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# Retrograde Activation of a Somatic Transcriptional Program Regulates Distal Axon Degeneration

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RETROGRADE ACTIVATION OF A SOMATIC TRANSCRIPTIONAL PROGRAM  
REGULATES DISTAL AXON DEGENERATION

A Thesis Presented to the Faculty of  
The Rockefeller University  
in Partial Fulfillment of the Requirements for  
the degree of Doctor of Philosophy

by

Jason Pitts

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# RETROGRADE ACTIVATION OF A SOMATIC TRANSCRIPTIONAL PROGRAM REGULATES DISTAL AXON DEGENERATION

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The Rockefeller University 2016

During development of the peripheral nervous system, sensory axons extend to the periphery in excess where they compete for limiting target-derived neurotrophic support. Local neurotrophin insufficiency triggers axon degeneration, resulting in the pruning of over half of all sensory axons during development. Although axon degeneration facilitates the essential sculpting of the developing nervous system, its improper activation may underlie several neurodegenerative disorders. This process can be modeled *in vitro* by culturing sensory neurons from mouse dorsal root ganglia in the presence of nerve growth factor either as explant cultures or in compartmented chambers that allow independent manipulation of cell bodies and axons. The mitochondrial apoptotic pathway and the effector caspases, Caspase-3 and Caspase-6, mediate axon degeneration in both this *in vitro* model system and *in vivo*, clearly indicating a role for classical apoptotic machinery in axon degeneration. However, the full mechanism executing this process has yet to be determined.

Previous studies proposed a model whereby the signaling mechanisms that driving axon degeneration in response to local deprivation resides completely within the axon itself. This view has been challenged by results

showing that transcriptional inhibition prevents caspase activation in axons, as does physically separating the axon from its cell body. We used the *in vitro* culture system described above to address this controversy and conclusively show that, although the apoptotic machinery is present in axons, the cell body is required for gating axonal caspase activation and axon degeneration in response to trophic factor withdrawal.

Use of selective pharmacological inhibitors and knockdown of candidate genes identifies a pathway whereby local trophic deprivation results in loss of Akt signaling and activation of DLK signaling, leading to activation of the JNK/c-jun signaling and a Foxo3a dependent transcriptional program. The cell body acts as a convergence point for these two pathways resulting in upregulation of the pro-apoptotic protein Puma, itself identified through a genetic knockout screen. We further show that although Puma is unexpectedly confined to the cell body, rising levels of somatic Puma in response to trophic deprivation overcome inhibition by pro-survival Bcl-xL and Bcl-w to initiate an anterograde, JNK-dependent, pro-degenerative program. Taken together, our results identify the cell body as a key arbiter of large-scale axon removal.

*To my family and my wife for always believing in me.*

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transforming what at the time was a loose collection of experimental results into a unified story. The work detailed below, all produced during our collaboration, is something I am truly proud of. Dave's passion and curiosity about the nature of biology exemplify the best parts of academic science and I owe him a great debt for facilitating my intellectual growth over the past several years.

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## List of Abbreviations

ALS	Amyotrophic Lateral Sclerosis
BDNF	Brain-Derived Neurotrophic Factor
BH	Bcl-2 homology
CNS	Central nervous system
CTB	Cholera toxin subunit B
da	Dendritic arborization
DRG	Dorsal root ganglia
DIV	Days <i>in vitro</i>
DLK	Dual lysine kinase
dSarm	<i>Drosophila</i> sterile a/Armadillo/Toll-interleukin receptor homology domain protein
DUBs	Deubiquitinating enzymes
IAP	Inhibitor of apoptosis
IPB	Infrapyramidal bundle
MB	Mushroom body
MOMP	Mitochondrial outer membrane permeabilization
NGF	Nerve growth factor
NMJ	Neuromuscular junction
NT-3	Neurotrophin-3
PBS-X	PBS containing 0.1% Triton-X- 100
PNS	Peripheral nervous system
RGC	Retinal Ganglion Cell
SC	Superior Colliculus
TD	Trophic deprivation
TNFR1	Tumor Necrosis Factor Receptor 1
UPS	Ubiquitin proteasome system
Wlds	Wallerian Degeneration slow

# **Chapter 1: Introduction and overview of the field**

## **Rationale for the current study**

Axon degeneration is a crucial component of normal development and plasticity, but aberrant degeneration is also a hallmark of neurodegenerative diseases, such as Alzheimer's and Amyotrophic Lateral Sclerosis (ALS), and direct injury (Luo and O'Leary, 2005; Neukomm and Freeman, 2014; Pease and Segal, 2014; Saxena and Caroni, 2007). Several triggers initiate this process, which, once started, leads to swelling or blebbing of the axonal process followed by fragmentation and engulfment by glia or macrophages (Saxena and Caroni, 2007). Despite garnering considerable research interest, understanding of mechanisms leading to degeneration remains incomplete. Elucidating key components of this process will yield key insights into the process of developmental pruning and plasticity, as well as shedding light on mechanisms of neurodegenerative disease progression and possible targets for therapeutic intervention.

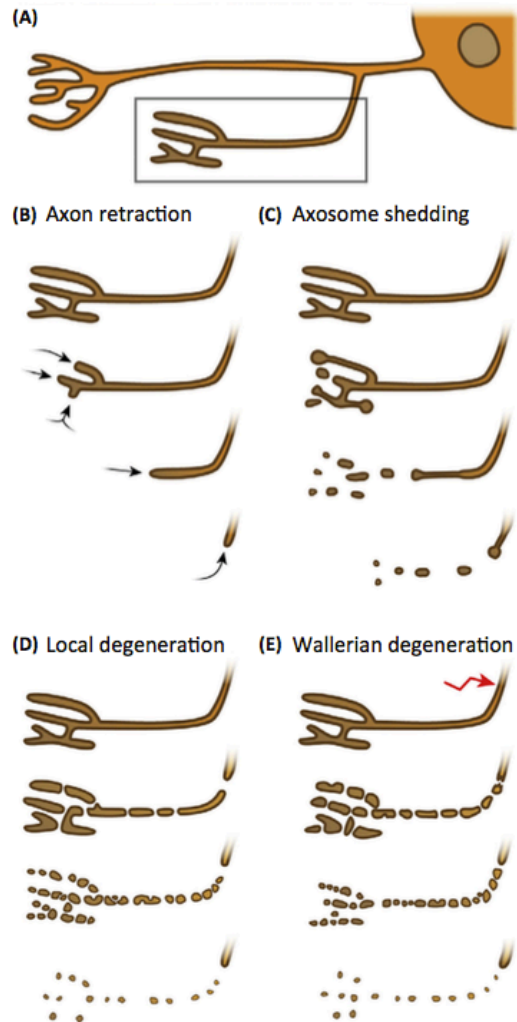
While exploring the process of axon degeneration, it is crucial to understand the different contexts during which it occurs. These contexts can be broadly divided into axon degeneration that occurs (1) during development, (2) following injury, or (3) during neurodegenerative disease. The full complement of factors operating in each context has yet to be discovered and, perhaps even more vital, how these factors interact and where in the cell they operate are

critical unanswered questions. These questions are the primary driving force behind the work detailed in this thesis.

### **Axon degeneration during development**

During development, axon pruning eliminates excess and misconnected axonal projections and this process often occurs in the absence of neuronal cell body death (Luo and O'Leary, 2005; Saxena and Caroni, 2007). This phenomenon is observed across multiple systems from *Drosophila* to humans, usually occurring during metamorphosis in *Drosophila* and during postnatