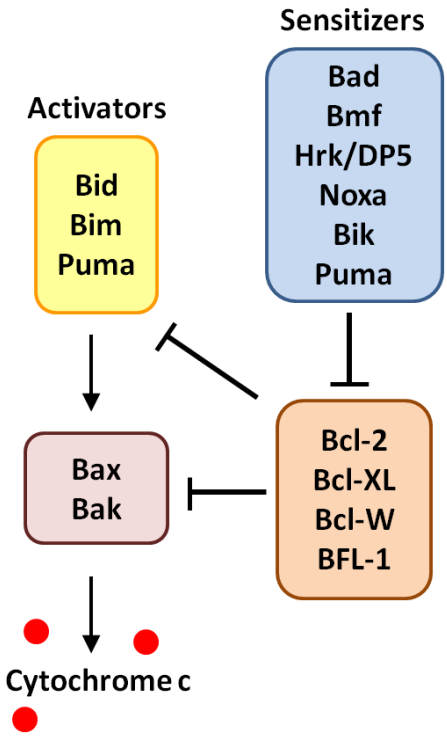

Sensitizers
Bad
Bmf
Hrk/DP5
Noxa
Bik
Puma
Activators
Bid
Bim
Puma
Bax
Bak
Bcl-2
Bcl-XL
Bcl-W
BFL-1
Cytochrome c

Figure 1.3. Formation of the apoptosome complex

Cytochrome *c* binds to the WD40 domain of Apaf-1, inducing a conformational change that releases auto-inhibition and recruits procaspase-9 to the CARD domain of Apaf-1.

Figure 1.3

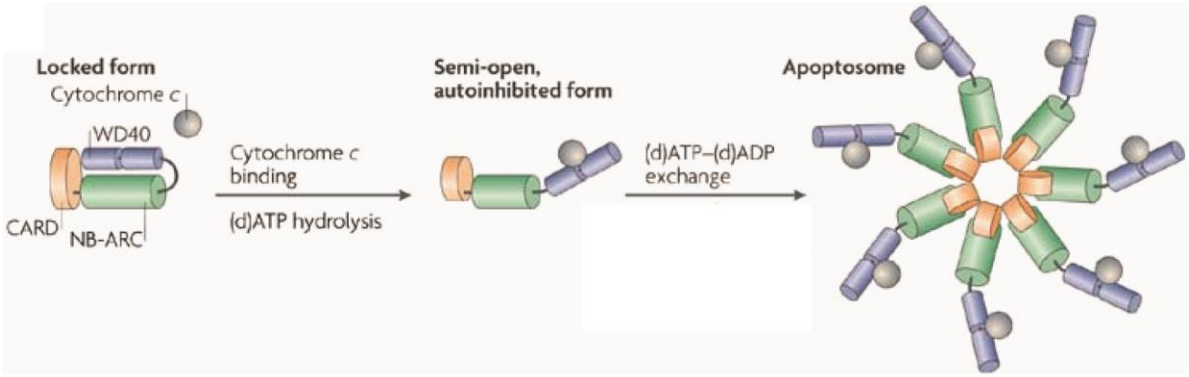


Figure adapted from Riedl and Salveson, Nature Reviews Mol Cell Bio (2007)

Figure 1.4. The Caspase family

The caspases were discovered by sequence homology analysis with nematode gene *ced-3*. Caspases have a primary role as initiators or executioners of apoptosis, or in inflammation. Executioner proteins lack an N-terminal protein interaction domain such as CARD or DED. The family of murine caspases is below.

Figure 1.4

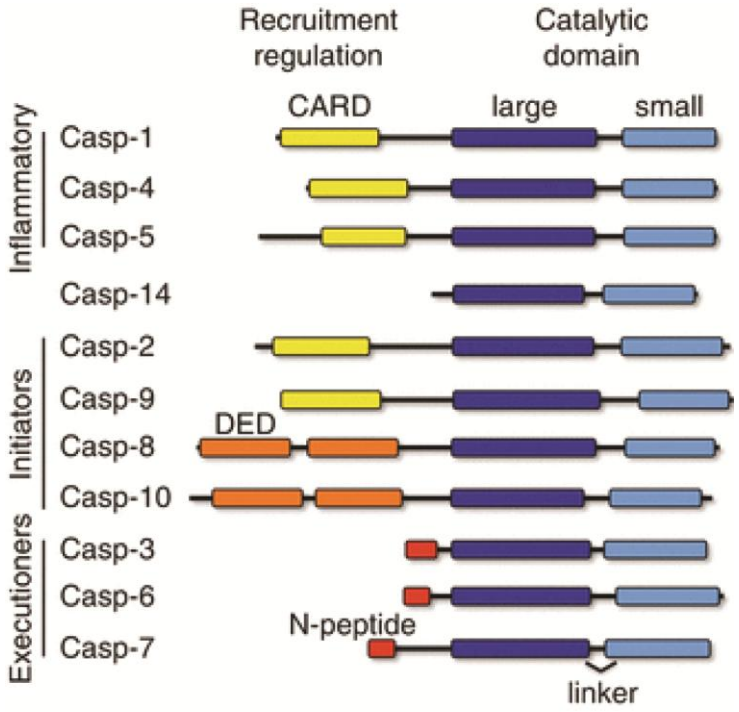
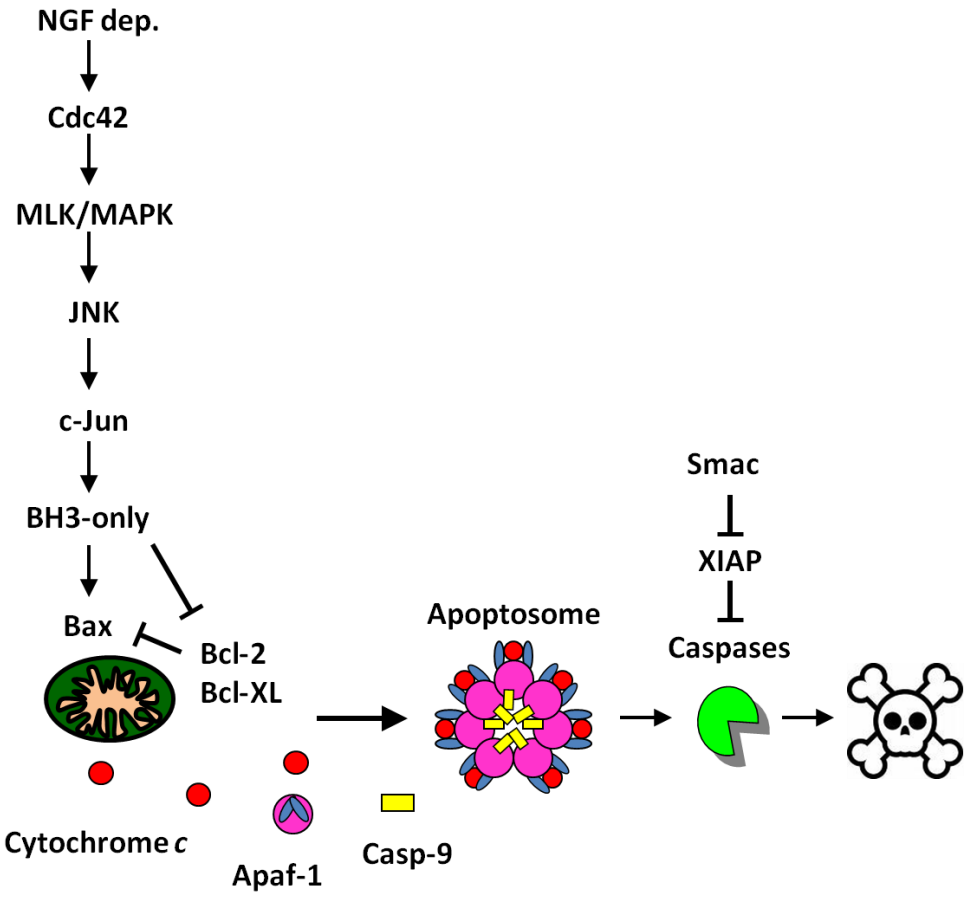


Figure adapted from Boucher and Denault, Encyclopedia of Signaling Molecules (2012)

Figure 1.5. The NGF deprivation apoptotic pathway

NGF absence activates Rho GTPase Cdc42, which activates MLK and MAPK pathways, phosphorylating JNK. JNK phosphorylates c-Jun, which upregulates the BH3-only genes. BH3-only genes block Bcl-2 and Bcl-XL and activate Bax. Bax oligomerizes at the mitochondrial membrane, forming a pore which releases cytochrome *c* into the cytosol. Cytochrome *c* binds the WD40 domain of Apaf-1, which induces apoptosome formation with procaspase-9, activating caspase-9. If caspases escape XIAP inhibition, caspase-9 initiates a positive feedback loop of caspase activation.

Figure 1.5


NGF dep.
Cdc42
MLK/MAPK
JNK
c-Jun
BH3-only
Bax
Bcl-2
Bcl-XL
Cytochrome c
Apaf-1
Casp-9
Apoptosome
Smac
XIAP
Caspases

CHAPTER II: INTERFERON-GAMMA PROTECTS SYMPATHETIC NEURONS FROM APOPTOSIS AT THE POINT OF CYTOCHROME *C* RELEASE

2.1 Overview

While immune responses during nervous system injury and disease are well studied, exactly how primary neurons respond to immune signals is still largely unknown. We find that primary sympathetic neurons respond unexpectedly to interferon-gamma (IFN-γ), a cytokine released by immune cells in response to infection. While IFN-γ induces apoptosis in many cell types, it has the opposite effect on sympathetic neurons by protecting them from apoptotic stimuli. We found that IFN-γ addition enabled sympathetic neurons to become resistant to nerve growth factor (NGF) deprivation- or pan-kinase inhibition-induced apoptosis. In investigating how IFN-γ modulates the apoptotic pathway, we discovered that c-Jun phosphorylation and Bim induction in response to NGF deprivation were unchanged with IFN-γ. Downstream of the mitochondria, however, IFN-γ blocked cytochrome *c* release and caspase-3 activation in NGF-deprived neurons. Microinjection of cytochrome *c* into XIAP$^{-/-}$ neurons revealed no difference in cell death with IFN-γ addition, demonstrating a role for IFN-γ at the point of mitochondria permeabilization. These results identify Bax activation as the likely point at which IFN-γ acts to inhibit neuronal apoptosis. Finally, microarray analysis revealed that sympathetic neurons respond to IFN-γ by upregulating interferon-inducible genes and several pro-apoptotic genes. Together, our

results show that IFN-γ is not only incapable of inducing apoptosis in neurons but remarkably enables neurons to become resistant to apoptosis. As sympathetic neurons become exposed to IFN-γ during infection or injury, the ability of IFN-γ to inhibit apoptosis in neurons is likely important for ensuring the long-term survival of neurons during situations of pathological stress.

2.2 Introduction

Overview of Interferons

A major mechanism of cellular host response to pathogenic infection is the secretion of interferons (IFNs). IFNs are upregulated and released by cells to inhibit pathogen replication and regulate activation of immune cells. In particular, IFNs are known to induce apoptosis of both infected cells and pathogens to limit the spread of infection (Dafny and Yang, 2005; Maher et al., 2007). Additionally, IFNs modulate angiogenesis and regulate cell growth, proliferation, and differentiation (Chawla-Sarkar et al., 2003; Maher et al., 2007). IFNs also exert these functions during injury and in tumor suppression (Chawla-Sarkar et al., 2003; Maher et al., 2007). IFNs are classified into different subtypes where Type I IFNs (IFN-α and IFN-β) are produced by almost all cell types, with hematopoietic cells being major producers of IFN-α and fibroblasts being the main source of IFN-β (Schroder et al., 2004; Maher et al., 2007). Th1 lymphocytes and natural killer (NK) cells are the major source of Type II IFN, IFN-γ (Chawla-Sarkar et al., 2003; Schroder et al., 2004; Dafny and Yang, 2005; Maher et al., 2007). Type III IFN, IFN-λ, was recently discovered through sequence homology analysis and is not well characterized (Maher et al., 2007).

The signaling pathway for IFN production has been well characterized in the context of the immune system. IRFs (IFN regulatory factors) induce interferon production in response to pathogen-associated molecular patterns or other signals (Maher et al., 2007). IFN-α/β bind IFNAR (IFN-α receptor) and IFN- γ binds to IFNGR (IFN-γ receptor), but both types phosphorylate and activate the JAK (Janus tyrosine kinase)/STAT (signal transducers and activators of transcription) pathway (Schroder et al., 2004). STATs bind to ISRE (IFN-

stimulated response element sequence in promoter) or GAS (gamma interferon-activated sequence) sequences to mediate the regulation of at least 300 interferon-stimulated genes (ISGs) (Dafny and Yang, 2005; Maher et al., 2007). The activation of the JAK/STAT pathway mediates the antiproliferative, antiviral, and immunomodulatory functions of interferons (Maher et al., 2007). Phosphorylation of JAK leads to downstream activation of the PI3K pathway (Akt, mTOR), insulin and IGF-1 receptor signaling, and also p38, JNK (c-Jun N-terminal kinases) and ERK (extracellular signal regulated kinases) MAPKs (mitogen-activated protein kinases) (Kalvakolanu and Roy, 2005; Maher et al., 2007; Hervas-Stubbs et al., 2011). Interferon activation can also lead to NF-κB activation (Hervas-Stubbs et al., 2011).

Interferons regulate apoptosis

In addition to inhibiting translation and transcription in cells to suppress expression of viral genes and proteins, interferons also induce apoptosis of infected or surrounding cells through gene regulation in a variety of cell types to limit spread of infection. Through upregulation of genes involved in the apoptotic pathway and other genes that induce cell death through unknown mechanisms (DAPs, IRFs, RIDs, RNaseL), the interferons induce apoptosis in many cell types including endothelial cells (Chawla-Sarkar et al., 2003), keratinocytes (Maher et al., 2007), hepatocytes (Barber, 2000; Chawla-Sarkar et al., 2003), oligodendrocytes (Baerwald and Popko, 1998), fibroblasts (Dijkmans et al., 1990), and many types of cancer cells (Barber, 2000; Becher et al., 2000; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005; Maher et al., 2007; Zhang et al., 2008; Das et al., 2009). Additionally, interferons can also sensitize cells to apoptosis by viral

infection or apoptotic stimuli through upregulation of apoptotic genes (Choi et al., 1999; Sedger et al., 1999; Nishikawa et al., 2001; Chawla-Sarkar et al., 2003; Clemens, 2003; Maher et al., 2007; Gysemans et al., 2008).

Although interferons are considered apoptosis-inducing cytokines (Chawla-Sarkar et al., 2003; Schroder et al., 2004) there is evidence that interferons may inhibit apoptosis in activated T cells and primary B cells (Barber, 2000; Clemens, 2003; Pokrovskaja et al., 2005). Mechanisms for an anti-apoptotic response include blocking cell cycle (p21 upregulation in macrophages (Barber, 2000; Chawla-Sarkar et al., 2003; Clemens, 2003), activation of NF-κB (Clemens, 2003; Hervas-Stubbs et al., 2011), PI3K activation (Clemens, 2003), or upregulation of *ifi202b* (interferon-inducible protein 202b), which modulates activity of multiple transcription factors (Koul et al., 1998; Clemens, 2003).

Interferons in neuronal apoptosis

While interferons overwhelmingly exert a pro-apoptotic effect on most cell types, there is evidence for a mixed outcome in neurons. IFN-γ transgenic HSV-infected mice displayed reduced neuronal apoptosis than wild-type mice, while IFN-$\gamma^{-/-}$ mice exhibited greater neuronal death and exacerbated encephalitis and ataxia. In both cases, increased IFN-γ signaling corresponded to increased neuronal expression of Bcl-2 and these effects were independent of viral replication and neuroinvasion (Geiger et al., 1994; Geiger et al., 1995; Geiger et al., 1997). Similarly, IFN-$\gamma^{-/-}$ mice infected with Borna disease virus (BDV) exhibited increased hippocampal neuronal apoptosis during CD8+ T cell responses than wild-type mice (Richter et al., 2009). Other research shows that IFN-α/β sensitizes

fibroblasts, but not dorsal root ganglia (DRG) neurons to cell death by poly(I:C) (Yordy et al., 2012).

Conversely, other research indicates that interferons can induce apoptosis in neurons. Type I IFN inhibited BDNF (brain-derived neurotrophic factor)-mediated survival and neurite outgrowth of primary mouse cortical neurons, which was associated with caspase activation and reduced TrkB, Akt, and ERK1/2 signaling (Dedoni et al., 2012). *In vitro*, IFN-γ either induced apoptosis in motoneurons (Smith et al., 2009; Aebischer et al., 2011) or had no effect (Mir et al., 2009), however *in vivo*, spinal cord motoneurons in untreated IFN-$\gamma^{-/-}$ mice were positive for apoptotic markers (TUNEL, active Casp3), unlike wild-type mice (Victorio et al., 2010). Another study found that IFN-γ does not induce death of cortical, hippocampal, DRG, or striatal neurons but protects motoneurons from death induced by mutant SOD astrocyte media (Aebischer et al., 2011). Thus, interferons' effects on neuron survival can be protective, neutral, or deleterious and further research is needed to elucidate the role of interferons in neuronal apoptosis.

Interferons and sympathetic neurons

Sympathetic neurons of the peripheral nervous system may be particularly exposed to infectious microorganisms and immune cells (Rottenberg and Kristensson, 2002), as some pathogens first invade sensory nerves and spread to the brain via centripetal axonal transport (Rottenberg and Kristensson, 2002). DRG (dorsal root ganglia) and SCG (sympathetic cervical ganglia) express interferon receptors and interferons protect peripheral neurons from viral and bacterial infection (Tsukamoto and Price, 1982; Chang et

al., 1990; Rottenberg and Kristensson, 2002; Burdeinick-Kerr et al., 2009; Mitchell et al., 2012). Additionally, Chang *et al*. found that rat sympathetic neurons exposed to IFN-γ are resistant to apoptosis induced by nerve growth factor (NGF) deprivation (Chang et al., 1990). In this study, we show that IFN-γ suppresses apoptosis in sympathetic neurons treated with nerve growth factor deprivation or pan-kinase inhibition. Importantly, we report that IFN-γ acts at the point of mitochondrial permeabilization to suppress the intrinsic apoptotic pathway in NGF-deprived neurons. Interestingly, IFN-γ exerts this protective effect on sympathetic neurons despite inducing the expression of several pro-apoptotic genes. These findings point to inhibition of Bax activation as the likely mechanism by which this immune cytokine inhibits sympathetic neuron apoptosis.

2.3 Methods

Cell Culture:

Primary sympathetic neurons were dissected from the superior cervical ganglia of P0-1 wild-type ICR or XIAP-deficient C57BL/6 mice, dissociated and maintained until P5 equivalent, as previously described (Potts et al., 2003). At P5-6, neurons were untreated or treated with NGF deprivation, pan-kinase inhibition with 100 nM staurosporine (STS), DNA damage with 20 μM etoposide, or ER stress with 2.5 μM tunicamycin (TUN). NGF deprived neurons were washed three times and then maintained in NGF-deficient media with anti-NGF neutralizing antibody. Unless otherwise indicated, 500 U/ml IFN-γ was added simultaneously with apoptotic insult, with boiled IFN-γ serving as a negative control.

Immunofluorescence:

Neurons were fixed with 0.4 % paraformaldehyde at 12 hours for phospho-c-Jun, 24 hours for cleaved caspase-3, or 48 hours for cytochrome *c*. Pan-caspase inhibitor 25 μM Q-VD-OPh (MP Biomedicals) was added during cytochrome *c* immunofluorescence experiments. Immunofluorescence staining was performed as previously described (Deshmukh and Johnson, 1998). Primary antibodies used were anti-cytochrome *c* (#556432, BD Biosciences; 1:100 dilution), anti-phospho-c-Jun-Ser63 (#9261, Cell Signaling; 1:1000 dilution), and cleaved caspase-3 (1:100, Cell Sig.). Secondary antibodies were Cy3 (1:400) or Alexa 488 (1:1000)-conjugated antibodies (Jackson Immunoresearch Laboratories Inc.; 1:400 dilution). Nuclei were counterstained with 4'6-diamino-2-phenylindole (DAPI) in mounting media (Vector Laboratories) or Hoechst 33258 (Molecular Probes).

Western blot analysis:

Mouse cerebella and tumors were homogenized in RIPA buffer and protein concentration was determined by Pierce BCA assay (ThermoScientific, Waltham, MA, USA). Immunoblotting was performed as previously described (Deshmukh et al., 2002) or by using a SNAP ID device (Millipore) per protocol. Blots were probed with antibodies to Apaf-1 (1:500, Alexis), XIAP (1:500, MBL), β-actin (Sigma, 1:10000), Bax (1:500, Santa Cruz Biotech), Bcl-XL (1:1000, Cell Sig.), Bim (1:1000, Cell Sig.), cleaved caspase-3 (1:500, Cell Sig.), procaspase-3 (1:1000,Stressgen), phospho-c-Jun (1:500, Cell Sig.), Puma (1:1000,Cell Sig.), alpha-tubulin (Sigma-Aldrich), and Smac (R&D). Antibody conjugates were visualized by chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK).

Microinjection:

$XIAP^{-/-}$ sympathetic neurons were injected with (8 µg/µL) rhodamine-dextran (Sigma) with or without 15 mg/ml bovine cytochrome *c* as previously described (Potts et al., 2003). 500 U/ml IFN-γ was added where indicated to plates 24 hours before injection.

Quantitation of cell survival:

Neuron survival was assessed by morphology immediately at treatment or microinjection and at various time points. Survival was assessed by counting clearly identifiable neurons with intact phase-bright cell bodies, whereas dead neurons were atrophied. Cell survival was expressed as a percentage of the original number of treated or

microinjected cells. This method of assessing survival correlates well with other cell survival assays such as staining with calcein AM or trypan blue exclusion (Potts et al., 2003). Data presented are mean ± s.e.m analyzed by Student's *t*-test. Experiments were done at least three times. Boiled 500 U/ml IFN-γ, boiled 5 min at 99˚C, serves as a negative control. All IFN-γ treatments are 500 U/ml unless otherwise stated.

Quantitative RT-PCR:

Total RNA was extracted from sympathetic neurons using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) or RNeasy mini kit (Qiagen, Germantown, MD, USA) as per protocol. 200-1000 ng RNA was converted to cDNA using previously described methods (Kole et al., 2011). Each 25 µl PCR reaction contained 25 ng cDNA, 400 nM of each primer, and PowerSYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Primers were designed using Primer Express software (Applied Biosystems) or obtained from the literature (sequences in Table 2.1). For each set of primers, post-amplification melting curves were performed to verify a single amplification product. Amplification was conducted in an ABI7500 system (Applied Biosystems) and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. 5-6 samples per group were run in triplicate with a negative control (no SuperScript II). Significance was determined by a one-tailed Mann-Whitney statistical test. Results were duplicated in at least three independent experiments and target gene expression was normalized to 18S or GAPDH levels.

Table 2.1. qRT-PCR primers (5'-3') used in this study.

Gene	Forward	Reverse
Ifi202b	GCAGTGGCATCCTAGAGATCAA	TTGGGCACTTCAATAATTTGGTT
18S	TTGACGGAAGGGCACCACCAG	GCACCACCACCCACGGAATCG (Sato et al., 2013)
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

Microarray analysis:

Total RNA was purified from sympathetic neurons treated with or without 500 U/ml IFN-γ for 24 hours using Trizol Reagent (Invitrogen) as per protocol. RNA integrity was assessed on a 2100 Bioanalyzer using an RNA6000 nano chip (Agilent Technologies, Sugar Land, TX, USA) and concentration was quantified using a NanoDrop 1000 spectrophotometer. 500 ng RNA per sample was labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies) per manufacturer instructions. 500 ng RNA from untreated P5 sympathetic cervical ganglia was amplified and labeled with Cy3 as a reference for each sample. Overnight hybridization of two-color whole mouse genome 4x44K microarrays (Agilent Technologies) and scanning on an Agilent G2505C microarray scanner were performed at the UNC Genomics Core according to manufacturer recommendations. 500 ng RNA from untreated P5 sympathetic neuron served as an internal reference for each slide. Raw data was processed and analyzed by GeneSpring GX Version 11.0 (Agilent Technologies). Genes with a corrected *P* value <0.05 following unpaired t-test and Benjamini and Hochberg false discovery rate procedure were considered differentially expressed.

Image acquisition and processing:

Images were acquired by an ORCA-ER digital B/W CCD camera (Hamamatsu) mounted on a DMI6000 B inverted fluorescence microscope (Leica) using MetaMorph version 7.6 software (Molecular Devices). Adobe Photoshop was used to adjust size and crop images to prepare the final figures.

2.4 Results

IFN-γ suppresses neuronal apoptosis induced by NGF deprivation and pan-kinase inhibition

IFN-α/β/γ induce apoptosis or increase sensitivity to apoptosis in many cell types (Dijkmans et al., 1990; Baerwald and Popko, 1998; Barber, 2000; Becher et al., 2000; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005; Maher et al., 2007; Zhang et al., 2008; Das et al., 2009), however, there is evidence that IFN-γ inhibits apoptosis in sympathetic neurons undergoing NGF deprivation (Chang et al., 1990). To determine whether interferons affect neuronal apoptosis, we exposed sympathetic neurons to apoptotic stimuli with or without IFN-γ. By 36 hours, 80% of sympathetic neurons died with NGF deprivation, however about 55% remained alive with addition of IFN-γ (Figure 2.1a). This antiapoptotic effect of IFN-γ was not specific to NGF deprivation as IFN-γ addition also inhibited sympathetic neuronal apoptosis in response to staurosporine (STS) addition. While fewer than 20% of sympathetic neurons alive at 72 hrs after STS addition, IFN-γ treatment promoted the survival of 60% of neurons at this timepoint (Figure 2.1b). 500 U/ml IFN-γ was observed to be the concentration yielding the highest, most consistent saving of sympathetic neurons (Supplemental Figure 2.1a). Remarkably, sympathetic neurons treated with NGF deprivation (or STS, images not shown) and IFN-γ resembled healthy control neurons, with intact neurites and smaller but phase-bright cell bodies (Figure 2.1c). While Kim *et al.* found that IFN-γ induces dendritic retraction and reduced neurite outgrowth in sympathetic neurons, we did not observe this in our model (Kim et al., 2002). Type I IFN at high concentrations also protects neurons

from apoptosis with NGF deprivation or STS (data not shown) (Chang et al., 1990). IFN-γ had no impact on apoptosis of sympathetic neurons treated with DNA damage or ER stress (Supplemental Figure 2.1b and c). Together, these data indicate that IFN-γ protects sympathetic neurons from apoptosis with NGF deprivation or pan-kinase inhibition. Chang *et al.* (Chang et al., 1990) previously reported reduced apoptosis in IFN-γ-treated sympathetic neurons with NGF deprivation, yet how IFN-γ alters the neuronal apoptotic pathway is unknown.

IFN-γ blocks apoptosis at the point of cytochrome c release in NGF-deprived neurons

To determine the precise effects of IFN-γ function on neuronal apoptosis, we investigated the result of IFN-γ treatment on specific steps of the apoptotic pathway induced by NGF deprivation. NGF withdrawal leads to the phosphorylation and activation of transcription factor c-Jun, which upregulates BH3-only proteins, resulting in mitochondrial cytochrome *c* release, caspase activation, and cell death (Figure 2.2a) (Eilers, Whitfield et al. 1998; Whitfield, Neame et al. 2001). We first examined phospho-c-Jun, which is upregulated and translocates to the nucleus upon activation. Robust staining of nuclear phospho-c-Jun was similar in sympathetic neurons treated with or without IFN-γ at 12 hours of NGF deprivation, indicating that IFN-γ did not affect c-Jun phosphorylation (Figure 2.2b).

Next, we tested the effect of IFN-γ on cytochrome *c* release from the mitochondria. Following NGF deprivation, cytochrome *c* staining is faint, indicating release into the cytosol (Deshmukh and Johnson, 1998). Neurons treated with IFN-γ, however, maintained

cytochrome *c* staining in the mitochondria, demonstrating IFN-γ blocks mitochondrial permeabilization in response to NGF deprivation (Figure 2.2c). IFN-γ also blocked caspase-3 activation downstream of cytochrome *c* release in response to NGF deprivation (Figure 2.2d). Consistent with these findings, Western blot analysis showed no change in phospho-c-Jun or BH3-only proteins, Bim and Puma, but a decrease in active caspase-3 with IFN-γ addition with NGF deprivation (Figure 2.2e). Levels of additional apoptosis-related proteins remained unchanged (Supplemental Figure 2.1). Thus, these findings demonstrate that IFN-γ inhibits apoptosis in neurons downstream of BH3 protein induction and upstream of cytochrome *c* release.

IFN-γ does not alter apoptosis downstream of cytochrome c release

Our data reveal a distinct suppression of active caspase-3 with IFN-γ treatment in NGF-deprived neurons. Previous research shows that interferons can regulate expression of caspases and caspase inhibitors to modulate apoptosis downstream of mitochondrial permeabilization (Chawla-Sarkar et al., 2003; Clemens, 2003; Pokrovskaja et al., 2005). To investigate whether IFN-γ also acts downstream of cytochrome *c* release, we microinjected cytochrome *c* into $XIAP^{-/-}$ sympathetic neurons with or without IFN-γ 24 hr pretreatment. Cytochrome *c* induced similar rates of cell death in treated neurons, demonstrating that IFN-γ does not suppress the apoptotic pathway downstream of cytochrome *c* release (Figure 2.3).

IFN-γ modulates gene expression in sympathetic neurons

Interferons stimulate expression of pro-apoptotic genes, including Fas, TRAIL, XAF-1, and caspases, in a variety of cell types, which induces apoptosis in these cells either with or without additional stressors (Dijkmans et al., 1990; Baerwald and Popko, 1998; Barber, 2000; Becher et al., 2000; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005; Maher et al., 2007; Zhang et al., 2008; Das et al., 2009). To first determine whether IFN-γ modulates gene expression in sympathetic neurons as it does in other cell types, we conducted quantitative RT-PCR on sympathetic neurons with or without IFN-γ. Levels of *ifi202b*, an interferon-responsive gene in the HIN-200 family, increased 30-fold with 24 hours of IFN-γ treatment (Figure 2.4). This finding reveals that IFN-γ modulates gene expression in sympathetic neurons, indicating it is possible IFN-γ exerts its anti-apoptotic effects in neurons by regulating gene expression.

To evaluate whether IFN-γ stimulates the expression of genes regulating apoptosis in neurons as it does in other cell types, we conducted a microarray experiment comparing gene expression of sympathetic neurons with or without 500 U/ml IFN-γ. 24 hours of IFN-γ exposure in neurons significantly upregulated many interferon-inducible genes including *Stat1* and the HIN-200 gene family, while downregulating only five genes (Table 2.2). Microarray data have been deposited in Gene Expression Omnibus under the accession number GSE48683. Surprisingly, although IFN-γ protects sympathetic neurons from apoptosis, pro-apoptotic gene *Trail* (*Tnfsf10*) is upregulated yet no anti-apoptotic genes were significantly altered by IFN-γ (Table 2.2). Among the pro-death genes that are upregulated by IFN-γ in other cell types, very few of these genes were induced by IFN-γ in

sympathetic neurons (Table 2.3). However, *Irf1*, *Irf8* (interferon-regulatory factor 1 and 8) and *PKR* (protein kinase, interferon-inducible double stranded RNA dependent), genes that promote cell death through the extrinsic pathway in other cell types (Barber, 2000; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005) were strongly upregulated by IFN-γ in these neurons (Table 2.3). Interestingly, *Ifi202b* has been shown to either promote or inhibit apoptosis, depending on the cell type, by binding and inhibiting transcription factors including c-Jun, c-Fos, c-Myc, NF-κB, E2F2, and p53 (Min et al., 1996; Asefa et al., 2004; Choubey and Panchanathan, 2008; Mondini et al., 2010). Although we found *ifi202b* to be strongly upregulated by IFN-γ in sympathetic neurons (Figure 2.4 and Table 2.3), microinjection of *ifi202b* plasmid or *sh-ifi202b* into neurons did not affect apoptosis with NGF deprivation (data not shown).

Together, these data demonstrate that, while IFN-γ prompts apoptosis in other cell types, IFN-γ exerts an anti-apoptotic effect on sympathetic neurons by blocking apoptosis at the point of cytochrome *c* release.

2.5 Discussion

In this study, we examined the effects of IFN-γ on sympathetic neuron survival. We found that, while sympathetic neuron survival was not affected by IFN-γ alone, IFN-γ inhibited neuronal apoptosis with NGF-deprivation or pan-kinase inhibition, but not with DNA damage or ER stress. IFN-γ treatment of NGF-deprived sympathetic neurons did not alter c-Jun phosphorylation or induction of BH-3 only genes, but decreased mitochondrial cytochrome *c* release and subsequent caspase 3 activation. Although some studies report upregulation of caspases and XAF-1 by interferons (Chawla-Sarkar et al., 2003; Clemens, 2003; Maher et al., 2007) cytochrome *c* injection experiments showed that IFN-γ did not affect neuronal apoptosis downstream of the mitochondria. Finally, we report that IFN-γ modulated gene expression in sympathetic neurons, including the upregulation of apoptosis-related genes, including *ifi202b*. While *ifi202b* is implicated in apoptosis inhibition in other cell types (Choubey and Panchanathan, 2008), *ifi202b* knockdown and overexpression did not alter neuronal apoptosis. These results demonstrate that despite IFN-γ inducing expression of several pro-apoptotic genes in sympathetic neurons, IFN-γ suppresses apoptosis at the point of mitochondrial permeabilization during NGF deprivation.

The ability of IFN-γ to suppress apoptosis in sympathetic neurons is quite intriguing. IFN-γ has been reported to activate p38 and JNK MAPK pathways (Xia et al., 1995; Bruckner et al., 2001; Kalvakolanu and Roy, 2005; Maher et al., 2007; Hervas-Stubbs et al., 2011), as well as suppress NF-κB activity (Hervas-Stubbs et al., 2011), either of which induce apoptosis in sympathetic neurons (Maggirwar et al., 1998; Bruckner et al., 2001). Thus,

interferon's pro-survival effect in sympathetic neurons suggests the inability of IFN-γ to activate JNK or suppress NF-κB in these neurons, which would promote apoptosis. Similarly, inhibition of transcription or translation inhibits apoptosis in sympathetic neurons (Martin et al., 1988; Xia et al., 1995). Although interferon inhibits protein synthesis through activation of EIF2α to limit viral replication (Xia et al., 1995; Chawla-Sarkar et al., 2003; Schroder et al., 2004; Pokrovskaja et al., 2005), induction of BH3-only protein was not suppressed in IFN-γ-treated NGF-deprived neurons (Figure 22). Interestingly, BH3-only protein induction is inhibited by miR-29 (Kole et al., 2011), which is upregulated by IFN-γ in melanoma cells (Schmitt et al., 2012). In this study, we found no suppression of BH3-only induction in interferon-treated sympathetic neurons with NGF deprivation.

While neurons upregulated several pro-apoptotic genes (*Trail, Irf1*, *Irf8, PKR*) in response to interferon, it was not sufficient to cause apoptosis in NGF-maintained or deprived sympathetic neurons. Previous research shows that sympathetic neuron survival is resistant to TNF-α (Marz et al., 1996) and there is no literature on the expression or ability of death receptor ligands to activate the extrinsic apoptotic pathway in sympathetic neurons. Therefore, it is possible that these neurons are insensitive to this gene upregulation (due to lack of death receptor expression), pro-apoptotic signals were insufficient to promote apoptosis, or a pro-survival mechanism(s) such as Mig (monokine induced by interferon-gamma) (Uwabe et al., 2005) overrode the pro-death signal.

Cytochrome *c* release is the point of divergence for IFN-treated neurons undergoing NGF deprivation, suggesting Bax activation as the likely molecule altering mitochondrial permeabilization. Our data at the protein level excludes main modulators of Bax activation,

Bcl-XL as well as the BH3-only proteins, however Bax-activating candidates Ku70 (Cohen et al., 2004) and phosphorylated Bim have not been excluded (Lei and Davis, 2003; Pokrovskaja et al., 2005). Inhibition of mitochondrial permeabilization by IFN-γ prevents damage to this important organelle, allowing maintenance of neuronal metabolism. Although some studies report upregulation of caspases and XIAP inhibitor, XAF-1, by interferons (Chawla-Sarkar et al., 2003; Clemens, 2003; Maher et al., 2007), cytochrome *c* injection experiments showed that IFN-γ did not affect neuronal apoptosis downstream of the mitochondria.

Bernabei *et al.* proposed that cells with higher levels of IFN-γ receptor (IFN-γR2) undergo rapid Stat1 activation of apoptosis in response to IFN-γ, while a slower Stat1 activation with lower levels of IFN-γ receptor does not induce apoptosis (Bernabei et al., 2001; Bernabei et al., 2001; Boselli et al., 2010). However, sympathetic neurons express IFN-γ receptors at a higher level than cortical neurons, which undergo apoptosis in response to IFN-γ (Rottenberg and Kristensson, 2002; Dedoni et al., 2012). Thus, expression of the IFN-γ receptor does not appear to be the determining factor of apoptosis in neurons.

An infection or injury stimulates interferon production and recruitment of macrophages, natural killer (NK) cells, and T lymphocytes (Chawla-Sarkar et al., 2003; Schroder et al., 2004). IFN-γ activates macrophages to release nitric oxide radicals, which can contribute to neuronal death (Rottenberg and Kristensson, 2002). Sympathetic neurons co-cultured with active, but not resting, macrophages triggered neurite loss and neuronal death (Arantes et al., 2000; Almeida-Leite et al., 2007). Similarly, activated T cells or NK cells following IFN-γ exposure can mediate neurite and DRG and hippocampal neuron

elimination (Rottenberg and Kristensson, 2002; Yong et al., 2007). In vivo, CD8+ T cells entering Borna disease virus (BDV) infected brain did not cause hippocampal neuron death unless the mice lacked IFN-γ (Richter et al., 2009). Therefore, IFN-γ's ability to promote neuronal survival may counteract the cytotoxic signals from immune cells in the neurons' milieu as well as protecting neurons from death due to viral infection (Burdeinick-Kerr et al., 2009).

Overall, these findings demonstrate that sympathetic neurons, unlike most cell types, respond to interferon by suppressing the apoptotic pathway at the point of mitochondrial permeabilization. Despite upregulation of several proapoptotic genes, neurons treated with IFN-γ display a remarkable ability to suppress cell death with NGF deprivation or pan-kinase inhibition. The ability of IFN-γ to promote sympathetic neuronal survival while inducing pathways that kill pathogens is likely a physiologically important mechanism which could ensure the long-term survival of these neurons during critical situations of pathogenic infection.

2.6 Figures and Legends

Figure 2.1. **IFN-γ protects neurons from apoptosis in response to nerve growth factor deprivation or pan-kinase inhibition.**

Neurons were treated with (**A**) nerve growth factor (NGF) deprivation or (**B**) staurosporine (pan-kinase inhibition; STS) with or without 500 U/ml IFN-γ and survival was assessed by morphology at 36 hours or 72 hours, respectively. Boiled 500 U/ml IFN-γ, boiled 5 min at 99˚C, served as a negative control. (**C**) Phase images were taken of neurons after 40 hours of NGF deprivation. Data represent mean +/- s.e.m. analyzed by Student's *t*-test. Experiments were done at least three times. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure 2.1

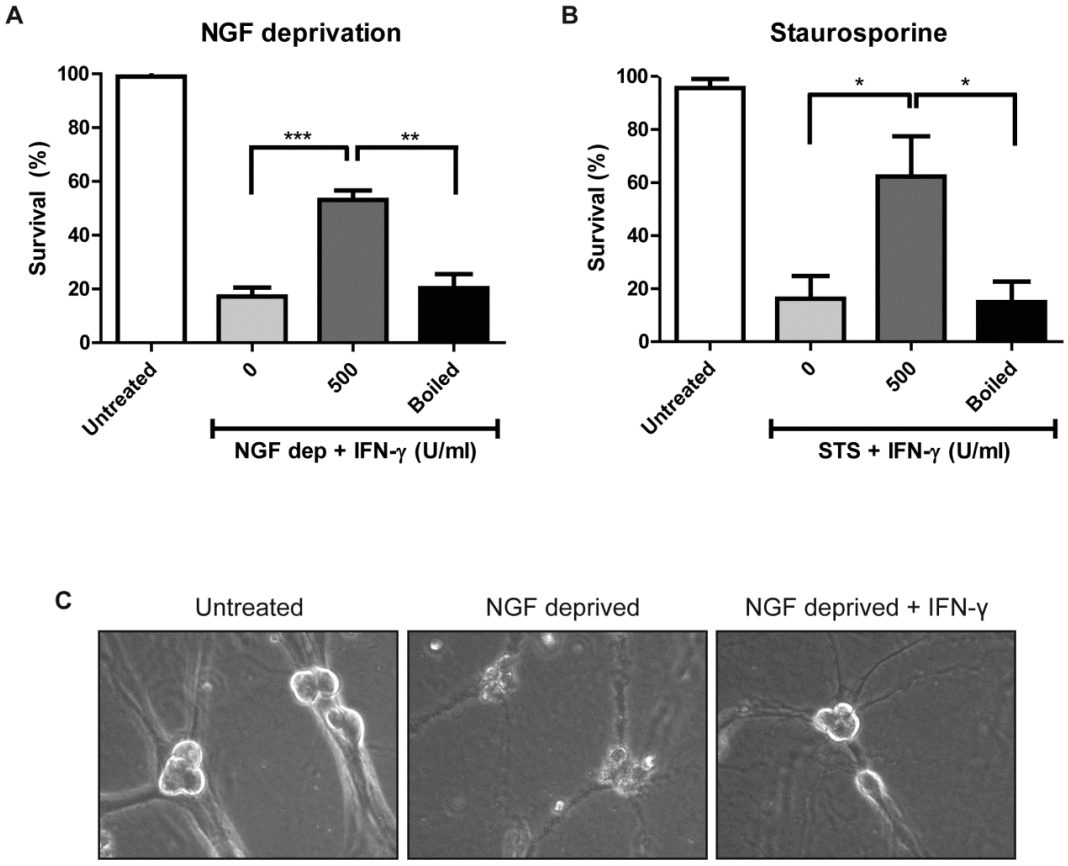

A
NGF deprivation
Survival (%)
100
80
60
40
20
0
***
**
Untreated
0
500
Boiled
NGF dep + IFN-γ (U/ml)
B
Staurosporine
Survival (%)
100
80
60
40
20
0
*
*
Untreated
0
500
Boiled
STS + IFN-γ (U/ml)
C
Untreated
NGF deprived
NGF deprived + IFN-γ

Figure 2.2. IFN-γ inhibits apoptosis at the point of cytochrome *c* release.

(**A**) Schematic representation of the apoptosis pathway induced after NGF deprivation. (**B**) Neurons were untreated or deprived of NGF with or without IFN-γ and fixed for phospho-c-Jun immunofluorescence at 12 hours. Percentage of cells with phospho-c-Jun nuclear staining and representative images of phospho-c-Jun staining in neurons. Data is mean ± std. dev. (**C**) Neurons were untreated or deprived of NGF with or without IFN-γ, in the presence of caspase inhibitor, QVD, and fixed for cytochrome *c* immunofluorescence at 48 hours. Data is mean percentage of cells with mitochondrial cytochrome *c* staining ± s.e.m. analyzed by the Student's *t*-test. **P*= 0.0279. Images are representative of neurons with cytochrome *c* staining. (**D**) Neurons were untreated or deprived of NGF with or without IFN-γ and fixed for cleaved-caspase-3 (c-Casp3) immunofluorescence at 24 hours. Data is mean percentage of cells with c-Casp3 staining ± std. dev. Images are representative of neurons with c-Casp3 staining. (**E**) Neurons were untreated, deprived of NGF, or deprived of NGF with 200 U/ml or 500 U/ml IFN-γ. Cell lysates were collected at 24 hours and examined by western blotting analysis. 500 U/ml IFN-γ reduces cleaved caspase-3 but does not alter levels of phospho-c-Jun or induction of BH3-only apoptotic proteins.

Figure 2.2

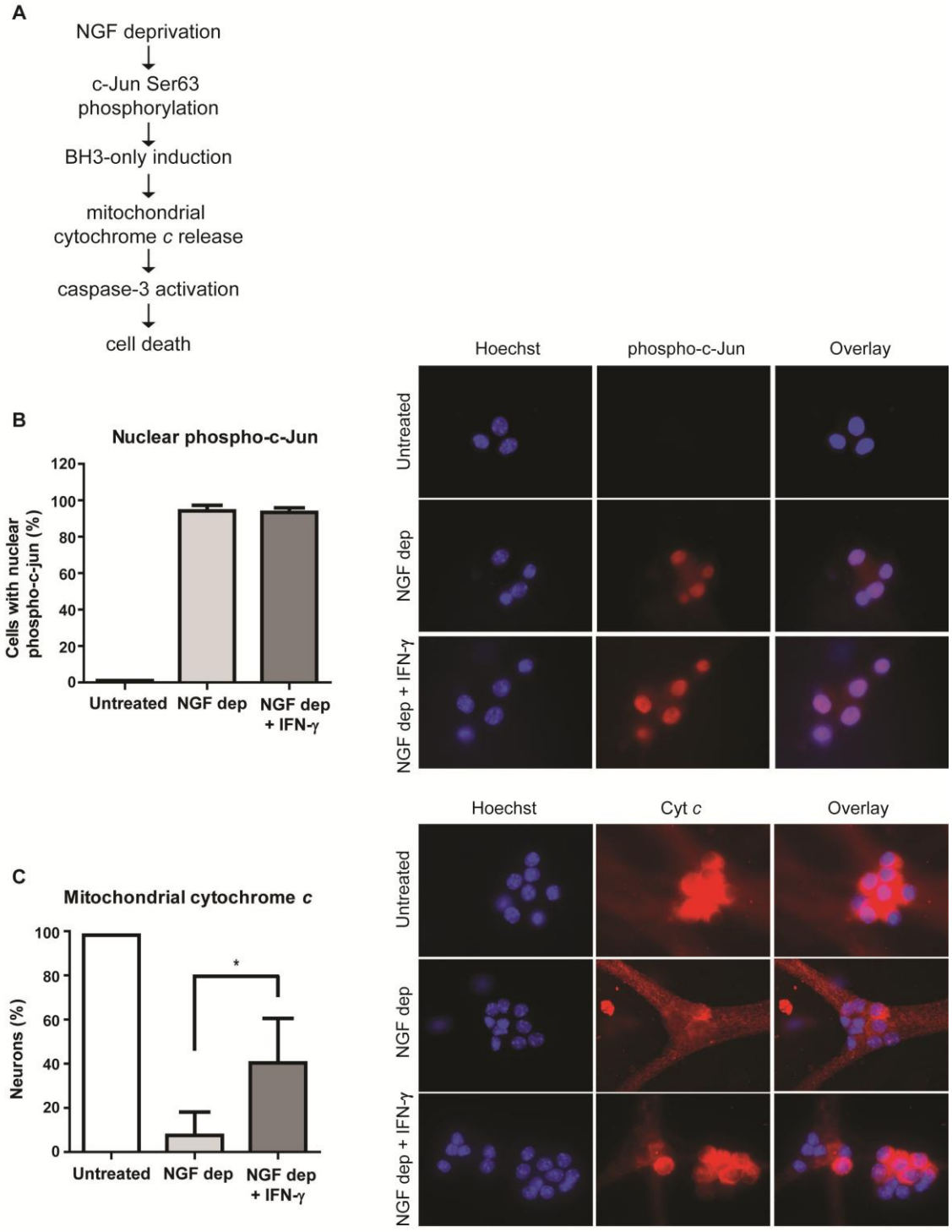
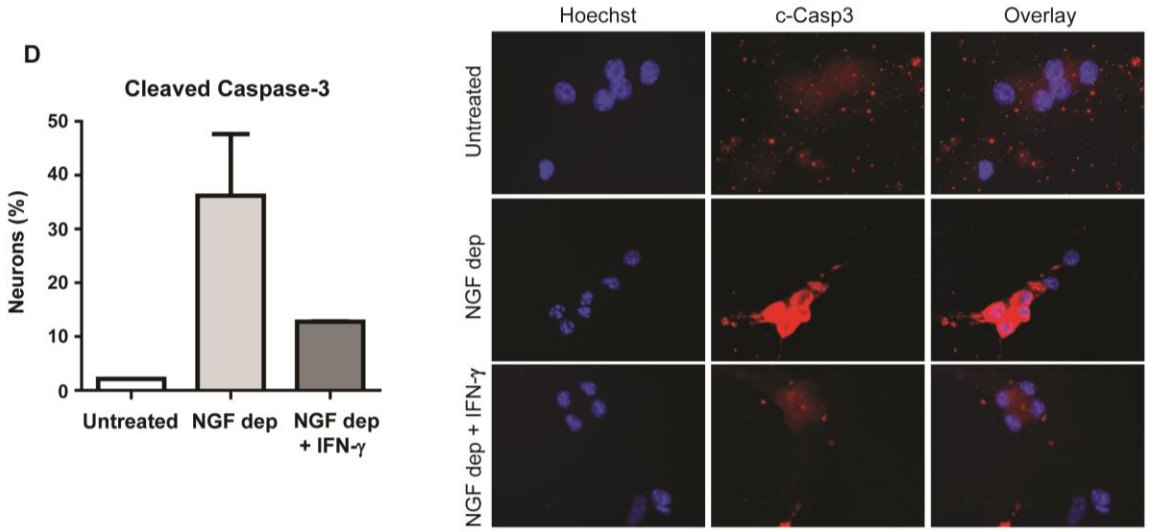

A
NGF deprivation
c-Jun Ser63 phosphorylation
BH3-only induction
mitochondrial cytochrome c release
caspase-3 activation
cell death
B
Nuclear phospho-c-Jun
Cells with nuclear phospho-c-jun (%)
120
100
80
60
40
20
0
Untreated
NGF dep
NGF dep + IFN-γ
Hoechst
phospho-c-Jun
Overlay
Untreated
NGF dep
NGF dep + IFN-γ
C
Mitochondrial cytochrome c
Neurons (%)
100
80
60
40
20
0
*
Untreated
NGF dep
NGF dep + IFN-γ
Hoechst
Cyt c
Overlay
Untreated
NGF dep
NGF dep + IFN-γ

Figure 2.2 continued



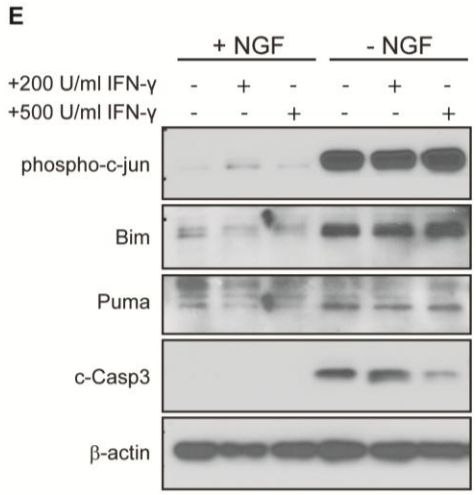


Figure 2.3. **IFN-γ does not alter the apoptotic pathway downstream of cytochrome *c* release**.

Cell death with microinjection of cytochrome *c* was equivalent with and without IFN-γ addition. XIAP$^{-/-}$ neurons pretreated with or without IFN-γ for 24 hours were microinjected with cytochrome *c* and survival was assessed by morphology. Data are mean ± s.e.m. of four independent experiments with at least 40 cells injected per experiment.

Figure 2.3

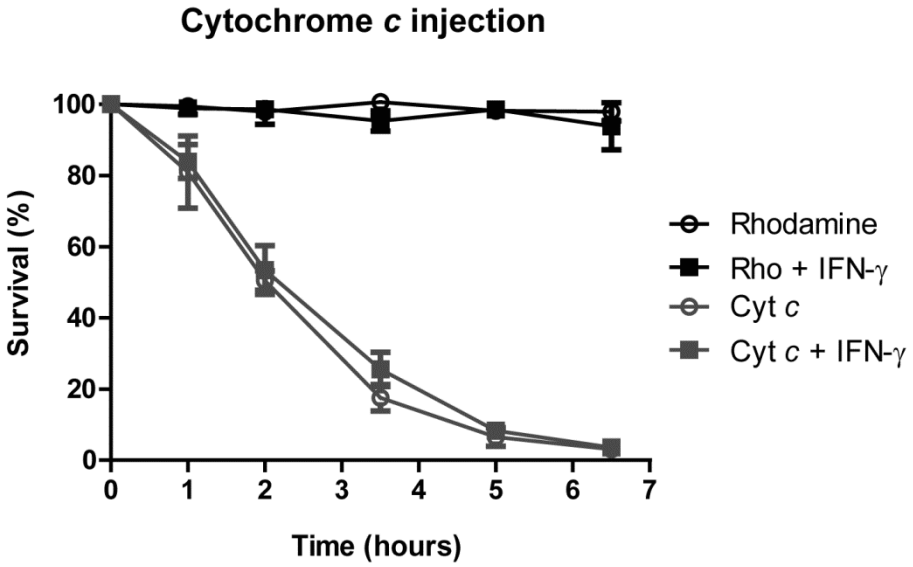

Cytochrome c injection
Survival (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Time (hours)
Rhodamine
Rho + IFN-γ
Cyt c
Cyt c + IFN-γ

Figure 2.4. **IFN-γ regulates gene expression in sympathetic neurons**.

Quantitative RT-PCR analysis demonstrating neurons exhibit upregulated expression of interferon-inducible *ifi202b* mRNA with 24 hours 500 U/ml IFN-γ treatment. *Ifi202b* expression is normalized to 18S levels. Data are mean of at least five samples per group ± s.e.m. analyzed by the Mann-Whitney test. ***P*=0.0043.

Figure 2.4

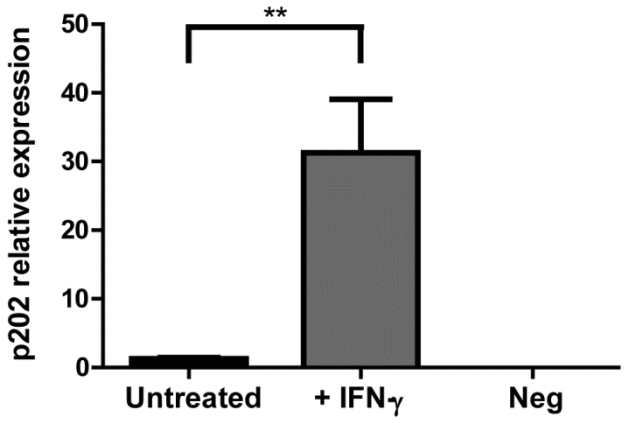


Table 2.2. IFN-γ modulates gene expression in sympathetic neurons.

Summary of genes differentially expressed with 24 hours 500 U/ml IFN-γ in sympathetic neurons (*P*<0.05 following unpaired *t*-test and Benjamini and Hochberg false discovery rate procedure).

Table 2.2

Symbol	Gene Name	Fold change
Up-regulated		
Gbp3	guanylate nucleotide binding protein 3	142
Gvin1	GTPase, very large interferon inducible 1	111
Ifi44	interferon-induced protein 44	101
Rtp4	receptor transporter protein 4	65
Batf2	basic leucine zipper transcription factor, ATF-like 2	59
Irgb10	interferon-gamma-inducible p47 GTPase	51
Igtp	interferon gamma induced GTPase	37
Tnfsf10 (Trail)	tumor necrosis factor (ligand) superfamily, member 10	34
Parp14	poly (ADP-ribose) polymerase family, member 14	23
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	23
Gbp6	guanylate binding protein 6	22
Stat1	signal transducer and activator of transcription 1	18
Mx2	myxovirus (influenza virus) resistance 2	18
RP23-14F5.8	similar to T-cell specific GTPase	18
Ifi204	interferon activated gene 204	17
Irgm	immunity-related GTPase family, M	17
Iigp2	interferon inducible GTPase 2	16
Isg15	ISG15 ubiquitin-like modifier	15
Gbp2	guanylate nucleotide binding protein 2	15
Ifih1	interferon induced with helicase C domain 1	14
Tap1	transporter 1, ATP-binding cassette (MDR/TAP)	14
Tgtp	T-cell specific GTPase	13
Ifi203	interferon activated gene 203	13
Fbxo39	F-box protein 39	12
Irf8	interferon regulatory factor 8	12
Bst1	bone marrow stromal cell antigen 1	12
Rsad2	radical S-adenosyl methionine domain containing 2	12
Parp9	poly (ADP-ribose) polymerase family, member 9	11
Ifi203	interferon activated gene 203	11
Oasl2	2'-5' oligoadenylate synthetase-like 2	9.9
RP23-14F5.7	hypothetical gene Rp23-14f5.7	9.8
Irf1	interferon regulatory factor 1	9.7
Oas1g	2'-5' oligoadenylate synthetase 1G	9.6
H2-T22	histocompatibility 2, T region locus 22	9.5
Psmb9	proteosome (prosome, macropain) subunit, beta type 9	9.4
Bst2	bone marrow stromal cell antigen 2	9.0
Il18bp	interleukin 18 binding protein	8.9
Gls	Glutaminase	8.1
Socs1	suppressor of cytokine signaling 1	7.8
Herc5	hect domain and RLD 5	7.6

Psmb8	proteosome (prosome, macropain) subunit, beta type 8	6.7
Cd274	CD274 antigen	6.6
Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta	6.2
Tap2	transporter 2, ATP-binding cassette (MDR/TAP)	6.2
Cd52	CD52 antigen	5.8
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	5.7
Tex14	testis expressed gene 14	5.7
Abhd8	abhydrolase domain containing 8	5.5
Samhd1	SAM domain and HD domain, 1	5.4
Parp12	poly (ADP-ribose) polymerase family, member 12	5.4
Psmb10	proteasome (prosome, macropain) subunit, beta type 10	5.0
B2m	beta-2 microglobulin	4.8
Arts1	type 1 TNF receptor shedding aminopeptidase regulator	4.6
Pnpla5	patatin-like phospholipase domain containing 5	3.9
Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	3.9
Ly75	lymphocyte antigen 75	3.8
Plec1	plectin 1	3.7
Ifit3	interferon-induced protein with tetratricopeptide repeats 3	3.7
Slamf8	SLAM family member 8	3.6
Psme2	proteasome (prosome, macropain) 28 subunit, beta	3.4
Pla1a	phospholipase A1 member A	3.3
Nmi	N-myc (and STAT) interactor	3.2
Tapbp	TAP binding protein	3.1
Tnfsf13b	tumor necrosis factor (ligand) superfamily, member 13b	3.1
Isgf3g	IFN dependent positive acting transcription factor 3 gamma	3.1
Ifi35	interferon-induced protein 35	3.0
Lap3	leucine aminopeptidase 3	2.9
Megf6	multiple EGF-like-domains 6	2.6
Ube2l6	ubiquitin-conjugating enzyme E2L 6	2.5
Pbef1	pre-B-cell colony-enhancing factor 1	2.5
Zfp313	zinc finger protein 313	2.4
H2-Oa	histocompatibility 2, O region alpha locus	2.1
Psme1	proteasome (prosome, macropain) 28 subunit, alpha	2.0
Spo11	sporulation protein, meiosis-specific, SPO11 homolog	2.0
Col23a1	procollagen, type XXIII, alpha 1	1.9
Rbm4	RNA binding motif protein 4	1.2
Down-regulated		
Olfr912	olfactory receptor 912	-2.3
Chrnb2	cholinergic receptor, nicotinic, beta polypeptide 2	-1.3
Tmem110	transmembrane protein 110	-1.2
Tktl2	transketolase-like 2	-1.2
Polr2e	polymerase (RNA) II (DNA directed) polypeptide E	-1.1

Table 2.3. Sympathetic neurons upregulate few pro-apoptotic genes in response to IFN-γ.

Expression of genes reported to be regulated by interferon in other cell types (Koul et al., 1998; Choi et al., 1999; Barber, 2000; Nishikawa et al., 2001; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005; Maher et al., 2007) were evaluated by gene array in sympathetic neurons treated with 24 hours 500 U/ml IFN-γ. Indicated *P* value is corrected *P* value following unpaired *t*-test and Benjamini and Hochberg false discovery rate procedure (ns= non-specific).

Table 2.3

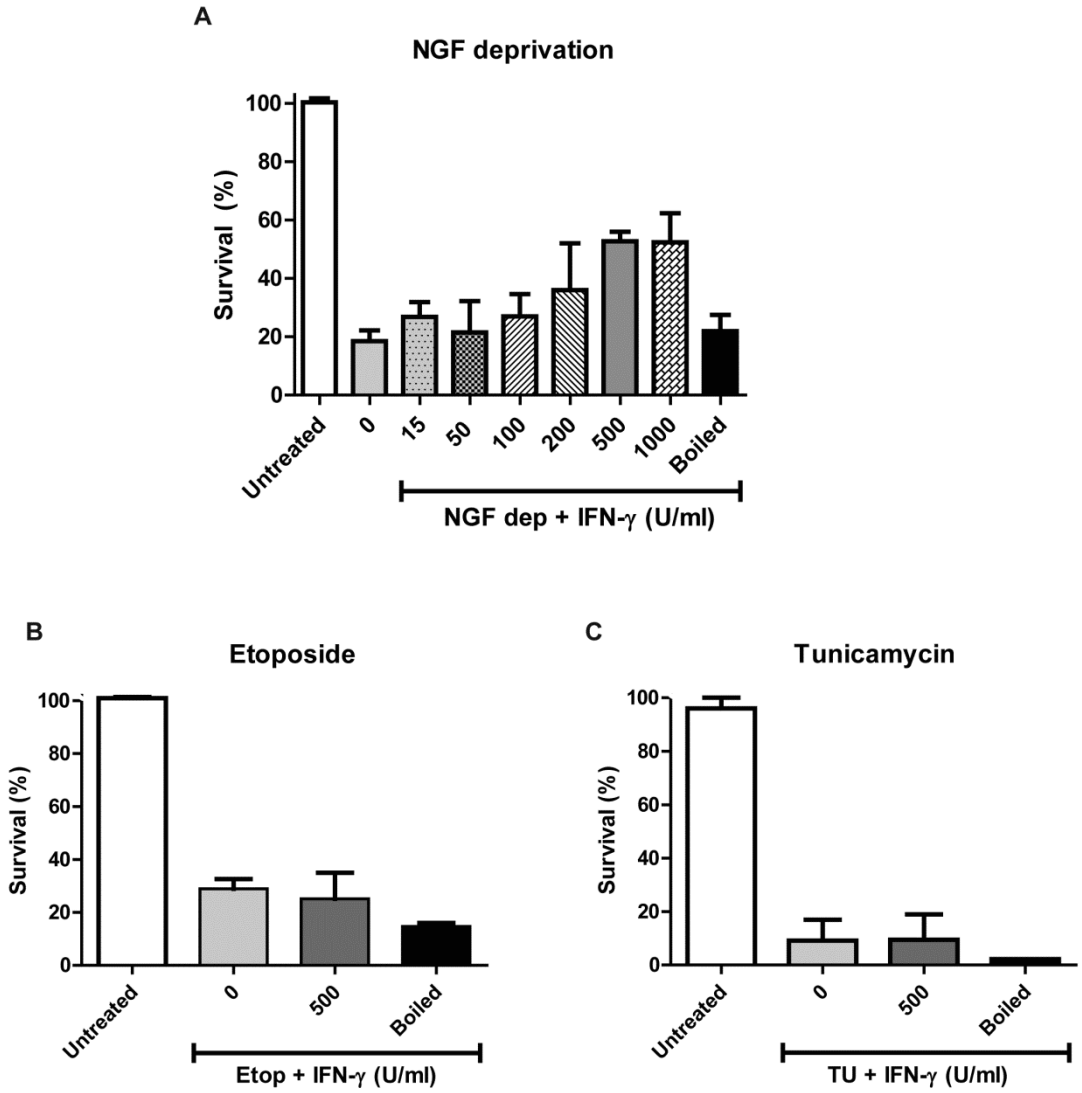
Symbol	Gene Name	Fold change	*P* value
Pro-apoptotic			
Fas	Fas (TNF receptor superfamily member)	2.1	ns
FasL	Fas ligand (TNF superfamily, member 6)	-1.0	ns
Trail	tumor necrosis factor (ligand) superfamily 10	34.1	0.0178
Trail receptor	TNF receptor superfamily, member 10b	-1.0	ns
TNF	tumor necrosis factor	2.5	ns
TNFα receptor	TNF receptor superfamily, member 1a	1.4	ns
Fadd	Fas (TNFRSF6)-associated via death domain	-1.0	ns
Daxx	Fas death domain-associated protein	1.2	ns
Bak1	BCL2-antagonist/killer 1	1.2	ns
Bax	Bcl2-associated X protein	1.1	ns
Casp1	caspase 1	5.3	ns
Casp3	caspase 3	1.1	ns
Casp4	caspase 4	3.1	ns
Casp7	caspase 7	3.0	ns
Casp8	caspase 8	1.4	ns
Casp9	caspase 9	-1.1	ns
RnaseL	ribonuclease L	-1.0	ns
Irf1	interferon regulatory factor 1	9.7	0.0177
Irf2	interferon regulatory factor 2	1.1	ns
Irf3	interferon regulatory factor 3	1.0	ns
Irf4	interferon regulatory factor 4	-1.6	ns
Irf5	interferon regulatory factor 5	1.8	ns
Irf6	interferon regulatory factor 6	-1.5	ns
Irf7	interferon regulatory factor 7	8.0	ns
Irf8	interferon regulatory factor 8	12.3	0.028
PKR	eukaryotic translation initiation factor 2-α kinase 2	3.5	0.054
OAS1	2'-5' oligoadenylate synthetase 1	3.1	ns
Oas3	2'-5' oligoadenylate synthetase 3	5.4	ns
Pml	promyelocytic leukemia	1.4	ns
Dap	death-associated protein	-1.2	ns
Dap3	death associated protein 3	1.0	ns
Dapk1	death associated protein kinase 1	-1.1	ns
Dapk2	death-associated kinase 2	1.8	ns
Dapk3	death-associated kinase 3	-1.1	ns
Hif1a	hypoxia inducible factor 1, alpha subunit	1.2	ns
Plscr1	phospholipid scramblase 1	1.5	ns
Plscr2	phospholipid scramblase 2	2.1	ns
Plscr3	phospholipid scramblase 3	1.1	ns
Plscr4	phospholipid scramblase 4	1.1	ns

p21	cyclin-dependent kinase inhibitor 1A (P21)	-1.5	ns
Ctsd	cathepsin D	-1.2	ns
Anti-apoptotic			
Bcl2	B-cell leukemia/lymphoma 2	-1.4	ns
Bcl-XL	Bcl2-like 1 (Bcl2l1)	-1.1	ns
XIAP	baculoviral IAP repeat-containing 4 (Birc4)	-1.0	ns
c-FLIP	CASP8 and FADD-like apoptosis regulator (Cflar)	1.2	ns
Survivin	baculoviral IAP repeat-containing 5 (Birc5)	2.5	ns
Pro- or anti-apoptotic			
Mcl1	myeloid cell leukemia sequence 1	1.1	ns
ifi202	interferon activated gene 202B	21.7	0.112

Supplemental Figure 2.1. **Varying concentrations of IFN-γ protect sympathetic neurons from apoptosis with NGF deprivation but IFN-γ does not protect neurons from DNA damage or ER stress.**

(**A**) Neurons were treated with NGF deprivation and with varying concentrations of IFN-γ and survival was assessed by morphology at 36 hours. (**B**) Neurons were subjected to DNA damage with etoposide or (**C**) ER stress with tunicamycin with or without 500 U/ml IFN-γ and survival was assessed by morphology at 72 hours or 48 hours, respectively. Boiled 500 U/ml IFN-γ, boiled 5 min at 99˚C, served as a negative control. Experiments were done at least three times.

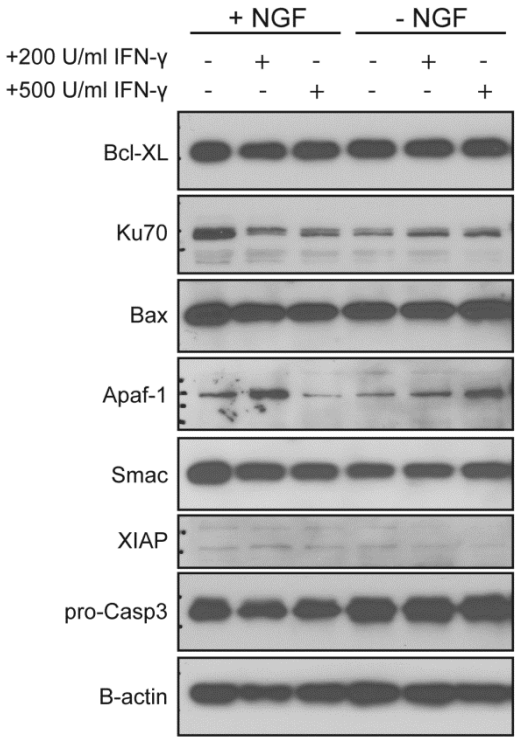
Supplemental Figure 2.1



Supplemental Figure 2.2. **IFN-γ doesn't alter pro-caspase-3 or other apoptotic proteins in neurons.**

Neurons were untreated, deprived of NGF, or deprived of NGF with 200 U/ml or 500 U/ml IFN-γ. Cell lysates were collected at 24 hours and examined by western blotting analysis. Levels of indicated pro- and anti- apoptotic proteins are unchanged by IFN-γ.

Supplemental Figure 2.2



CHAPTER III: ASC DEFICIENCY SUPPRESSES PROLIFERATION AND PREVENTS MEDULLOBLASTOMA INCIDENCE

3.1 Overview

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is silenced by promoter methylation in many types of tumors, yet ASC's role in most cancers remains unknown. Here, we show that ASC is highly expressed in a model of medulloblastoma, the most common malignant pediatric brain cancer. Importantly, while ASC deficiency did not affect normal cerebellar development, ASC knock-out mice in the Smoothened (ND2:SmoA1) transgenic model of medulloblastoma exhibited a profound reduction in medulloblastoma incidence and delayed tumor onset. Additionally, ASC deficiency increased age at tumor onset in a second model of medulloblastoma, GFAP-Cre:SmoM2 mice. Premalignant lesions in cerebella of $ASC^{-/-}$;ND2:SmoA1 mice displayed a striking decrease in number of ectopic progenitors. While proliferation rates decreased with ASC deletion, apoptosis and differentiation markers remained unchanged. Interestingly, ASC deficiency disrupted expression of genes in the TGF-β pathway and increased the level of nuclear Smad3 in this medulloblastoma model. Together, these results demonstrate an unexpected requirement for ASC in Sonic hedgehog-driven

medulloblastoma tumorigenesis, thus identifying ASC as a promising novel target for anti-tumor therapy.

3.2 Introduction

Medulloblastoma, a tumor of cerebellar progenitors, is the most common malignant brain cancer in children (Hatten and Roussel, 2011). During normal development, proliferation of progenitors in the cerebellum extends into the early postnatal period, as cerebellar granule neuron progenitors (CGNPs) undergo rapid division in the external granule layer (EGL), then differentiate and migrate to the internal granule layer (IGL) (Hatten and Heintz, 1995). CGNPs proliferate in response to endogenous Sonic hedgehog (Shh; mouse), and mutations that activate SHH (human) signaling cause predisposition to medulloblastoma in humans with Gorlin Syndrome, and in genetically engineered mouse models (Polkinghorn and Tarbell, 2007; Hatten and Roussel, 2011). These models, which operate through either *Patched* deletion or insertion of constitutively active alleles of *Smoothened* (*Smo*), consistently implicate CGNPs as the cells of origin for Shh-driven medulloblastoma (Hallahan, Pritchard et al. 2004; Yang, Ellis et al. 2008). Importantly, while advances in treatment have increased the survival of patients with medulloblastoma, mortality remains significantly high and debilitating cognitive and endocrine side effects result from current treatment regimens (Polkinghorn and Tarbell, 2007). Thus, advancing the understanding of the genetic components of medulloblastoma tumorigenesis is needed to develop improved targeted therapies.

ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain; also known as TMS-1, target of methylation-induced silencing-1) is expressed in many human tissues, however, is silenced by promoter hypermethylation in many tumor types, including glioblastoma (Stone et al., 2004), neuroblastoma (Alaminos et al., 2004),

breast cancer (Conway et al., 2000), melanoma (Guan et al., 2003), and lung cancer (Machida et al., 2006). Ectopic ASC expression sensitizes pancreatic, breast, and colon cancer cells to apoptosis (Conway et al., 2000; Ohtsuka et al., 2004; Ohtsuka et al., 2006; Parsons and Vertino, 2006; Ramachandran et al., 2010; Hong et al., 2013), while knocking down endogenous ASC inhibits cell death of osteosarcoma cells (Ohtsuka et al., 2004), colon cells (Hong et al., 2013), and breast epithelial cells (Parsons et al., 2009). Additionally, ASC is the adaptor protein of the inflammasome, a cytosolic complex that senses pathogen-associated molecules and subsequently responds by activating pro-inflammatory substrates, pro-interleukin-1β (pro-IL-1β) and pro-IL-18, which recruit and activate immune cells (Fernandes-Alnemri et al., 2009; Franchi et al., 2009; Davis et al., 2011). Consistent with the expectation that ASC inactivation promotes tumorigenesis, ASC-deficiency has been shown to enhance polyp formation in a colitis-associated colon cancer mouse model (Allen et al., 2010; Zaki et al., 2010). Together, these studies point to ASC as a functional tumor suppressor.

In this study, we examined the role of ASC in medulloblastoma using the ND2:SmoA1 transgenic mouse model. Our results reveal the unexpected finding that ASC promotes tumorigenesis in medulloblastoma. ASC was highly expressed in these tumors and the genetic deletion of ASC markedly reduced proliferation, hyperplasia, and mortality. These findings identify a novel role for ASC in promoting tumorigenesis in medulloblastoma.

3.3 Results and Discussion

ASC is highly expressed in medulloblastomas

To investigate the role of ASC in cerebellar development and medulloblastoma, we evaluated ASC expression during mouse cerebellum development. ASC protein levels were high in postnatal-day 7 (P7) cerebellum, which corresponds to the peak period of CGNP cell proliferation, and decreased with cerebellar maturation (Figure 3.1a). ASC is reported to enhance apoptosis upon expression and is often silenced via methylation in a variety of cancers (Conway et al., 2000; Ohtsuka et al., 2004; Ohtsuka et al., 2006; Parsons and Vertino, 2006; Allen et al., 2010; Ramachandran et al., 2010). To determine whether ASC was also silenced in cancer of cerebellar origin, we examined ASC expression in medulloblastoma. We used the ND2:SmoA1 (SmoA1) mouse model of medulloblastoma which expresses the activated allele of *Smo* in CGNPs, resulting in CGNP hyperproliferation and tumor growth as a consequence of the constitutive activation of the Sonic hedgehog pathway (Hallahan et al., 2004). In contrast to the reports in other cancer types, we unexpectedly detected high levels of ASC in medulloblastoma (Figure 3.1a). Also, ASC mRNA levels closely matched protein levels throughout development and in the tumor (Figure 3.1b). These data reveal the surprising observation that ASC is not subjected to silencing but is induced in a mouse model of medulloblastoma.

In human medulloblastoma, publicly available gene expression profiling data in the Oncomine database reveals ASC to be expressed in human medulloblastomas, with increased expression in desmoplastic *versus* classic tumors (Figure 3.1c) (Kool et al., 2008;

Fattet et al., 2009). Since SHH-associated medulloblastomas are generally characterized by a desmoplastic histology (Thompson et al., 2006; Cho et al., 2011; Fellay et al., 2011), this data suggests that ASC expression is more upregulated in medulloblastomas with SHH pathway disruption than other subtypes. Thus, in SHH-driven medulloblastomas ASC is upregulated in both human and mouse tumors.

ASC deficiency does not affect normal cerebellum development

To investigate whether ASC expression is important in medulloblastoma tumorigenesis, we first evaluated whether ASC deficiency affected normal cerebellar development. Cerebellar development occurs postnatally with rapid CGNP proliferation in the EGL peaking at P7. As CGNPs terminally differentiate into cerebellar granule neurons (CGNs), they migrate to the IGL to form the mature cerebellum by P20 (Hatten and Heintz, 1995). We therefore compared cerebellar architecture of $ASC^{+/+}$ and $ASC^{-/-}$ mice during this key developmental period. H&E staining revealed no differences in gross morphology between wild-type and ASC-deficient cerebella throughout development (Figure 3.1d). To specifically examine whether proliferation was altered by ASC deficiency, we compared expression of proliferation markers cyclin D2 and phospho-histone H3 (pH3) in $ASC^{+/+}$ and $ASC^{-/-}$ P7 cerebellum. No differences in cyclin D2 levels and pH3 staining were observed between wild-type and ASC-deficient cerebella (Figures 3.1e and f).

ASC deficiency suppresses SmoA1- and SmoM2-induced medulloblastoma

Next, to determine whether ASC deficiency affects medulloblastoma, we generated $ASC^{+/+}$, $ASC^{+/-}$, and $ASC^{-/-}$ SmoA1 mice and compared the time of tumor emergence in each group. In this Smo transgenic medulloblastoma model, signs of tumors typically emerge at 3-8 months and precede death from the disease by only a few days; thus, once signs emerge, mice are in a moribund state (Hallahan et al., 2004). Surprisingly, ASC deficiency conferred a striking reduction in tumor incidence and a delay in tumor onset in the SmoA1 transgenic mice (Figure 3.2a). Overall tumor incidence by P300 was significantly reduced in mice lacking ASC: while 12 of 15 $ASC^{+/+}$;SmoA1 mice exhibited tumors, only 5 of 18 $ASC^{-/-}$;SmoA1 mice developed medulloblastoma (Figure 3.2b). Additionally, ASC deficiency markedly delayed latency to tumor formation in $ASC^{-/-}$;SmoA1 mice compared to $ASC^{+/+}$;SmoA1 and $ASC^{+/-}$;SmoA1 mice (Figure 3.2a). ASC heterozygous SmoA1 mice also exhibited a delayed rate of tumorigenesis when compared to wild-type mice (Figure 3.2a). These results demonstrate a profound suppression of medulloblastoma tumorigenesis with ASC deletion.

To test whether ASC deficiency affects tumorigenesis in another model of medulloblastoma, we utilized the SmoM2 model. SmoM2 is a constitutively active Shh-driving *Smo* mutation discovered to aggressively induce medulloblastoma in mice by P20 (Xie et al., 1998; Jeong et al., 2004; Mao et al., 2006). We generated $ASC^{+/+}$, $ASC^{+/-}$, and $ASC^{-/-}$ GFAP-Cre:SmoM2 (SmoM2) mice and evaluated time to tumor emergence and moribund status. ASC deficiency markedly reduced tumorigenesis in the SmoM2 model, with loss of one or two ASC alleles extending survival (Figure 3.2c). While all mice

developed medulloblastoma in this model, the average age at which mice became moribund was delayed in ASC heterozygous and knock-out mice (Figure 3.2d). Thus, ASC deficiency profoundly reduced tumorigenesis and extended survival in two mouse models of medulloblastoma.

In addition to other functions, ASC acts as the adaptor protein for the inflammasome, a complex that, when triggered by intracellular signals, cleaves proinflammatory substrate pro-IL-1β into its mature form (Fernandes-Alnemri et al., 2009; Franchi et al., 2009; Davis et al., 2011). IL-1β has been reported to have several functions promoting cancer, although it has not been studied in medulloblastoma (Apte et al., 2006). To determine whether ASC requires IL-1β for medulloblastoma development, we evaluated tumorigenesis in IL-1β deficient mice in the SmoA1 model. IL-1β deficiency did not affect medulloblastoma onset or incidence (Figures 3.2e and f).

To determine the point at which ASC deficiency blocks tumorigenesis in medulloblastoma, we compared wild-type and ASC-deficient SmoA1 cerebella at multiple time points during tumor development. In this tumor model, ectopic CGNP proliferation can be detected by P20 and progresses into tumor in adulthood. To determine whether ASC deficiency affects CGNP hyperproliferation during the pre-tumor stages (P20, P60), histological H&E stained sections of $ASC^{+/+}$ and $ASC^{-/-}$ SmoA1 cerebella were compared. $ASC^{-/-}$;SmoA1 cerebella exhibited equivalent ectopic CGNP proliferation and EGL architecture as seen in $ASC^{+/+}$;SmoA1 mice at P7 and P20 (Figure 3.3a). However, by P60, ASC deletion significantly reduced CGNP hyperproliferation and reduced the proportion and thickness of EGL in the cerebella (Figures 3.3a and b). Thus, ASC deficiency did not block the

initial stages of CGNP hyperproliferation but markedly diminished ectopic EGL in the cerebellum by P60.

ASC deficient SmoA1 cerebella exhibit reduced proliferation

Tumorigenesis can be regulated by the balance of proliferation, differentiation, and apoptosis. We sought to determine which factors were responsible for the requirement of ASC in Shh-driven medulloblastoma. To test whether proliferation was altered in the ASC knock-out cerebella in the medulloblastoma model, we performed Western blot analysis for cyclin D2 and immunohistochemistry for the mitotic marker pH3 in wild-type and ASC-deficient SmoA1 mice. Cyclin D2 levels were unchanged at P7, but were reduced by P20 with ASC deficiency. This difference was even more striking by P60, at which point cyclin D2 levels were virtually undetectable in the ASC-deficient mice but were sustained in the wild-type mice on the SmoA1 background (Figure 3.3d). Thus, even though gross reduction in hyperplasia was not detected by P20 in the $ASC^{-/-}$;SmoA1 cerebella, markers of proliferation showed an emerging trend of reduction at this time point. To directly test whether ASC expression affected proliferation of the CGNPs in the EGL, we conducted pH3 immunohistochemistry. Consistent with the reduction in cyclin D2 levels, pH3 staining was also reduced by nearly 50 % at P20, revealing a lower mitotic frequency in the EGL with ASC deletion (Figures 3.3e and f). These results identify a role for ASC in regulating ectopic proliferation of CGNPs in medulloblastoma.

ASC RNA and protein levels were both high during the peak period of CGNP proliferation at P7 and decreased with cerebellar maturation during normal development

(Figure 3.1a), but continued to be expressed throughout development in the medulloblastoma model where CGNP proliferation persists (Figure 3.3d). Previous studies have reported a decrease in proliferation with ASC deficiency in stimulated CD4+ and CD8+ T cells, splenocytes, and lymph node cells (Kolly et al., 2009; Ippagunta et al., 2010; Narayan et al., 2011). However, other studies have reported either no difference in proliferation of ASC knock-out splenocytes or an increase in proliferation with ASC deletion in keratinocytes (Shaw et al., 2010; Drexler et al., 2012). Together, these data point to a complex role for ASC in regulating cell proliferation, with our results showing that ASC deficiency markedly reduces proliferation in medulloblastoma.

Numerous studies have shown that ASC is proapoptotic when overexpressed in cancer cell lines (Conway et al., 2000; Ohtsuka et al., 2004; Ohtsuka et al., 2006; Parsons and Vertino, 2006; Ramachandran et al., 2010; Hong et al., 2013). Furthermore, knockdown of endogenous ASC reduces apoptosis of breast epithelial cells (Parsons et al., 2009), colon cancer cells (Hong et al., 2013), and osteosarcoma cells (Ohtsuka et al., 2004). We examined whether ASC altered apoptosis or differentiation during tumorigenesis and found no statistical differences in the differentiation marker NeuN or the apoptosis marker cleaved caspase-3 between wild-type and ASC-deficient P20 SmoA1 EGL (Figures 3.3e and f). Likewise, other groups have found no difference in apoptosis with loss of endogenous ASC in keratinocytes or splenocytes (Kolly et al., 2009; Drexler et al., 2012). Together, these results identify reduced proliferation as the likely key factor suppressing medulloblastoma tumorigenesis in ASC deficient mice, as no significant differences in cell differentiation or apoptosis were observed between wild-type and ASC-deficient cerebella.

ASC deficient SmoA1 cerebella exhibit increased TGF-β signaling

To determine the mechanism by which ASC deficiency inhibits proliferation in medulloblastoma, we conducted a microarray analysis comparing gene expression of $ASC^{-/-}$ versus $ASC^{+/+}$ cerebella on the SmoA1 background. We focused on P20 because the differences in the $ASC^{-/-}$ and $ASC^{+/+}$ cerebellar phenotypes begin to emerge at this timepoint. Microarray data are available in Gene Expression Omnibus under the accession number GSE48682. Microarray analysis revealed only 3 genes (in addition to ASC) to be differentially expressed between $ASC^{-/-}$ and $ASC^{+/+}$ SmoA1 cerebellum at this timepoint (Table 3.1). Of these, *Tgfb1i1* (transforming growth factor beta-1-induced transcript 1), which was 2.5 fold higher in the ASC knock-out samples, is known to be upregulated with *TGF-β* (*transforming growth factor-β*) (Shibanuma et al., 1993). Interestingly, *Tgfb1i1* (also known as *Hic-5*) has been reported to inhibit cell proliferation when overexpressed in cells (Shibanuma and Nose, 1998; Dabiri et al., 2008). We next examined whether the TGF-β pathway was altered in the absence of ASC. Indeed, quantitative PCR analysis confirmed dysregulation of TGF-β pathway genes in the knock-out, with *Tgfb1i1* significantly upregulated while the TGF-β inhibitor *Tgif1* was significantly downregulated in ASC-deficient P20 SmoA1 mice. Furthermore, cell cycle genes *cyclin D2* and *Cdk2* were decreased in the ASC knock-out SmoA1 cerebella (Figure 3.4a).

Dysregulation of the TGF-β pathway has been recognized in a subgroup of medulloblastomas (Cho et al., 2011; Aref et al., 2012; Northcott et al., 2012). To investigate whether TGF-β signaling is altered in $ASC^{-/-}$ transgenic cerebella, we evaluated cellular

phospho-Smad3 localization using immunohistochemistry. Interestingly, we found a greater percentage of cells with nuclear Smad3 in the ASC deficient SmoA1 transgenic cerebella (Figure 3.4b). These data reveal an unexpected link between ASC and the TGF-β pathway whereby ASC deficiency results in an increase in signaling via the TGF-β pathway in a mouse model of medulloblastoma.

Interestingly, TGF-β activation has a dual role in cancer, as the pathway promotes epithelial-mesenchymal transition and metastasis, but early TGF-β activation restricts tumorigenesis by inducing apoptosis and reducing cell proliferation (Rich, 2003; Massague, 2008). The dichotomous effects of the TGF-β pathway are evident in medulloblastoma. One group found disruption of TGF-β pathway genes in one of four subsets of medulloblastoma, namely deletion of TGF-β inhibitors and amplification of Type II activin receptors and *TGFBR1* (Northcott et al., 2012). In contrast, we observed an association between increased TGF-β pathway gene expression and signaling and decreased CGNP proliferation in the ASC knock-out SmoA1 P20 cerebella. Consistent with our results, increasing TGF-β signaling with Smad5 overexpression in the presence of Shh stimulation reduces CGNP proliferation *in vitro* (Rios et al., 2004). Furthermore, increased TGF-β signaling (e.g. increased nuclear Smad3) in SHH-related human medulloblastomas is correlated with a more favorable prognosis (Aref et al., 2012).

While these results in medulloblastoma identify a clear role of ASC in promoting tumorigenesis, ASC has also been described as a tumor suppressor in specific cancer models. ASC suppresses tumorigenesis in a colitis-associated colon cancer mouse model, where ASC deficiency enhanced polyp formation and clinical exacerbation, resulting in

reduced survival (Allen et al., 2010; Zaki et al., 2010). In melanoma, ASC has a complex role in which selective deletion of ASC in bone marrow derived macrophages suppressed tumor number but ASC ablation in keratinocytes promoted tumorigenesis (Drexler et al., 2012). When ASC was deleted from the whole animal, ASC knock-out mice showed no differences from wild-type in melanoma susceptibility (Drexler et al., 2012). A recent study found that ASC-deficient metastatic melanoma cells injected into nude mice displayed reduced tumor growth as compared to wild-type tumor cell implantation. In contrast, ASC-deficient primary melanoma implants hastened tumor growth versus wild-type controls (Liu et al., 2013). Thus, the role of ASC in carcinogenesis appears to depend on cancer type and the stage of cancer progression.

In summary, our results identify a critical function of ASC in driving proliferation and tumorigenesis in Shh pathway-driven models of medulloblastoma. Improved therapeutics for this cancer are needed due to the high mortality and devastating side effects of current treatment. Our results show that targeting ASC may be a promising strategy for preventing tumor progression of medulloblastoma.

3.4 Figures and Legends

Figure 3.1. **ASC is expressed in cerebellum and medulloblastoma but ASC deficiency does not affect normal cerebellum development.**

(**a**) Western blot analysis of cerebellar lysates reveals ASC expression during normal cerebellum development and increased ASC expression in medulloblastoma. (Tumor sample shown is from postnatal 101-day-old, P101, mouse.) Densitometric quantification reveals ASC levels are increased in medulloblastomas. Data are mean of at least three separate experiments +/- s.e.m. analyzed by Student's *t*-test. ASC levels are normalized to loading controls and average P60 levels were set to 1.0 for each experiment. **P*<0.05. Cerebella were collected at specified ages and lysates were probed with antibodies against ASC (Alexis Biochemicals, Lausen, Switzerland; Adipogen, San Diego, CA, USA), Cyclin D2 (CD2; Cell Signaling, Danvers, MA, USA) and α-tubulin (Sigma-Aldrich, St. Louis, MO, USA). (**b**) Quantitative RT-PCR analysis demonstrates ASC mRNA decreases during normal cerebellum development and is induced in medulloblastoma. Cerebella were collected at the ages specified (n≥4 per age). Tumors were collected at an average age of P138 (P101-P205; n=8). qRT-PCR was conducted similarly as previously described (Kole et al., 2011), using ASC primers *Pycard*-FW: 5'-GACCAGCACAGGCAAGCA-3', *Pycard*-Rev: 5'-TCCAGCACTCCGTCCACTTC-3', *Gapdh*-FW: 5'-TGTGTCCGTCGTGGATCTGA-3', and *Gapdh*-Rev: 5'-CCTGCTTCACCACCTTCTTGA-3', and normalizing to GAPDH levels. Data are mean ± s.e.m analyzed by the Mann-Whitney test. Experiments were done in triplicate at least three

times. $**P<0.01$; $*P<0.05$. (**c**) Oncomine analysis of independent gene profiling studies, with data from Kool *et al.* (Kool et al., 2008) and Fattet *et al*. (Fattet et al., 2009) used to compare ASC mRNA expression levels in classic versus desmoplastic human primary medulloblastomas. Each dataset had equivalent proportions of desmoplastic tumors (22%) and classic tumors (78%) and F-test analysis determined the datasets did not have unequal variance (F=0.054). Each dataset mean was adjusted to zero to correct for technical bias between datasets. Data are median, minimum to maximum values (*, $P=0.025$, two-tailed *t*-test, F-test=0.054. n=74 classic; n=21 desmoplastic). ($P=0.047$ in comparison of unadjusted data.) (**d**) Representative images of H&E stained sagittal sections of $ASC^{+/+}$ and $ASC^{-/-}$ cerebella at specified ages show no differences in gross cerebellar architecture with development between genotypes (n≥3 per group). Scale bars represent 1 mm and 200 µm (inset). (**e**) $ASC^{+/+}$ and $ASC^{-/-}$ cerebellar lysates at specified ages were immunoblotted as described above. (**f**) Immunohistochemistry (IHC) for phospho-histone H3 (pH3; Cell Signaling) in P7 $ASC^{+/+}$ and $ASC^{-/-}$ sagittal cerebellar sections. Quantitative comparison of cells expressing pH3 in $ASC^{+/+}$ and $ASC^{-/-}$ EGL (n≥3 per group). The EGL region was manually annotated using Aperio ImageScope V12 and analyzed with Aperio Nuclear V9 Algorithm (Aperio Technologies, Inc., Vista, CA, USA). The data represent mean +/- s.e.m. analyzed by Student's *t*-test. Scale bar represents 200 µm.

Figure 3.1

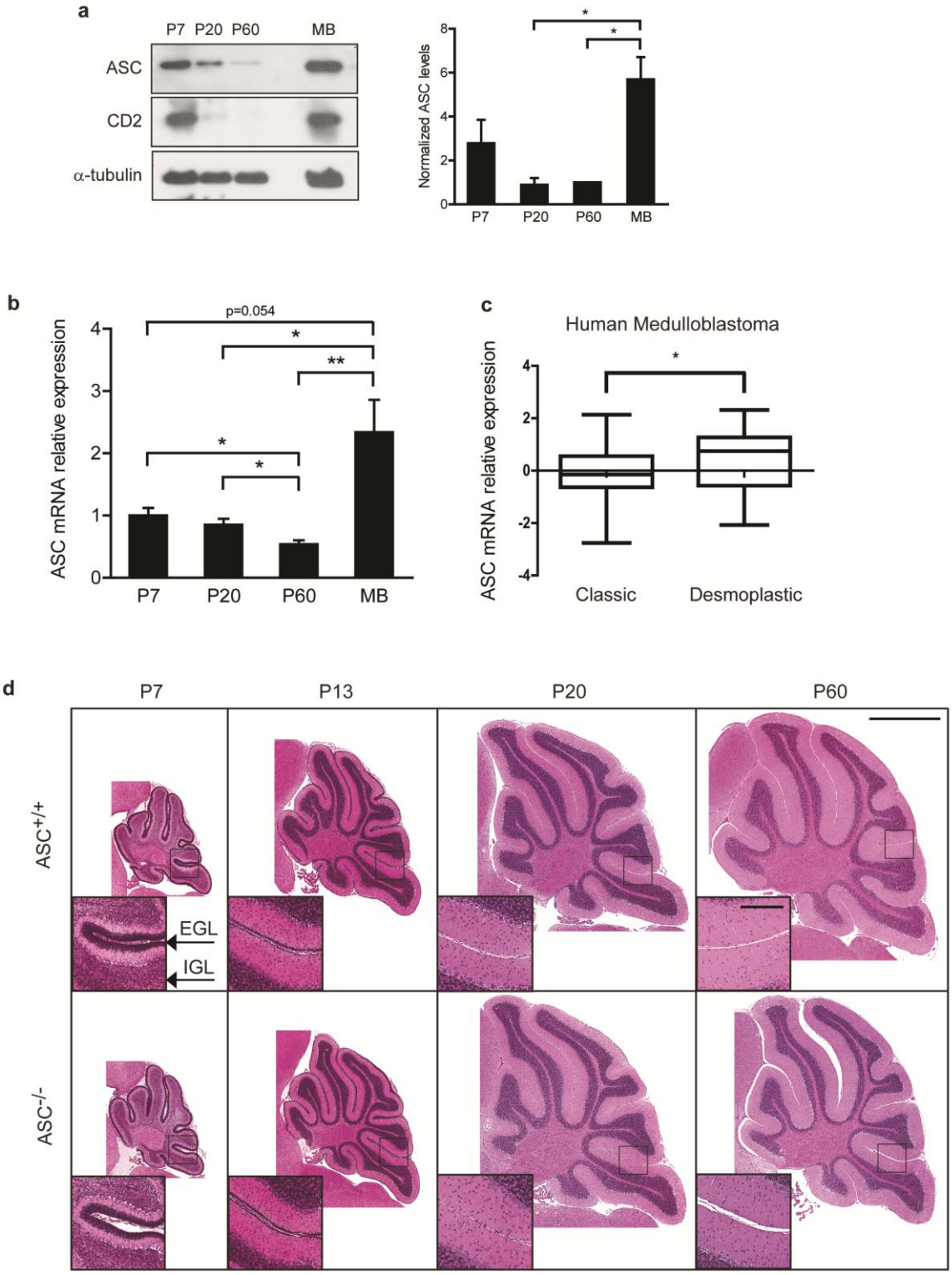

a
P7 P20 P60 MB
ASC
CD2
α-tubulin
Normalized ASC levels
P7 P20 P60 MB
b
p=0.054
ASC mRNA relative expression
P7 P20 P60 MB
c
Human Medulloblastoma
ASC mRNA relative expression
Classic Desmoplastic
d
P7 P13 P20 P60
ASC+/+
ASC-/-
EGL
IGL

Figure 3.1 continued

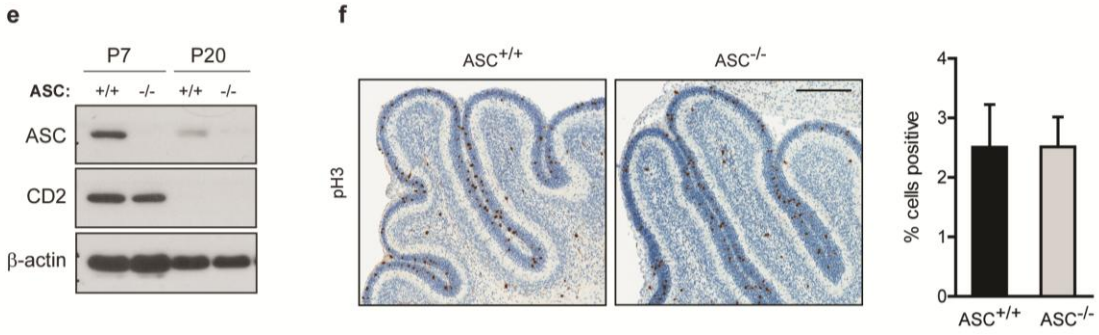


Figure 3.2. ASC deficiency suppresses medulloblastoma tumorigenesis in the SmoA1 and SmoM2 mouse models.

(**a**) Kaplan-Meier analysis of $ASC^{+/+}$;SmoA1 mice (n=15), $ASC^{+/-}$;SmoA1 mice (n=34), and $ASC^{-/-}$;SmoA1 mice (n=18) reveals a significant difference in tumor incidence with ASC expression (*P*=0.0019; Log-Rank test). ASC deficiency significantly decreased tumor frequency and increased tumor latency when compared to $ASC^{+/+}$;SmoA1 (*P*=0.0004; Log-Rank test) and $ASC^{+/-}$;SmoA1 mice (*P*=0.0077; Log-Rank test). (*P*=0.1108 between $ASC^{+/-}$;SmoA1 and $ASC^{+/+}$;SmoA1 mice; Log-Rank test.) (**b**) Overall incidence of medulloblastoma by P270 is reduced in $ASC^{-/-}$;SmoA1 versus $ASC^{+/+}$;SmoA1 (*P*=0.0049) and $ASC^{+/-}$;SmoA1 (*P*=0.0088) mice; ***P*<0.01 (Fisher's exact test). (**c**) Kaplan-Meier analysis of $ASC^{+/+}$;SmoM2 mice (n=4), $ASC^{+/-}$;SmoM2 mice (n=9), and $ASC^{-/-}$;SmoM2 mice (n=6) reveals a significant difference in tumor incidence with ASC expression (*P*= 0.0004; Log-Rank test). $ASC^{+/+}$;SmoM2 mice displayed shorter tumor latency than either $ASC^{-/-}$;SmoM2 (*P*=0.0025; Log-Rank test) or $ASC^{+/-}$;SmoM2 (*P*=0.0003; Log-Rank test) mice. (*P*=0.7317 between $ASC^{+/-}$;SmoM2 and $ASC^{-/-}$;SmoM2 mice; Log-Rank test.) (**d**) Average age mice become moribund is increased with ASC deficiency in $ASC^{-/-}$;SmoM2 mice and $ASC^{+/-}$;SmoM2 mice. ***P*=0.004; **P*=0.025 (Student's *t*-test). (**e**) IL-1β deficiency does not affect tumorigenesis in the SmoA1 medulloblastoma model. Kaplan-Meier analysis of $IL\text{-}1\beta^{+/+}$;SmoA1 mice (n=19), $IL\text{-}1\beta^{+/-}$;SmoA1 mice (n=59), and $IL\text{-}1\beta^{-/-}$;SmoA1 mice (n=11) reveals no difference in tumorigenesis with IL-1β expression (*P*=0.792; Log-Rank test). (**f**) Overall incidence of medulloblastoma by P270 is unaffected by IL-1β deficiency (Fisher's exact test).

Figure 3.2

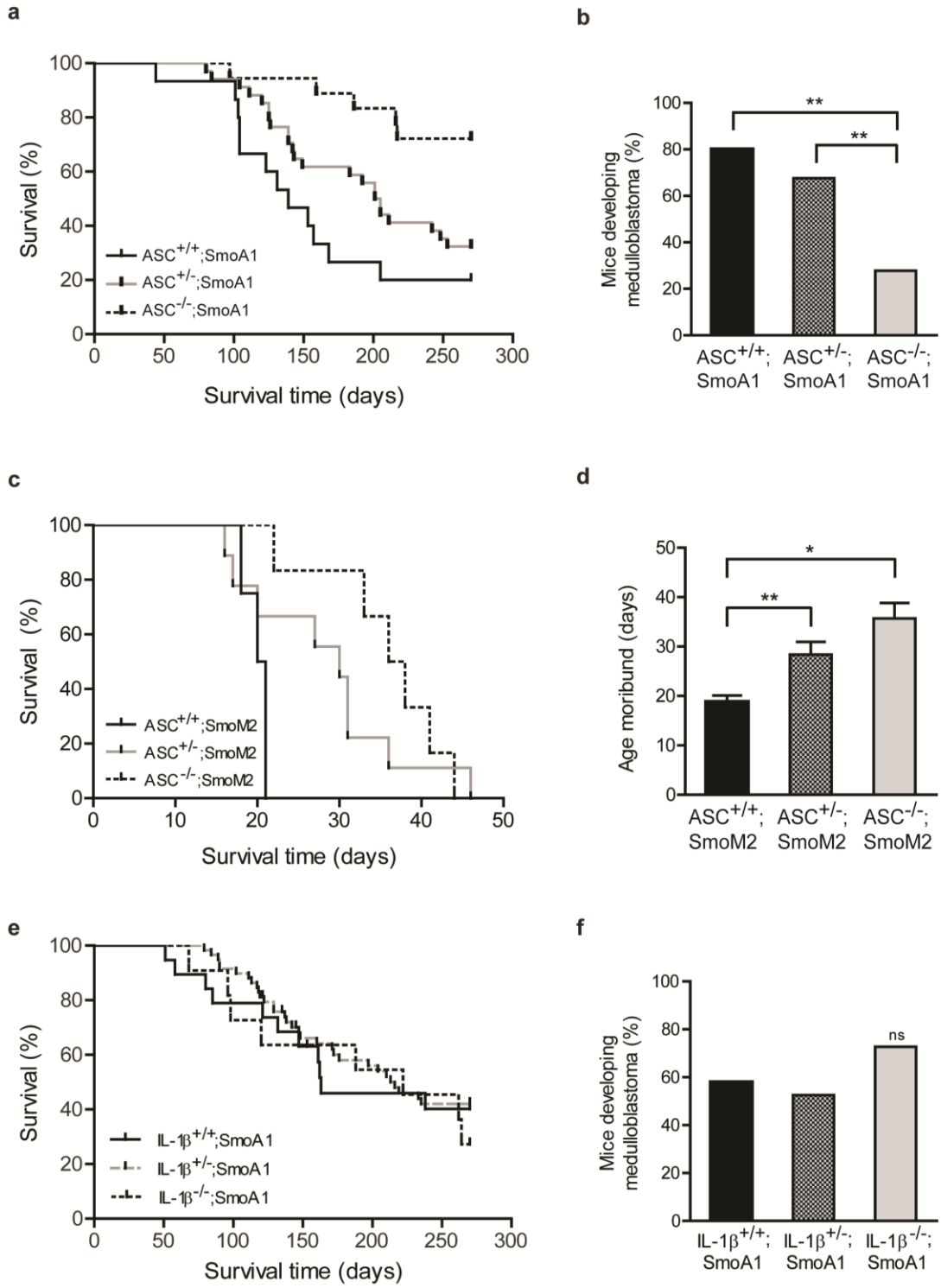

a
Survival (%)
Survival time (days)
ASC+/+;SmoA1
ASC+/-;SmoA1
ASC-/-;SmoA1
b
Mice developing medulloblastoma (%)
**
**
ASC+/+; SmoA1
ASC+/-; SmoA1
ASC-/-; SmoA1
c
Survival (%)
Survival time (days)
ASC+/+;SmoM2
ASC+/-;SmoM2
ASC-/-;SmoM2
d
Age moribund (days)
*
**
ASC+/+; SmoM2
ASC+/-; SmoM2
ASC-/-; SmoM2
e
Survival (%)
Survival time (days)
IL-1β+/+;SmoA1
IL-1β+/-;SmoA1
IL-1β-/-;SmoA1
f
Mice developing medulloblastoma (%)
ns
IL-1β+/+; SmoA1
IL-1β+/-; SmoA1
IL-1β-/-; SmoA1

Figure 3.3. ASC deficiency reduces proliferation in the SmoA1 cerebella.

(**a**) Representative H&E stained sagittal sections of $ASC^{+/+}$;SmoA1 and $ASC^{-/-}$;SmoA1 cerebella at specified ages reveals reduced EGL with ASC deficiency. Scale bars represent 1 mm and 200 μm (inset). EGL was quantified at P7, P20, and P60 by (**b**) EGL area in proportion to cerebellum (CB) area and (**c**) maximum EGL thickness in $ASC^{+/+}$;SmoA1 and $ASC^{-/-}$;SmoA1 cerebella at the specified ages (n≥3 per group). ASC deficiency reduced EGL area proportion and maximum EGL thickness in P60 cerebella. $^{**}P$=0.0049;$^{*}P$=0.045 (Student's *t*-test). ASC status significantly altered both EGL proportion and thickness over all timepoints (*P*=0.035 and *P*=0.04 respectively; two-way ANOVA). (**d**) Western blot analysis of $ASC^{+/+}$;SmoA1 and $ASC^{-/-}$;SmoA1 cerebellar lysates at the specified ages as described above. Densitometric quantification at P60 reveals Cyclin D2 (CD2) levels are reduced with ASC deficiency. Data are mean +/- s.e.m. analyzed by Student's *t*-test (n≥5 per group). $^{***}P$=0.00039. (**e**) IHC for pH3, NeuN (Millipore, Billerica, MA, USA), and cleaved caspase-3 (CC3; Biocare Medical, Concord, CA, USA) of P20 $ASC^{+/+}$;SmoA1 and $ASC^{-/-}$;SmoA1 cerebella (n≥3 per group). Scale bar represents 200 μm. (**f**) Quantification of positively stained cells in the P20 EGL reveals a significant decrease in pH3 staining with ASC deficiency. $^{*}P$= 0.017. The data represent mean +/- s.e.m. analyzed by Student's *t*-test (n≥3 per group).

Figure 3.3

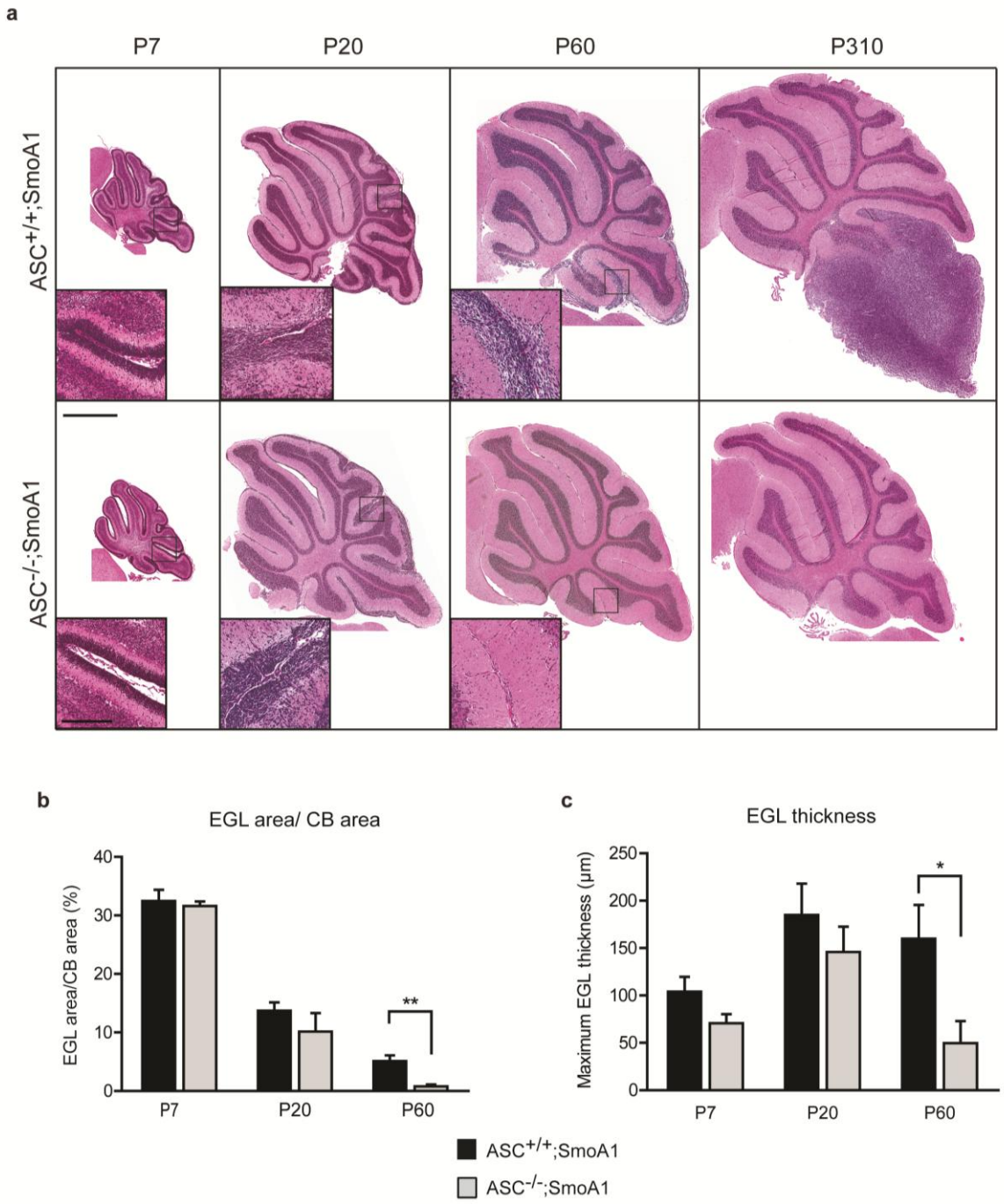

a
P7
P20
P60
P310
ASC+/+;SmoA1
ASC-/-;SmoA1
b
EGL area/ CB area
EGL area/CB area (%)
40
30
20
10
0
P7
P20
P60
**
c
EGL thickness
Maximum EGL thickness (µm)
250
200
150
100
50
0
*
ASC+/+;SmoA1
ASC-/-;SmoA1

Figure 3.3 continued

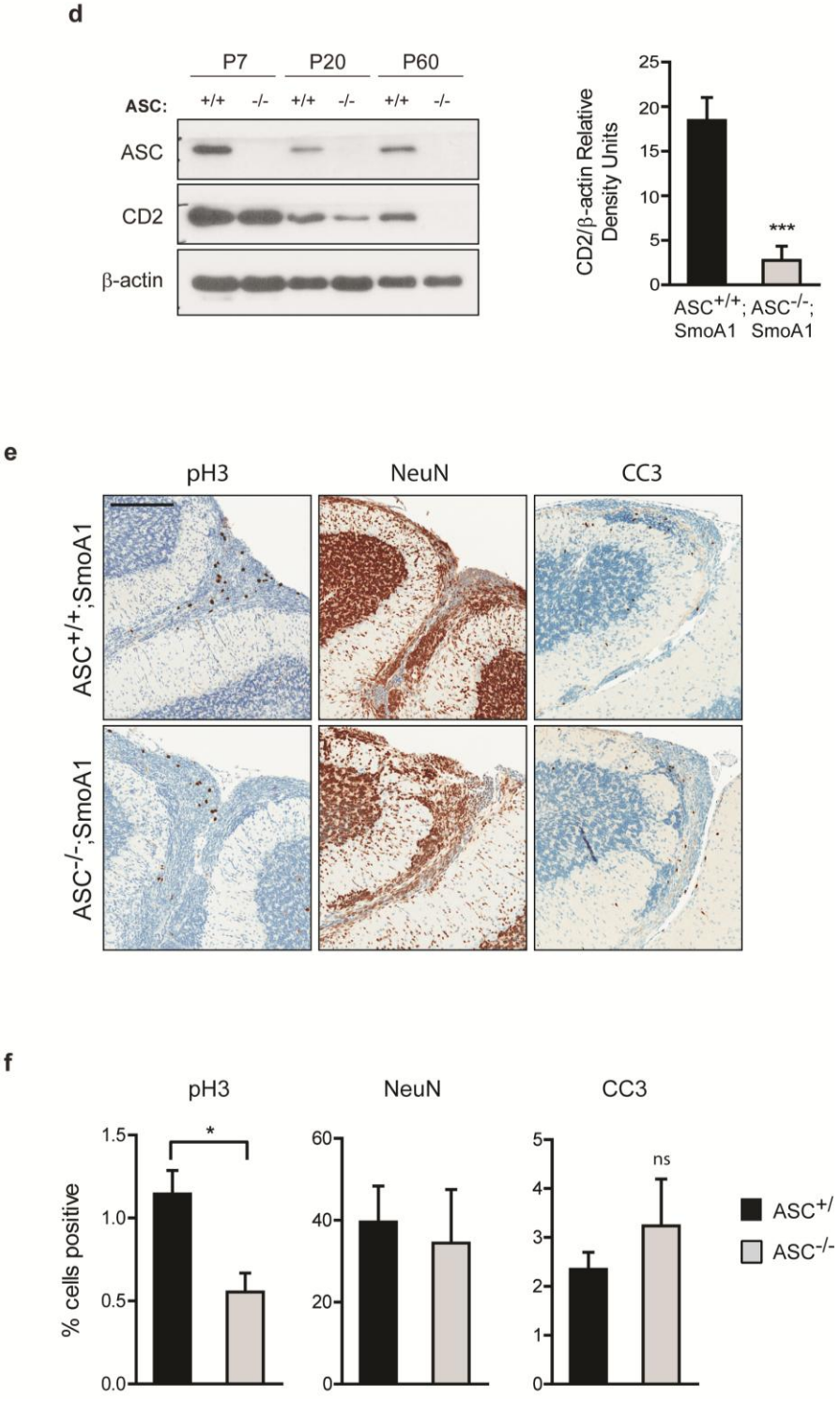


Table 3.1. Summary of genes differentially expressed with ASC deficiency in P20 SmoA1 cerebella.

Total RNA was purified from P20 SmoA1 cerebella (n=5, $ASC^{+/+}$;SmoA1; n=4, $ASC^{-/-}$;SmoA1) and 1000 ng RNA per sample was labeled using the Quick Amp Labeling Kit (Agilent Technologies, Sugar Land, TX, USA) and hybridized on two-color whole mouse genome 4x44K microarrays (Agilent Technologies). 1000 ng RNA from pooled P16 wild-type cerebella was amplified and labeled with Cy3 as a reference for each sample. Raw data was processed and analyzed by GeneSpring GX Version 11.0 (Agilent Technologies). Genes with a corrected *P* value <0.05 following unpaired *t*-test and Benjamini and Hochberg false discovery rate procedure were considered differentially expressed.

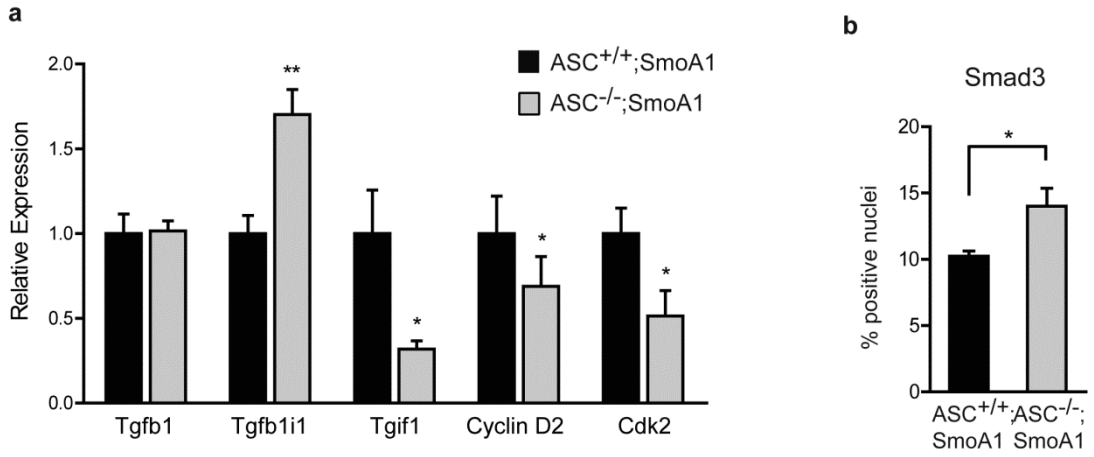
Table 3.1

Symbol	RefSeq	Gene Name	Fold change	*P* value
Up-regulated				
Gdpd3	NM_024228	Glycerophosphodiester phospho-diesterase domain containing 3	+5.914	0.0268
Tgfb1i1	NM_009365	Transforming growth factor beta 1 induced transcript 1	+2.504	0.0494
Down-regulated				
Sec23ip	NM_001029982	Sec23-interacting protein	-1.492	0.0268

Figure 3.4. ASC deficiency alters the TGF-β pathway in SmoA1 cerebella.

(**a**) Quantitative RT-PCR analysis reveals several genes in the TGF-β pathway are differentially expressed in $ASC^{-/-}$ versus $ASC^{+/+}$ P20 SmoA1 cerebella. qRT-PCR was conducted similarly as previously described (Kole et al., 2011), using primers *Tgfb1*-FW: 5'-ACCATGCCAACTTCTGTCTG-3' and *Tgfb1*-Rev: 5'-CGGGTTGTGTTGGTTGTAGA-3'; *Tgfb1i1*-FW: 5'-CCTTTTCGCCCCGAGTGCTA-3' and *Tgfb1i1*-Rev: 5'-CGGATGGGTTGGTTACAGAAG-3'; *Tgif1*-FW: 5'-GAGGATGAAGACAGCATGGA-3' and *Tgif1*-Rev: 5'-TTCTCAGCATGTCAGGAAGG-3'; *Cyclin D2*-FW: 5'-TCGATGATTGCAACTGGAAG-3' and *Cyclin D2*-Rev: 5'-AGAGCTTCGATTTGCTCCT-3'; and *Cdk2*-FW: 5'-TCATGGATGCCTCTGCTCTCAC-3' and *Cdk2*-Rev: 5'-TGAAGGACACGGTGAGAATGGC-3'; *Gapdh*-FW: 5'-TGTGTCCGTCGTGGATCTGA-3' and *Gapdh*-Rev: 5'-CCTGCTTCACCACCTTCTTGA-3', and normalizing to GAPDH levels. Data are mean ± s.e.m analyzed by the Mann-Whitney test. Experiments were done in triplicate at least three times (n≥3 per genotype). $^{**}P<0.01$; $^{*}P<0.05$. (**b**) Quantification of Smad3 IHC (Abcam, Cambridge, MA, USA) in P20 $ASC^{+/+}$;SmoA1 and $ASC^{-/-}$;SmoA1 cerebella reveals an increase in cells positive for nuclear Smad3. 50 μm wide bands of the outer internal granular layer and molecular layer were analyzed and Purkinje cells were excluded from the analysis. $^{*}P<0.05$. The data represent mean +/- s.e.m. analyzed by Student's *t*-test (n≥3 per group).

Figure 3.4


a
ASC+/+;SmoA1
ASC-/-;SmoA1
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
*
*
*
Tgfb1
Tgfb1i1
Tgif1
Cyclin D2
Cdk2
b
Smad3
% positive nuclei
20
15
10
5
0
*
ASC+/+; SmoA1
ASC-/-; SmoA1

CHAPTER IV: DISCUSSION

4.1 Summary of Findings

While the brain has long been considered an immunoprivileged region, our perceptions are shifting to recognize that immune genes play important roles in neurons and the nervous system. Research is revealing the unexpected results that neurons express immune genes such as complement and toll-like receptors (TLRs), and now, the NLRs and inflammasome genes. Surprisingly, the role of immune molecules in neurons varies from neuronal or immune functions to no known function. For instance, complement proteins direct synapse elimination in developing brain (Stevens et al., 2007) and TLR-3 activation can suppress axonal growth (Cameron et al., 2007) or reduce HSV-1 infection (Zhou et al., 2009). Furthermore, cytokines produced by immune cells can influence neuron-specific characteristics, such as IL-2 regulating neurite outgrowth of sympathetic neurons (Haugen and Letourneau, 1990). In turn, sympathetic nerves of the peripheral nervous system can regulate the immune system by modulating cytokine release and immune cell recruitment into target organs (Madden et al., 1994; Li et al., 2004). Finally, immune genes can regulate tumorigenesis, as has been shown with ASC promoting metastatic melanoma (Liu et al., 2013) and colon cancer (Allen et al., 2010). This dissertation work is the first to investigate the role of ASC expression on a cancer of the nervous system and also the first to elucidate

how the sympathetic neuron apoptotic pathway is affected by interferon-gamma. Thus, we are in a nascent stage in a burgeoning field at the crossroads of neurobiology and immunology. The findings from this dissertation are summarized below.

Interferon-gamma protects sympathetic neurons from apoptosis at the point of cytochrome c release:

1. Whereas IFN-γ induces apoptosis in many cell types, IFN-γ protects sympathetic neurons from apoptosis induced by nerve growth factor (NGF) deprivation or pan-kinase inhibition.
2. The inhibition of apoptosis by IFN-γ occurs at the point of mitochondrial permeabilization.
3. IFN-γ does not change neuron death with cytochrome *c* injection, revealing that it does not directly act downstream of mitochondrial permeabilization.
4. Despite inhibiting apoptosis in neurons, IFN-γ induces upregulation of interferon-stimulated genes and proapoptotic genes in sympathetic neurons.
5. Many proapoptotic genes that are induced by interferon in other cell types are not induced by IFN-γ in sympathetic neurons.

ASC deficiency suppresses proliferation and prevents medulloblastoma incidence:

1. ASC, which is silenced by promoter methylation in many cancers, is upregulated at the RNA and protein level in a Sonic hedgehog-driven model of medulloblastoma.
2. ASC is expressed in human medulloblastomas and its expression is increased in Sonic hedgehog-driven tumors over classic tumors.
3. ASC knock-out cerebella on a non-tumor background display gross architecture and proliferation markers similar to wild-type.
4. ASC deficient ND2:SmoA1 mice exhibit a profound reduction in medulloblastoma incidence and a delayed tumor onset.
5. ASC heterozygous and knock-out GFAP-Cre:SmoM2 mice display a markedly extended survival.
6. IL-1β deficiency does not affect tumorigenesis in the SmoA1 model.
7. Premalignant lesions in cerebella of $ASC^{-/-}$;ND2:SmoA1 mice displayed a striking decrease in number of ectopic progenitors.
8. Markers of proliferation, but not apoptosis or differentiation, decreased with ASC deletion in ND2:SmoA1 cerebella.
9. ASC deficiency disrupted expression of genes in the TGF-β pathway and increased the level of nuclear Smad3 in the SmoA1 model.

4.2 Discussion of the Major Findings and Future Directions

Interferon-gamma protects sympathetic neurons from apoptosis at the point of cytochrome c release

During infection, injury, or tumors, interferons (IFNs) are produced and released by cells to inhibit pathogen replication, regulate immune cells, modulate cell growth, proliferation, and differentiation, and induce cell death (Chawla-Sarkar et al., 2003; Maher et al., 2007). Interferons are considered apoptosis-inducing cytokines (Schroder et al., 2004) and induce apoptosis and other forms of cell death in many cell types (Dijkmans et al., 1990; Baerwald and Popko, 1998; Barber, 2000; Becher et al., 2000; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005; Maher et al., 2007; Zhang et al., 2008; Das et al., 2009) to limit the spread of infection (Dafny and Yang, 2005; Maher et al., 2007). Sympathetic neurons of the peripheral nervous system may be particularly exposed to infectious microorganisms and immune cells, as some pathogens first invade sensory nerves and spread to the brain via centripetal axonal transport (Rottenberg and Kristensson, 2002).

In this research, we show that the effect of IFN-γ on sympathetic neurons is completely the opposite of what is seen in other cell types. Not only does IFN-γ fail to induce apoptosis, but it suppresses apoptosis in sympathetic neurons treated with NGF deprivation or pan-kinase inhibition (Fig. 2.1). We have probed the apoptotic pathway in IFN-γ-treated sympathetic neurons and found that IFN-γ inhibited the events that occur after the point of mitochondrial permeabilization including cytochrome *c* release and

caspase-3 activation but didn't change the early events of c-Jun phosphorylation or BH3-only protein induction (Fig. 2.2). Thus, IFN-γ acts at the point of mitochondrial permeabilization, likely through inhibition of Bax activation, to suppress the intrinsic apoptotic pathway in NGF-deprived sympathetic neurons. Cytochrome *c* injection experiments also revealed that IFN-γ does not inhibit apoptosis after the point of mitochondrial permeabilization in sympathetic neurons (Fig. 2.3). Interestingly, gene array analysis showed that IFN-γ upregulates several proapoptotic genes in sympathetic neurons, but this induction is insufficient to cause apoptosis in these cells (Table 2.3). Finally, IFN-γ did not induce expression of a number of proapoptotic genes in sympathetic neurons as it does in other cell types (e.g. *Fas*, *FADD*, *caspases*) (Table 2.3). Overall, these findings demonstrate that sympathetic neurons, unlike most cell types, respond to interferon by suppressing the apoptotic pathway. Thus, IFN-γ promotes survival of these terminally differentiated sympathetic neurons, which must persist for the lifetime of the organism.

The data presented in this dissertation pinpoints mitochondrial permeabilization as the step in the apoptotic pathway which IFN-γ inhibits in NGF-deprived sympathetic neurons. Our hypothesis is that IFN-γ acts directly at the point of Bax activation to inhibit neuronal apoptosis. One approach to test this hypothesis is as follows. In the extrinsic apoptotic pathway, Bid is cleaved by active caspase-8 into tBid, which then induces the oligomerization of Bax at the mitochondria, forming a pore in the outer membrane (Eskes et al., 2000; Grinberg et al., 2002). Thus, tBid can be used as a tool to directly trigger Bax activation in cells. We will perform microinjection of truncated Bid (tBid) into sympathetic neurons with or without IFN-γ and assess cytochrome *c* release. If IFN-γ suppresses Bax

activation, tBid injection will result in reduced Bax activation and cell death with IFN-γ. An alternative approach to this question would be to inject GFP-Bax plasmid into neurons with pan-caspase inhibitor QVD-OPH. GFP-Bax translocates from a diffuse cytosolic expression to a punctate pattern at the mitochondria upon activation (Wolter et al., 1997). Microinjection of this plasmid into sympathetic neurons treated with or without IFN-γ will result in similar or differential Bax localization and activation, revealing whether IFN-γ suppresses Bax activation.

Our data show that levels of Bax, Bcl-XL, Bim, and Puma are unchanged with IFN-γ (Fig. 2.2), thus future research is needed to determine the exact mechanism by which IFN-γ suppresses mitochondrial permeabilization in sympathetic neurons. Several candidates not yet examined here have previously been shown to affect Bax activation. For example, we will examine levels of another BH3-only protein, Mcl-1, which may be regulated by protein kinase casein kinase 2 (Chang and Chao, 2013). Another candidate is Ku70, which binds Bax to sequester it from the mitochondria and prevent mitochondrial permeabilization (Cohen et al., 2004). Furthermore, while Bim and Bmf are induced by phospho-c-Jun during apoptosis, phosphorylation of these BH3-only proteins may free them from sequestration at dynein motor complexes, allowing engagement in the apoptotic pathway (Lei and Davis, 2003). Thus, we will examine whether IFN-γ decreases the phosphorylation of Bim and Bmf, reducing their ability to activate Bax.

We found HIN-200 gene *ifi202b* to be highly induced in sympathetic neurons with IFN-γ treatment (Fig. 2.4). *ifi202b* encodes p202, which binds and inhibits several transcription factors, including c-Jun, c-Fos, c-Myc, NF-κB, E2F2, and p53 (Min et al., 1996;

Asefa et al., 2004; Choubey and Panchanathan, 2008; Mondini et al., 2010). p202 can enhance or diminish apoptosis in cells depending on which transcription factors are active in each cell type (Choubey and Panchanathan, 2008). While c-Jun induction and phosphorylation was unchanged by IFN-γ in NGF-deprived sympathetic neurons (Fig. 2.2), it is possible IFN-γ could act through p202 to regulate the activity of other transcription factors, such as NF-κB or E2Fs, which regulate sympathetic neuron survival (Maggirwar et al., 1998; Wright et al., 2007). We found that microinjection of *ifi202b* plasmid or *sh-ifi202b* into neurons did not affect apoptosis with NGF deprivation (data not shown). However, since levels of p202 are constitutively high in neurons compared to other cell types (data not shown), overexpression or IFN-γ induction of p202 may not confer additional protection. Methodologically, the individual cell assay of microinjection experiments only allows for evaluation of cellular expression by immunofluorescence, and the p202 antibody had not been tested in knock-out cells. Future experiments should utilize sympathetic neurons from a p202 knock-out organism to assess the importance of p202 in modulating neuronal survival.

The data presented here show that sympathetic neurons respond to IFN-γ by upregulating an array of genes including interferon-stimulated genes and also several genes (e.g. *Trail, Irf1*, *Irf8,* and *PKR*), which promote activation of the extrinsic apoptotic pathway (Table 2.2 and 2.3) (Barber, 2000; Chawla-Sarkar et al., 2003; Clemens, 2003; Schroder et al., 2004; Pokrovskaja et al., 2005). The induction of proapoptotic genes was insufficient to reduce survival of sympathetic neurons, however, perhaps due to these cells being resistant to apoptosis induced by death receptor ligands (Marz et al., 1996). No anti-apoptotic genes

were identified in the unbiased gene array (Table 2.2). Importantly, sympathetic neurons did not upregulate many of the proapoptotic genes reported to be induced by interferon in other cell types, revealing a differential response in these neurons (Table 2.3). We are currently quantitatively comparing IFN-γ-induced gene expression between sympathetic neurons and other cell types using publically available microarray data on Gene Expression Omnibus (GEO). The findings will reveal which of the tens of thousands of genes are differentially expressed in neurons versus other cell types, illuminating neuron-specific gene induction in response to IFN-γ.

IFN-γ promoting neuronal survival: possible physiological implications

Sympathetic neurons are vulnerable to apoptosis during development but resistant during maturity (Kole et al., 2011). The data presented here indicate that IFN-γ protects developing sympathetic neurons from apoptosis. The ability of IFN-γ to inhibit neuronal apoptosis could be a physiological mechanism that protects neurons from the harmful effects of immune cell activation, infection, or injury during development. Sympathetic neurons of the peripheral nervous system may be particularly exposed to infectious microorganisms, as some pathogens first invade sensory nerves and spread to the brain via centripetal axonal transport (Rottenberg and Kristensson, 2002). In addition to inhibiting replication of neurotrophic viruses and bacteria, such as *Listeria monocytogenes* (Rottenberg and Kristensson, 2002), IFN-γ protects against neuronal death from herpes simplex virus-1 (Geiger et al., 1997), Sindbis virus (Burdeinick-Kerr et al., 2009), and Borna disease virus (Richter et al., 2009). IFN-γ is secreted by macrophages, NK cells, and T

lymphocytes, which are recruited to the site of infection or injury (Chawla-Sarkar et al., 2003; Schroder et al., 2004). IFN-γ activates macrophages, which then release molecules such as nitric oxide radicals that can kill pathogens, but could also inadvertently trigger sympathetic neuron death (Arantes et al., 2000; Rottenberg and Kristensson, 2002; Almeida-Leite et al., 2007). Similarly, stimulated T or NK cells can promote DRG and hippocampal neuron death (Rottenberg and Kristensson, 2002; Yong et al., 2007). IFN-γ protects neurons from activated macrophages, T lymphocytes, and NK cells, indicating that IFN-γ counteracts cytotoxic signals from the immune cells in the neurons' milieu (Burdeinick-Kerr et al., 2009; Richter et al., 2009). Thus, control of infection depends on recruitment and activation of immune cells which produce IFN-γ that both inhibits microorganisms and protects neurons from death. Disruption of IFN-γ signaling can therefore render the nervous system vulnerable to infection. For example, immunodeficiency disorders such as Human Immunodeficiency Virus (HIV) are characterized by disruption of immune cells, leading to lower IFN-γ and increased susceptibility to infections (Koirala et al., 2008). Therefore, clinical administration of IFN-γ may be advantageous in certain immunodeficient individuals to prevent infection and protect developing sympathetic neurons from cell death. Since IFN-γ can induce cell death of many cell types, including cortical neurons (Dedoni et al., 2012), IFN-γ administration should be localized to the site of infection in treated individuals. Finally, it remains to be seen whether IFN-γ can be an effective therapeutic for disorders of mature neuron degeneration, for instance in HIV-related neuropathies of the peripheral nervous system (Kamerman et al.,

2012), which are characterized by macrophage-induced loss of DRG neurons (Pardo et al., 2001).

ASC deficiency suppresses proliferation and prevents medulloblastoma incidence:

ASC, also known as TMS-1, target of methylation-induced silencing-1, is silenced by promoter methylation in many types of cancers (Conway et al., 2000; Guan et al., 2003; Alaminos et al., 2004; Stone et al., 2004; Machida et al., 2006) Ectopic ASC expression induces cell death (Conway et al., 2000; Ohtsuka et al., 2004; Ohtsuka et al., 2006; Parsons and Vertino, 2006; Ramachandran et al., 2010; Hong et al., 2013), while knock down of endogenous ASC inhibits apoptosis in several cell types (Ohtsuka et al., 2004; Parsons et al., 2009; Hong et al., 2013). Furthermore, ASC is the adaptor protein for the inflammasome, which when activated can induce cell death (Satoh et al., 2013). Due to ASC's pro-death functions, ASC has been considered a tumor suppressor in several cancers.

The data presented here reveal the unexpected results that ASC potently promotes tumorigenesis in two Sonic hedgehog-driven models of medulloblastoma, the most common malignant pediatric brain cancer (Hatten and Roussel, 2011). ASC was expressed at high levels in tumors and ASC deficiency robustly suppressed medulloblastoma incidence and delayed age of tumor onset (Fig. 3.1, 3.2). ASC deficient mice displayed reduced premalignant lesions as well as EGL proliferation, while EGL apoptosis and differentiation remained unchanged (Fig. 3.3). Furthermore, ASC deficiency led to an increase in cerebellar TGF-β pathway expression and signaling (Fig. 3.4).

Interestingly, although ASC had a profound effect on medulloblastoma incidence, ectopic EGL volume, and proliferation, there was no discernible difference between ASC

knock-out and wild-type on the non-tumor background in gross cerebellar architecture or proliferation (Fig. 3.1). Thus, ASC is not required for normal cerebellum development but is necessary for cerebellum cancer development. These findings reveal a requirement of ASC in hyperproliferation but not normal proliferation of CGNPs. Further research is needed to elucidate the mechanisms underlying these differences.

ASC promotes proliferation markers in the ectopic EGL in the medulloblastoma model. To examine whether the effects of ASC in CGNP proliferation was cell autonomous, we cultured CGNPs from ASC wild-type or ASC knock-out mice and compared cyclin D2 expression using Western blotting. Surprisingly we found that cyclin D2 levels were equivalent regardless of ASC expression in this cell culture model (data not shown). This finding suggests that ASC expression may not have a cell-autonomous effect on CGNP proliferation. ASC is a ubiquitously expressed gene and is known to be expressed in astrocytes and microglia. Therefore, other cell types that secrete factors to regulate CGNP proliferation may be impacted by ASC expression. For instance, astrocyte-secreted GDNF (glial cell line- derived neurotrophic factor) increases proliferation of neural progenitors in the hippocampus (Chen et al., 2005).

Our research shows that ASC modulates expression of genes in the TGF- β pathway (Table 3.1). ASC has a CARD and PYRIN domain, which are protein interacting domains that allow it to bind caspases, NLRs, and other proteins via homotypic CARD or PYRIN domain binding. Through protein-protein interactions with IKKα and IKKβ through the PYRIN domain, ASC has been shown to bind and enhance or inhibit transcription factor activity of NF-κB (Stehlik et al., 2002). Thus, there is precedent for ASC regulating gene expression

indirectly. Determining how ASC is regulating expression of TGF-β pathway genes is a promising area for further research. Furthermore, TGF-β signaling is dysregulated in many disorders, including muscular dystrophy (Ceco and McNally, 2013), glaucoma (Prendes et al., 2013), and many cancers, where it promotes epithelial-mesenchymal transition (EMT) to drive metastasis (Katsuno et al., 2013). It will be interesting to see if ASC modulates the TGF-β pathway in these disease states as well.

In addition to other functions, ASC is the adaptor protein of the inflammasome, a cytosolic complex that responds to pathogen or danger-associated molecules by activating Caspase-1, which then activates its substrates including IL-1β and IL-18. To test whether ASC's role in promoting medulloblastoma was inflammasome-dependent, we tested whether Caspase-1 or IL-1β expression altered tumor incidence similarly to ASC expression. IL-1β knock-out ND2:SmoA1 mice exhibited equivalent tumor incidence and rate of tumorigenesis as IL-1$\beta^{+/+}$;ND2:SmoA1 mice (data not shown), demonstrating that ASC's effects on medulloblastoma are independent of IL-1β. Results for Caspase-1 are pending and will determine whether ASC requires Caspase-1 and the inflammasome in this model. If ASC is acting via the inflammasome to promote tumorigenesis in medulloblastoma, clinical consideration should be given to avoid chemotherapeutics such as gemcitabine and 5-fluorouracil which activate the inflammasome (Bruchard et al., 2013).

Tumors consist of regions of apoptotic and necrotic cells which release ATP, triggering activation of the NLRP3 inflammasome (Ghiringhelli et al., 2009). Inflammasome activation stimulates and recruits immune cells to the site of activation. It is possible that dying cells in medulloblastomas also trigger inflammasome activation, which may result in

immune cell recruitment. Our research reveals that medulloblastoma tumors are characterized by enhanced astrocyte and microglial recruitment and activation, as compared to non-tumor cerebellum. We found no difference in astrocyte or microglial activation in fully developed $ASC^{-/-}$ tumors, however there was reduced astrocyte activation and fewer microglia in the P20 ASC knock-out ND2:SmoA1 EGL (data not shown). The influence of these cell types has not been studied in medulloblastoma, thus how the activation of these cells influence medulloblastoma progression remains an unexplored and intriguing area for future study.

We found ASC deficiency decreased markers of proliferation, cyclin D2 and pH3, in ectopic EGL in a medulloblastoma model (Fig. 3.3), however, ASC deletion has varied effects on tumor growth in other cancers. In a model of melanoma, ASC deficiency of metastatic melanoma cells was determined to decrease tumor growth upon implantation into nude mice (Liu et al., 2013). However, the same study found ASC knock down in primary melanoma cells to hasten tumor growth versus wild-type controls (Liu et al., 2013). Furthermore, ASC deletion in macrophages suppressed tumor number but ASC silencing in keratinocytes spurred tumorigenesis (Drexler et al., 2012). In colitis-associated colon cancer, ASC deficiency clearly enhanced tumor formation and progression (Allen et al., 2010; Zaki et al., 2010). These studies reveal a complex role for ASC in tumorigenesis which depends on the cancer, the type of cells expressing ASC, and the stage of tumor progression. These findings reveal that ASC, like TGF-β, can either suppress or promote tumorigenesis.

The discovery of a gene that has such a stark effect on medulloblastoma tumorigenesis is very rare and promising. Based on the data presented here, we propose ASC as a promising target for cancer therapeutics. Current treatments of medulloblastoma have devastating side effects and, even with treatment, mortality remains high for this disease (Polkinghorn and Tarbell, 2007). Targeted gene therapy to knock down ASC expression in tumors and surrounding areas may prove effective in reducing growth or metastasis of medulloblastoma.

REFERENCES

Acehan, D., X. Jiang, D. G. Morgan, J. E. Heuser, X. Wang and C. W. Akey (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell* **9**(2): 423-432.

Aebischer, J., P. Cassina, B. Otsmane, A. Moumen, D. Seilhean, V. Meininger, L. Barbeito, B. Pettmann and C. Raoul (2011). IFNgamma triggers a LIGHT-dependent selective death of motoneurons contributing to the non-cell-autonomous effects of mutant SOD1. *Cell Death Differ* **18**(5): 754-768.

Alaminos, M., V. Davalos, N. K. Cheung, W. L. Gerald and M. Esteller (2004). Clustering of gene hypermethylation associated with clinical risk groups in neuroblastoma. *J Natl Cancer Inst* **96**(16): 1208-1219.

Allen, I. C., E. M. TeKippe, R.-M. T. Woodford, J. M. Uronis, E. K. Holl, A. B. Rogers, H. H. Herfarth, C. Jobin and J. P. Y. Ting (2010). The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med* **207**(5): 1045-1056.

Almeida-Leite, C. M., L. M. Galvao, L. C. Afonso, F. Q. Cunha and R. M. Arantes (2007). Interferon-gamma induced nitric oxide mediates in vitro neuronal damage by Trypanosoma cruzi-infected macrophages. *Neurobiol Dis* **25**(1): 170-178.

Apte, R. N., Y. Krelin, X. Song, S. Dotan, E. Recih, M. Elkabets, Y. Carmi, T. Dvorkin, R. M. White, L. Gayvoronsky, S. Segal and E. Voronov (2006). Effects of micro-environment- and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour-host interactions. *European J Cancer* **42**(6): 751-759.

Arantes, R. M., S. Lourenssen, C. R. Machado and M. G. Blennerhassett (2000). Early damage of sympathetic neurons after co-culture with macrophages: a model of neuronal injury in vitro. *Neuroreport* **11**(1): 177-181.

Aref, D., C. J. Moffatt, S. Agnihotri, V. Ramaswamy, A. M. Dubuc, P. A. Northcott, M. D. Taylor, A. Perry, J. M. Olson, C. G. Eberhart and S. E. Croul (2012). Canonical TGF-beta Pathway Activity Is a Predictor of SHH-Driven Medulloblastoma Survival and Delineates Putative Precursors in Cerebellar Development. *Brain Pathol* **23**(2): 178-191.

Asefa, B., K. D. Klarmann, N. G. Copeland, D. J. Gilbert, N. A. Jenkins and J. R. Keller (2004). The interferon-inducible p200 family of proteins: a perspective on their roles in cell cycle regulation and differentiation. *Blood Cells Mol Dis* **32**(1): 155-167.

Baerwald, K. D. and B. Popko (1998). Developing and mature oligodendrocytes respond differently to the immune cytokine interferon-gamma. *J Neurosci Res* **52**(2): 230-239.

Barber, G. N. (2000). The interferons and cell death: guardians of the cell or accomplices of apoptosis? *Semin Cancer Biol* **10**(2): 103-111.

Becher, B., A. Prat and J. P. Antel (2000). Brain-immune connection: immuno-regulatory properties of CNS-resident cells. *Glia* **29**(4): 293-304.

Bernabei, P., A. Allione, L. Rigamonti, M. Bosticardo, G. Losana, I. Borghi, G. Forni and F. Novelli (2001). Regulation of interferon-gamma receptor (INF-gammaR) chains: a peculiar way to rule the life and death of human lymphocytes. *Eur Cytokine Netw* **12**(1): 6-14.

Bernabei, P., E. M. Coccia, L. Rigamonti, M. Bosticardo, G. Forni, S. Pestka, C. D. Krause, A. Battistini and F. Novelli (2001). Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. *J Leukoc Biol* **70**(6): 950-960.

Boselli, D., J. Ragimbeau, L. Orlando, P. Cappello, M. Capello, C. Ambrogio, R. Chiarle, G. Marsili, A. Battistini, M. Giovarelli, S. Pellegrini and F. Novelli (2010). Expression of IFNgammaR2 mutated in a dileucine internalization motif reinstates IFNgamma signaling and apoptosis in human T lymphocytes. *Immunol Lett* **134**(1): 17-25.

Boucher, D. and J. Denault (2012). Caspase Family. Sherbrooke, Springer.

Bruchard, M., G. Mignot, V. Derangere, F. Chalmin, A. Chevriaux, F. Vegran, W. Boireau, B. Simon, B. Ryffel, J. L. Connat, J. Kanellopoulos, F. Martin, et al. (2013). Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat Med* **19**(1): 57-64.

Bruckner, S. R., S. P. Tammariello, C. Y. Kuan, R. A. Flavell, P. Rakic and S. Estus (2001). JNK3 contributes to c-Jun activation and apoptosis but not oxidative stress in nerve growth factor-deprived sympathetic neurons. *J Neurochem* **78**(2): 298-303.

Brunet, A., S. R. Datta and M. E. Greenberg (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* **11**(3): 297-305.

Burdeinick-Kerr, R., D. Govindarajan and D. E. Griffin (2009). Noncytolytic clearance of sindbis virus infection from neurons by gamma interferon is dependent on Jak/STAT signaling. *J Virology* **83**(8): 3429-3435.

Buss, R. R., W. Sun and R. W. Oppenheim (2006). Adaptive roles of programmed cell death during nervous system development. *Annu Rev Neurosci* **29**: 1-35.

Cain, K., S. B. Bratton and G. M. Cohen (2002). The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie* **84**(2-3): 203-214.

Cameron, J. S., L. Alexopoulou, J. A. Sloane, A. B. DiBernardo, Y. Ma, B. Kosaras, R. Flavell, S. M. Strittmatter, J. Volpe, R. Sidman and T. Vartanian (2007). Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals. *J Neurosci* **27**(47): 13033-13041.

Carson, D. A. and J. M. Ribeiro (1993). Apoptosis and disease. *Lancet* **341**(8855): 1251-1254.

Cecconi, F., G. Alvarez-Bolado, B. I. Meyer, K. A. Roth and P. Gruss (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**(6): 727-737.

Ceco, E. and E. M. McNally (2013). Modifying muscular dystrophy through transforming growth factor-beta. *FEBS J* **280**(17): 4198-4209.

Chang, C. M. and C. C. Chao (2013). Protein kinase CK2 enhances Mcl-1 gene expression through the serum response factor-mediated pathway in the rat hippocampus. *J Neurosci Res* **91**(6): 808-817.

Chang, J. Y., D. P. Martin and E. M. Johnson, Jr. (1990). Interferon suppresses sympathetic neuronal cell death caused by nerve growth factor deprivation. *J Neurochem* **55**(2): 436-445.

Chao, D. T. and S. J. Korsmeyer (1998). BCL-2 family: regulators of cell death. *Annu Rev Immunol* **16**: 395-419.

Chawla-Sarkar, M., D. Lindner, Y. F. Liu, B. Williams, G. Sen, R. Silverman and E. Borden (2003). Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* **8**(3): 237-249.

Chen, Y., Y. Ai, J. R. Slevin, B. E. Maley and D. M. Gash (2005). Progenitor proliferation in the adult hippocampus and substantia nigra induced by glial cell line-derived neurotrophic factor. *Exp Neurol* **196**(1): 87-95.

Chipuk, J. E. and D. R. Green (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* **18**(4): 157-164.

Cho, Y. J., A. Tsherniak, P. Tamayo, S. Santagata, A. Ligon, H. Greulich, R. Berhoukim, V. Amani, L. Goumnerova, C. G. Eberhart, C. C. Lau, J. M. Olson, et al. (2011). Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. *J Clin Oncol* **29**(11): 1424-1430.

Choi, C., J. Y. Park, J. Lee, J. H. Lim, E. C. Shin, Y. S. Ahn, C. H. Kim, S. J. Kim, J. D. Kim, I. S. Choi and I. H. Choi (1999). Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-alpha, or IFN-gamma. *J Immunol* **162**(4): 1889-1895.

Choubey, D. and R. Panchanathan (2008). Interferon-inducible Ifi200-family genes in systemic lupus erythematosus. *Immunol Lett* **119**(1-2): 32-41.

Clarke, P. G. and S. Clarke (1996). Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol* **193**(2): 81-99.

Clemens, M. J. (2003). Interferons and apoptosis. *J Interferon Cytokine Res* **23**(6): 277-292.

Cohen, H. Y., S. Lavu, K. J. Bitterman, B. Hekking, T. A. Imahiyerobo, C. Miller, R. Frye, H. Ploegh, B. M. Kessler and D. A. Sinclair (2004). Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* **13**(5): 627-638.

Conway, K. E., B. B. McConnell, C. E. Bowring, C. D. Donald, S. T. Warren and P. M. Vertino (2000). TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Res* **60**(22): 6236-6242.

Cory, S. and J. M. Adams (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**(9): 647-656.

Creagh, E. M., H. Conroy and S. J. Martin (2003). Caspase-activation pathways in apoptosis and immunity. *Immunol Rev* **193**: 10-21.

Dabiri, G., D. A. Tumbarello, C. E. Turner and L. Van de Water (2008). TGF-beta1 slows the growth of pathogenic myofibroblasts through a mechanism requiring the focal adhesion protein, Hic-5. *J Invest Dermatol* **128**(2): 280-291.

Dafny, N. and P. B. Yang (2005). Interferon and the central nervous system. *Eur J Pharmacol* **523**(1-3): 1-15.

Danial, N. N. and S. J. Korsmeyer (2004). Cell death: critical control points. *Cell* **116**(2): 205-219.

Das, A., N. L. Banik and S. K. Ray (2009). Molecular mechanisms of the combination of retinoid and interferon-gamma for inducing differentiation and increasing apoptosis in human glioblastoma T98G and U87MG cells. *Neurochem Res* **34**(1): 87-101.

Davidescu, M., M. Sciaccaluga, L. Macchioni, R. Angelini, P. Lopalco, M. G. Rambotti, R. Roberti, A. Corcelli, E. Castigli and L. Corazzi (2012). Bromopyruvate mediates autophagy and cardiolipin degradation to monolyso-cardiolipin in GL15 glioblastoma cells. *J Bioenerg Biomembr* **44**(1): 51-60.

Davies, A. M. (2003). Regulation of neuronal survival and death by extracellular signals during development. *EMBO J* **22**(11): 2537-2545.

Davis, B. K., H. Wen and J. P. Ting (2011). The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* **29**: 707-735.

Deckwerth, T. L., R. M. Easton, C. M. Knudson, S. J. Korsmeyer and E. M. Johnson, Jr. (1998). Placement of the BCL2 family member BAX in the death pathway of sympathetic neurons activated by trophic factor deprivation. *Exp Neurol* **152**(1): 150-162.

Dedoni, S., M. Olianas, A. Ingianni and P. Onali (2012). Type I interferons impair BDNF-induced cell signaling and neurotrophic activity in differentiated human SH-SY5Y neuroblastoma cells and mouse primary cortical neurons. *J Neurochem* **122**(1): 58-71.

Deng, J., N. Carlson, K. Takeyama, P. Dal Cin, M. Shipp and A. Letai (2007). BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* **12**(2): 171-185.

Deshmukh, M., C. Du, X. Wang and E. M. Johnson, Jr. (2002). Exogenous smac induces competence and permits caspase activation in sympathetic neurons. *J Neurosci* **22**(18): 8018-8027.

Deshmukh, M. and E. M. Johnson, Jr. (1997). Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. *Mol Pharmacol* **51**(6): 897-906.

Deshmukh, M. and E. M. Johnson, Jr. (1998). Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome c. *Neuron* **21**(4): 695-705.

Dijkmans, R., J. Van Damme, F. Cornette, H. Heremans and A. Billiau (1990). Bacterial lipopolysaccharide potentiates gamma interferon-induced cytotoxicity for normal mouse and rat fibroblasts. *Infect Immun* **58**(1): 32-36.

Drexler, S. K., L. Bonsignore, M. Masin, A. Tardivel, R. Jackstadt, H. Hermeking, P. Schneider, O. Gross, J. Tschopp and A. S. Yazdi (2012). Tissue-specific opposing functions of the inflammasome adaptor ASC in the regulation of epithelial skin carcinogenesis. *PNAS* **109**(45): 18384-18389.

Dumitru, R., V. Gama, B. M. Fagan, J. J. Bower, V. Swahari, L. H. Pevny and M. Deshmukh (2012). Human embryonic stem cells have constitutively active Bax at the Golgi and are primed to undergo rapid apoptosis. *Mol Cell* **46**(5): 573-583.

Duprez, L., E. Wirawan, T. Vanden Berghe and P. Vandenabeele (2009). Major cell death pathways at a glance. *Microbes Infect* **11**(13): 1050-1062.

Eckelman, B. P., G. S. Salvesen and F. L. Scott (2006). Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep* **7**(10): 988-994.

Ellis, H. M. and H. R. Horvitz (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell* **44**(6): 817-829.

Ellison, D. W. (2010). Childhood medulloblastoma: novel approaches to the classification of a heterogeneous disease. *Acta Neuropathol* **120**(3): 305-316.

Erwig, L. P. and P. M. Henson (2007). Immunological consequences of apoptotic cell phagocytosis. *Am J Pathol* **171**(1): 2-8.

Eskes, R., S. Desagher, B. Antonsson and J. C. Martinou (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* **20**(3): 929-935.

Fadeel, B. and S. Orrenius (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med* **258**(6): 479-517.

Fattet, S., C. Haberler, P. Legoix, P. Varlet, A. Lellouch-Tubiana, S. Lair, E. Manie, M. A. Raquin, D. Bours, S. Carpentier, E. Barillot, J. Grill, et al. (2009). Beta-catenin status in paediatric medulloblastomas: correlation of immunohistochemical expression with mutational status, genetic profiles, and clinical characteristics. *J Pathol* **218**(1): 86-94.

Fellay, C., D. Frappaz, M. Sunyach, E. Franceschi, A. Brandes and R. Stupp (2011). Medulloblastomas in adults: prognostic factors and lessons from paediatrics. *Curr Opin Neurol* **24**(6): 626-632.

Fernandes-Alnemri, T., J. W. Yu, P. Datta, J. Wu and E. S. Alnemri (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* **458**(7237): 509-513.

Fink, S. L. and B. T. Cookson (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun* **73**(4): 1907-1916.

Franchi, L., T. Eigenbrod, R. Munoz-Planillo and G. Nunez (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nature Immunol* **10**(3): 241-247.

Geiger, K., E. L. Howes and N. Sarvetnick (1994). Ectopic expression of gamma interferon in the eye protects transgenic mice from intraocular herpes simplex virus type 1 infections. *J Virol* **68**(9): 5556-5567.

Geiger, K. D., M. S. Lee, C. Baugh and N. E. Sarvetnick (1995). Protective effects of interferon-gamma in intraocular herpes simplex type 1 infection do not depend on

major histocompatibility complex class I or class II expression. *J Neurovirol* **1**(5-6): 405-409.

Geiger, K. D., T. C. Nash, S. Sawyer, T. Krahl, G. Patstone, J. C. Reed, S. Krajewski, D. Dalton, M. J. Buchmeier and N. Sarvetnick (1997). Interferon-gamma protects against herpes simplex virus type 1-mediated neuronal death. *Virology* **238**(2): 189-197.

Ghiringhelli, F., L. Apetoh, A. Tesniere, L. Aymeric, Y. Ma, C. Ortiz, K. Vermaelen, T. Panaretakis, G. Mignot, E. Ullrich, J. L. Perfettini, F. Schlemmer, et al. (2009). Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* **15**(10): 1170-1178.

Gibson, P., Y. Tong, G. Robinson, M. C. Thompson, D. S. Currle, C. Eden, T. A. Kranenburg, T. Hogg, H. Poppleton, J. Martin, D. Finkelstein, S. Pounds, et al. (2010). Subtypes of medulloblastoma have distinct developmental origins. *Nature* **468**(7327): 1095-1099.

Gilbertson, R. J. and D. W. Ellison (2008). The origins of medulloblastoma subtypes. *Annu Rev Pathol* **3**: 341-365.

Glebova, N. O. and D. D. Ginty (2005). Growth and survival signals controlling sympathetic nervous system development. *Annu Rev Neurosci* **28**: 191-222.

Gorman, A. M. (2008). Neuronal cell death in neurodegenerative diseases: recurring themes around protein handling. *J Cell Mol Med* **12**(6A): 2263-2280.

Green, D. R. and G. I. Evan (2002). A matter of life and death. *Cancer Cell* **1**(1): 19-30.

Grinberg, M., R. Sarig, Y. Zaltsman, D. Frumkin, N. Grammatikakis, E. Reuveny and A. Gross (2002). tBID Homooligomerizes in the mitochondrial membrane to induce apoptosis. *J Biol Chem* **277**(14): 12237-12245.

Guan, X., J. Sagara, T. Yokoyama, Y. Koganehira, M. Oguchi, T. Saida and S. Taniguchi (2003). ASC/TMS1, a caspase-1 activating adaptor, is downregulated by aberrant methylation in human melanoma. *Int J Cancer* **107**(2): 202-208.

Gysemans, C., H. Callewaert, L. Overbergh and C. Mathieu (2008). Cytokine signalling in the beta-cell: a dual role for IFNgamma. *Biochem Soc Trans* **36**(Pt 3): 328-333.

Hallahan, A. R., J. I. Pritchard, S. Hansen, M. Benson, J. Stoeck, B. A. Hatton, T. L. Russell, R. G. Ellenbogen, I. D. Bernstein, P. A. Beachy and J. M. Olson (2004). The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. *Cancer Res* **64**(21): 7794-7800.

Hara, H., K. Fink, M. Endres, R. M. Friedlander, V. Gagliardini, J. Yuan and M. A. Moskowitz (1997). Attenuation of transient focal cerebral ischemic injury in transgenic mice

expressing a mutant ICE inhibitory protein. *J Cereb Blood Flow Metab* **17**(4): 370-375.

Hara, H., R. M. Friedlander, V. Gagliardini, C. Ayata, K. Fink, Z. Huang, M. Shimizu-Sasamata, J. Yuan and M. A. Moskowitz (1997). Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *PNAS* **94**(5): 2007-2012.

Hatten, M. and M. Roussel (2011). Development and cancer of the cerebellum. *Trends Neurosc* **34**(3): 134-142.

Hatten, M. E. and N. Heintz (1995). Mechanisms of neural patterning and specification in the developing cerebellum. *Annu Rev Neurosci* **18**: 385-408.

Haugen, P. K. and P. C. Letourneau (1990). Interleukin-2 enhances chick and rat sympathetic, but not sensory, neurite outgrowth. *J Neurosci Res* **25**(4): 443-452.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature* **407**(6805): 770-776.

Hengartner, M. O., R. E. Ellis and H. R. Horvitz (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature* **356**(6369): 494-499.

Hengartner, M. O. and H. R. Horvitz (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* **76**(4): 665-676.

Hervas-Stubbs, S., J. L. Perez-Gracia, A. Rouzaut, M. F. Sanmamed, A. Le Bon and I. Melero (2011). Direct effects of type I interferons on cells of the immune system. *Clin Cancer Res* **17**(9): 2619-2627.

Honda, H. M. and P. Ping (2006). Mitochondrial permeability transition in cardiac cell injury and death. *Cardiovasc Drugs Ther* **20**(6): 425-432.

Hong, S., I. Hwang, Y. S. Lee, S. Park, W. K. Lee, T. Fernandes-Alnemri, E. S. Alnemri, Y. S. Kim and J. W. Yu (2013). Restoration of ASC expression sensitizes colorectal cancer cells to genotoxic stress-induced caspase-independent cell death. *Cancer Lett* **331**(2): 183-191.

Honig, L. S. and R. N. Rosenberg (2000). Apoptosis and neurologic disease. *Am J Med* **108**(4): 317-330.

Ippagunta, S. K., D. D. Brand, J. Luo, K. L. Boyd, C. Calabrese, R. Stienstra, F. L. Van de Veerdonk, M. G. Netea, L. A. Joosten, M. Lamkanfi and T. D. Kanneganti (2010). Inflammasome-independent role of apoptosis-associated speck-like protein containing a CARD (ASC) in T cell priming is critical for collagen-induced arthritis. *J Biol Chem* **285**(16): 12454-12462.

Jeong, J., J. Mao, T. Tenzen, A. H. Kottmann and A. P. McMahon (2004). Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev* **18**(8): 937-951.

Jones, D. T., N. Jager, M. Kool, T. Zichner, B. Hutter, M. Sultan, Y. J. Cho, T. J. Pugh, V. Hovestadt, A. M. Stutz, T. Rausch, H. J. Warnatz, et al. (2012). Dissecting the genomic complexity underlying medulloblastoma. *Nature* **488**(7409): 100-105.

Jung, J. E., G. S. Kim, H. Chen, C. M. Maier, P. Narasimhan, Y. S. Song, K. Niizuma, M. Katsu, N. Okami, H. Yoshioka, H. Sakata, C. E. Goeders, et al. (2010). Reperfusion and neurovascular dysfunction in stroke: from basic mechanisms to potential strategies for neuroprotection. *Mol Neurobiol* **41**(2-3): 172-179.

Kalvakolanu, D. V. and S. K. Roy (2005). CCAAT/enhancer binding proteins and interferon signaling pathways. *J Interferon Cytokine Res* **25**(12): 757-769.

Kamerman, P. R., P. J. Moss, J. Weber, V. C. Wallace, A. S. Rice and W. Huang (2012). Pathogenesis of HIV-associated sensory neuropathy: evidence from in vivo and in vitro experimental models. *J Peripher Nerv Syst* **17**(1): 19-31.

Katsuno, Y., S. Lamouille and R. Derynck (2013). TGF-beta signaling and epithelial-mesenchymal transition in cancer progression. *Curr Opin Oncol* **25**(1): 76-84.

Kerr, J. F., A. H. Wyllie and A. R. Currie (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**(4): 239-257.

Kim, I. J., H. N. Beck, P. J. Lein and D. Higgins (2002). Interferon gamma induces retrograde dendritic retraction and inhibits synapse formation. *J Neurosci* **22**(11): 4530-4539.

Koirala, J., A. Adamski, L. Koch, D. Stueber, M. El-Azizi, N. M. Khardori, M. Ghassemi and R. M. Novak (2008). Interferon-gamma receptors in HIV-1 infection. *AIDS Res Hum Retroviruses* **24**(8): 1097-1102.

Kole, A. J., V. Swahari, S. M. Hammond and M. Deshmukh (2011). miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis. *Genes Dev* **25**(2): 125-130.

Kolly, L., M. Karababa, L. A. Joosten, S. Narayan, R. Salvi, V. Petrilli, J. Tschopp, W. B. van den Berg, A. K. So and N. Busso (2009). Inflammatory role of ASC in antigen-induced arthritis is independent of caspase-1, NALP-3, and IPAF. *J Immunol* **183**(6): 4003-4012.

Kool, M., J. Koster, J. Bunt, N. E. Hasselt, A. Lakeman, P. van Sluis, D. Troost, N. S. Meeteren, H. N. Caron, J. Cloos, A. Mrsic, B. Ylstra, et al. (2008). Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. *PLoS One* **3**(8): e3088.

Kostic, V., V. Jackson-Lewis, F. de Bilbao, M. Dubois-Dauphin and S. Przedborski (1997). Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* **277**(5325): 559-562.

Koul, D., R. Lapushin, H. J. Xu, G. B. Mills, J. U. Gutterman and D. Choubey (1998). p202 prevents apoptosis in murine AKR-2B fibroblasts. *Biochem Biophys Res Commun* **247**(2): 379-382.

Kuida, K., T. F. Haydar, C. Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M. S. Su, P. Rakic and R. A. Flavell (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**(3): 325-337.

Kuida, K., T. S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic and R. A. Flavell (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**(6607): 368-372.

Lamkanfi, M., N. Festjens, W. Declercq, T. Vanden Berghe and P. Vandenabeele (2007). Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* **14**(1): 44-55.

Lau, J., C. Schmidt, S. L. Markant, M. D. Taylor, R. J. Wechsler-Reya and W. A. Weiss (2012). Matching mice to malignancy: molecular subgroups and models of medulloblastoma. *Childs Nerv Syst* **28**(4): 521-532.

Lei, K. and R. J. Davis (2003). JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *PNAS* **100**(5): 2432-2437.

Letai, A., M. C. Bassik, L. D. Walensky, M. D. Sorcinelli, S. Weiler and S. J. Korsmeyer (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**(3): 183-192.

Levi-Montalcini, R. and B. Booker (1960). Destruction of the Sympathetic Ganglia in Mammals by an Antiserum to a Nerve-Growth Protein. *PNAS* **46**(3): 384-391.

Li, X., S. Taylor, B. Zegarelli, S. Shen, J. O'Rourke and R. E. Cone (2004). The induction of splenic suppressor T cells through an immune-privileged site requires an intact sympathetic nervous system. *J Neuroimmunol* **153**(1-2): 40-49.

Liu, W., Y. Luo, J. H. Dunn, D. A. Norris, C. A. Dinarello and M. Fujita (2013). Dual Role of Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) in Tumorigenesis of Human Melanoma. *J Invest Dermatol* **133**(2): 518-527.

Machida, E. O., M. V. Brock, C. M. Hooker, J. Nakayama, A. Ishida, J. Amano, M. A. Picchi, S. A. Belinsky, J. G. Herman, S. Taniguchi and S. B. Baylin (2006). Hypermethylation of ASC/TMS1 is a sputum marker for late-stage lung cancer. *Cancer Res* **66**(12): 6210-6218.

Madden, K. S., J. A. Moynihan, G. J. Brenner, S. Y. Felten, D. L. Felten and S. Livnat (1994). Sympathetic nervous system modulation of the immune system. III. Alterations in T and B cell proliferation and differentiation in vitro following chemical sympathectomy. *J Neuroimmunol* **49**(1-2): 77-87.

Maggirwar, S. B., P. D. Sarmiere, S. Dewhurst and R. S. Freeman (1998). Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. *J Neurosci* **18**(24): 10356-10365.

Maher, S. G., A. L. Romero-Weaver, A. J. Scarzello and A. M. Gamero (2007). Interferon: cellular executioner or white knight? *Curr Med Chem* **14**(12): 1279-1289.

Mao, J., K. L. Ligon, E. Y. Rakhlin, S. P. Thayer, R. T. Bronson, D. Rowitch and A. P. McMahon (2006). A novel somatic mouse model to survey tumorigenic potential applied to the Hedgehog pathway. *Cancer research* **66**(20): 10171-10178.

Marino, S. (2005). Medulloblastoma: developmental mechanisms out of control. *Trends Mol Med* **11**(1): 17-22.

Marsden, V. S. and A. Strasser (2003). Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. *Annu Rev Immunol* **21**: 71-105.

Martin, D. P., R. E. Schmidt, P. S. DiStefano, O. H. Lowry, J. G. Carter and E. M. Johnson, Jr. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J Cell Biol* **106**(3): 829-844.

Martin, G. S. (2003). Cell signaling and cancer. *Cancer Cell* **4**(3): 167-174.

Martinon, F. and J. Tschopp (2007). Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* **14**(1): 10-22.

Marz, P., R. A. Gadient and U. Otten (1996). Expression of interleukin-6 receptor (IL-6R) and gp130 mRNA in PC12 cells and sympathetic neurons: modulation by tumor necrosis factor alpha (TNF-alpha). *Brain Res* **706**(1): 71-79.

Massague, J. (2008). TGFbeta in Cancer. *Cell* **134**(2): 215-230.

Milligan, C. E., D. Prevette, H. Yaginuma, S. Homma, C. Cardwell, L. C. Fritz, K. J. Tomaselli, R. W. Oppenheim and L. M. Schwartz (1995). Peptide inhibitors of the ICE protease family arrest programmed cell death of motoneurons in vivo and in vitro. *Neuron* **15**(2): 385-393.

Min, W., S. Ghosh and P. Lengyel (1996). The interferon-inducible p202 protein as a modulator of transcription: inhibition of NF-kappa B, c-Fos, and c-Jun activities. *Mol Cell Biol* **16**(1): 359-368.

Mir, M., V. J. Asensio, L. Tolosa, M. Gou-Fabregas, R. M. Soler, J. Llado and G. Olmos (2009). Tumor necrosis factor alpha and interferon gamma cooperatively induce oxidative stress and motoneuron death in rat spinal cord embryonic explants. *Neuroscience* **162**(4): 959-971.

Mitchell, A. J., B. Yau, J. A. McQuillan, H. J. Ball, L. K. Too, A. Abtin, P. Hertzog, S. L. Leib, C. A. Jones, S. K. Gerega, W. Weninger and N. H. Hunt (2012). Inflammasome-dependent IFN-gamma drives pathogenesis in Streptococcus pneumoniae meningitis. *J Immunol* **189**(10): 4970-4980.

Mondini, M., S. Costa, S. Sponza, F. Gugliesi, M. Gariglio and S. Landolfo (2010). The interferon-inducible HIN-200 gene family in apoptosis and inflammation: implication for autoimmunity. *Autoimmunity* **43**(3): 226-231.

Nakamura, T., D. H. Cho and S. A. Lipton (2012). Redox regulation of protein misfolding, mitochondrial dysfunction, synaptic damage, and cell death in neurodegenerative diseases. *Exp Neurol* **238**(1): 12-21.

Narayan, S., L. Kolly, A. So and N. Busso (2011). Increased interleukin-10 production by ASC-deficient CD4+ T cells impairs bystander T-cell proliferation. *Immunology* **134**(1): 33-40.

Nhan, T. Q., W. C. Liles and S. M. Schwartz (2006). Physiological functions of caspases beyond cell death. *Am J Pathol* **169**(3): 729-737.

Nishikawa, Y., M. Mishima, H. Nagasawa, I. Igarashi, K. Fujisaki, H. Otsuka and T. Mikami (2001). Interferon-gamma-induced apoptosis in host cells infected with Neospora caninum. *Parasitology* **123**(Pt 1): 25-31.

Northcott, P. A., D. J. Shih, J. Peacock, L. Garzia, A. S. Morrissy, T. Zichner, A. M. Stutz, A. Korshunov, J. Reimand, S. E. Schumacher, R. Beroukhim, D. W. Ellison, et al. (2012). Subgroup-specific structural variation across 1,000 medulloblastoma genomes. *Nature* **488**(7409): 49-56.

Ohtsuka, T., X. F. Liu, Y. Koga, Y. Kitajima, Y. Nakafusa, C. W. Ha, S. W. Lee and K. Miyazaki (2006). Methylation-induced silencing of ASC and the effect of expressed ASC on p53-mediated chemosensitivity in colorectal cancer. *Oncogene* **25**(12): 1807-1811.

Ohtsuka, T., H. Ryu, Y. A. Minamishima, S. Macip, J. Sagara, K. I. Nakayama, S. A. Aaronson and S. W. Lee (2004). ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol* **6**(2): 121-128.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu Rev Neurosci* **14**: 453-501.

Ozoren, N. and W. S. El-Deiry (2003). Cell surface Death Receptor signaling in normal and cancer cells. *Semin Cancer Biol* **13**(2): 135-147.

Pardo, C. A., J. C. McArthur and J. W. Griffin (2001). HIV neuropathy: insights in the pathology of HIV peripheral nerve disease. *J Peripher Nerv Syst* **6**(1): 21-27.

Parsons, M. J., P. Patel, D. J. Brat, L. Colbert and P. M. Vertino (2009). Silencing of TMS1/ASC promotes resistance to anoikis in breast epithelial cells. *Cancer Res* **69**(5): 1706-1711.

Parsons, M. J. and P. M. Vertino (2006). Dual role of TMS1/ASC in death receptor signaling. *Oncogene* **25**(52): 6948-6958.

Pokrovskaja, K., T. Panaretakis and D. Grander (2005). Alternative signaling pathways regulating type I interferon-induced apoptosis. *J Interferon Cytokine Res* **25**(12): 799-810.

Polkinghorn, W. R. and N. J. Tarbell (2007). Medulloblastoma: tumorigenesis, current clinical paradigm, and efforts to improve risk stratification. *Nat Clin Pract Oncol* **4**(5): 295-304.

Potts, P. R., S. Singh, M. Knezek, C. B. Thompson and M. Deshmukh (2003). Critical function of endogenous XIAP in regulating caspase activation during sympathetic neuronal apoptosis. *J Cell Biol* **163**(4): 789-799.

Prendes, M. A., A. Harris, B. M. Wirostko, A. L. Gerber and B. Siesky (2013). The role of transforming growth factor beta in glaucoma and the therapeutic implications. *Br J Ophthalmol* **97**(6): 680-686.

Putcha, G. V., C. A. Harris, K. L. Moulder, R. M. Easton, C. B. Thompson and E. M. Johnson, Jr. (2002). Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice. *J Cell Biol* **157**(3): 441-453.

Ramachandran, K., H. Miller, E. Gordian, C. Rocha-Lima and R. Singal (2010). Methylation-mediated silencing of TMS1 in pancreatic cancer and its potential contribution to chemosensitivity. *Anticancer Res* **30**(10): 3919-3925.

Ren, D., H. C. Tu, H. Kim, G. X. Wang, G. R. Bean, O. Takeuchi, J. R. Jeffers, G. P. Zambetti, J. J. Hsieh and E. H. Cheng (2010). BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science* **330**(6009): 1390-1393.

Rich, J. N. (2003). The role of transforming growth factor-beta in primary brain tumors. *Front Biosci* **8**: e245-260.

Richter, K., J. Hausmann and P. Staeheli (2009). Interferon-gamma prevents death of bystander neurons during CD8 T cell responses in the brain. *Am J Pathol* **174**(5): 1799-1807.

Riedl, S. J., W. Li, Y. Chao, R. Schwarzenbacher and Y. Shi (2005). Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* **434**(7035): 926-933.

Riedl, S. J. and Y. Shi (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* **5**(11): 897-907.

Rios, I., R. Alvarez-Rodriguez, E. Marti and S. Pons (2004). Bmp2 antagonizes sonic hedgehog-mediated proliferation of cerebellar granule neurones through Smad5 signalling. *Development* **131**(13): 3159-3168.

Rottenberg, M. and K. Kristensson (2002). Effects of interferon-gamma on neuronal infections. *Viral Immunol* **15**(2): 247-260.

Roussel, M. and M. Hatten (2011). Cerebellum development and medulloblastoma. *Curr Top Dev Biol* **94**: 235-282.

Rubin, L. L. (1997). Neuronal cell death: when, why and how. *Br Med Bull* **53**(3): 617-631.

Sato, T., D. Kotake, M. Hiratsuka and N. Hirasawa (2013). Enhancement of inflammatory protein expression and nuclear factor Kappab (NF-Kappab) activity by trichostatin A (TSA) in OP9 preadipocytes. *PLoS One* **8**(3): e59702.

Satoh, T., N. Kambe and H. Matsue (2013). NLRP3 activation induces ASC-dependent programmed necrotic cell death, which leads to neutrophilic inflammation. *Cell Death Dis* **4**: e644.

Schmitt, M. J., D. Philippidou, S. E. Reinsbach, C. Margue, A. Wienecke-Baldacchino, D. Nashan, I. Behrmann and S. Kreis (2012). Interferon-gamma-induced activation of Signal Transducer and Activator of Transcription 1 (STAT1) up-regulates the tumor suppressing microRNA-29 family in melanoma cells. *Cell Commun Signal* **10**(1): 41.

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**(2): 163-189.

Sedger, L. M., D. M. Shows, R. A. Blanton, J. J. Peschon, R. G. Goodwin, D. Cosman and S. R. Wiley (1999). IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. *J Immunol* **163**(2): 920-926.

Selznick, L. A., T. S. Zheng, R. A. Flavell, P. Rakic and K. A. Roth (2000). Amyloid beta-induced neuronal death is bax-dependent but caspase-independent. *J Neuropathol Exp Neurol* **59**(4): 271-279.

Sharma, R. K., P. Tuli, S. S. Makkar and A. Parashar (2009). End-of-skin grafts in syndactyly release: description of a new flap for web space resurfacing and primary closure of finger defects. *Hand* **4**(1): 29-34.

Shaw, P. J., J. R. Lukens, S. Burns, H. Chi, M. A. McGargill and T. D. Kanneganti (2010). Cutting edge: critical role for PYCARD/ASC in the development of experimental autoimmune encephalomyelitis. *J Immunol* **184**(9): 4610-4614.

Shibanuma, M., J. Mashimo, A. Mita, T. Kuroki and K. Nose (1993). Cloning from a mouse osteoblastic cell line of a set of transforming-growth-factor-beta 1-regulated genes, one of which seems to encode a follistatin-related polypeptide. *Eur J Biochem* **217**(1): 13-19.

Shibanuma, M. and K. Nose (1998). Forced expression of hic-5, a senescence-related gene, potentiates a differentiation process of RCT-1 cells induced by retinoic acid. *Int J Biochem Cell Biol* **30**(1): 39-45.

Siegel, R. M., F. K. Chan, H. J. Chun and M. J. Lenardo (2000). The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* **1**(6): 469-474.

Smith, J. A., R. Zhang, A. K. Varma, A. Das, S. K. Ray and N. L. Banik (2009). Estrogen partially down-regulates PTEN to prevent apoptosis in VSC4.1 motoneurons following exposure to IFN-gamma. *Brain Res* **1301**: 163-170.

Srinivasula, S. M., M. Ahmad, T. Fernandes-Alnemri and E. S. Alnemri (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* **1**(7): 949-957.

Stehlik, C., L. Fiorentino, A. Dorfleutner, J. M. Bruey, E. M. Ariza, J. Sagara and J. C. Reed (2002). The PAAD/PYRIN-family protein ASC is a dual regulator of a conserved step in nuclear factor kappaB activation pathways. *J Exp Med* **196**(12): 1605-1615.

Stevens, B., N. J. Allen, L. E. Vazquez, G. R. Howell, K. S. Christopherson, N. Nouri, K. D. Micheva, A. K. Mehalow, A. D. Huberman, B. Stafford, A. Sher, A. M. Litke, et al. (2007). The classical complement cascade mediates CNS synapse elimination. *Cell* **131**(6): 1164-1178.

Stone, A. R., W. Bobo, D. J. Brat, N. S. Devi, E. G. Van Meir and P. M. Vertino (2004). Aberrant methylation and down-regulation of TMS1/ASC in human glioblastoma. *Am J Pathol* **165**(4): 1151-1161.

Sun, Y. F., L. Y. Yu, M. Saarma and U. Arumae (2003). Mutational analysis of N-Bak reveals different structural requirements for antiapoptotic activity in neurons and proapoptotic activity in nonneuronal cells. *Mol Cell Neurosci* **23**(1): 134-143.

Taylor, R. C., S. P. Cullen and S. J. Martin (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* **9**(3): 231-241.

Thoenen, H. and D. Edgar (1985). Neurotrophic factors. *Science* **229**(4710): 238-242.

Thompson, M., C. Fuller, T. Hogg, J. Dalton, D. Finkelstein, C. Lau, M. Chintagumpala, A. Adesina, D. Ashley, S. Kellie, M. Taylor, T. Curran, et al. (2006). Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. *J Clin Oncol* **24**(12): 1924-1931.

Thornberry, N. A. and Y. Lazebnik (1998). Caspases: enemies within. *Science* **281**(5381): 1312-1316.

Tsukamoto, L. F. and R. W. Price (1982). Interferon protects neurons in culture infected with vesicular stomatitis and herpes simplex viruses. *J Neurol Sci* **56**(1): 115-128.

Uo, T., Y. Kinoshita and R. S. Morrison (2005). Neurons exclusively express N-Bak, a BH3 domain-only Bak isoform that promotes neuronal apoptosis. *J Biol Chem* **280**(10): 9065-9073.

Uwabe, K., M. Matsumoto and K. Nagata (2005). Monokine induced by interferon-gamma acts as a neurotrophic factor on PC12 cells and rat primary sympathetic neurons. *J Biol Chem* **280**(40): 34268-34277.

Vaughn, A. E. and M. Deshmukh (2007). Essential postmitochondrial function of p53 uncovered in DNA damage-induced apoptosis in neurons. *Cell Death Differ* **14**(5): 973-981.

Vaughn, A. E. and M. Deshmukh (2008). Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. *Nat Cell Biol* **10**(12): 1477-1483.

Vaux, D. L. and S. J. Korsmeyer (1999). Cell death in development. *Cell* **96**(2): 245-254.

Victorio, S. C., L. A. Havton and A. L. Oliveira (2010). Absence of IFNgamma expression induces neuronal degeneration in the spinal cord of adult mice. *J Neuroinflammation* **7**: 77.

Vinken, P. and B. Bruyn, Eds. (1999). The Autonomic Nervous System. Handbook of Clinical Neurology. Amsterdam, Elsevier.

Wang, S. and W. S. El-Deiry (2003). TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* **22**(53): 8628-8633.

Wolter, K. G., Y. T. Hsu, C. L. Smith, A. Nechushtan, X. G. Xi and R. J. Youle (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* **139**(5): 1281-1292.

Wright, K. M. and M. Deshmukh (2006). Restricting apoptosis for postmitotic cell survival and its relevance to cancer. *Cell Cycle* **5**(15): 1616-1620.

Wright, K. M., M. W. Linhoff, P. R. Potts and M. Deshmukh (2004). Decreased apoptosome activity with neuronal differentiation sets the threshold for strict IAP regulation of apoptosis. *J Cell Biol* **167**(2): 303-313.

Wright, K. M., M. I. Smith, L. Farrag and M. Deshmukh (2007). Chromatin modification of Apaf-1 restricts the apoptotic pathway in mature neurons. *J Cell Biol* **179**(5): 825-832.

Wright, K. M., A. E. Vaughn and M. Deshmukh (2007). Apoptosome dependent caspase-3 activation pathway is non-redundant and necessary for apoptosis in sympathetic neurons. *Cell Death Differ* **14**(3): 625-633.

Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis and M. E. Greenberg (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**(5240): 1326-1331.

Xie, J., M. Murone, S. M. Luoh, A. Ryan, Q. Gu, C. Zhang, J. M. Bonifas, C. W. Lam, M. Hynes, A. Goddard, A. Rosenthal, E. H. Epstein, Jr., et al. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391**(6662): 90-92.

Yakovlev, A. G., S. M. Knoblach, L. Fan, G. B. Fox, R. Goodnight and A. I. Faden (1997). Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. *J Neurosci* **17**(19): 7415-7424.

Yong, V. W., F. Giuliani, M. Xue, A. Bar-Or and L. M. Metz (2007). Experimental models of neuroprotection relevant to multiple sclerosis. *Neurology* **68**(22 Suppl 3): S32-37; discussion S43-54.

Yordy, B., N. Iijima, A. Huttner, D. Leib and A. Iwasaki (2012). A neuron-specific role for autophagy in antiviral defense against herpes simplex virus. *Cell Host Microbe* **12**(3): 334-345.

Yu, J. and L. Zhang (2003). No PUMA, no death: implications for p53-dependent apoptosis. *Cancer Cell* **4**(4): 248-249.

Zaki, M. H., P. Vogel, M. Body-Malapel, M. Lamkanfi and T.-D. Kanneganti (2010). IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J Immunology* **185**(8): 4912-4920.

Zhang, R., N. L. Banik and S. K. Ray (2008). Combination of all-trans retinoic acid and interferon-gamma upregulated p27(kip1) and down regulated CDK2 to cause cell cycle arrest leading to differentiation and apoptosis in human glioblastoma LN18 (PTEN-proficient) and U87MG (PTEN-deficient) cells. *Cancer Chemother Pharmacol* **62**(3): 407-416.

Zhou, Y., L. Ye, Q. Wan, L. Zhou, X. Wang, J. Li, S. Hu, D. Zhou and W. Ho (2009). Activation of Toll-like receptors inhibits herpes simplex virus-1 infection of human neuronal cells. *J Neurosci Res* **87**(13): 2916-2925.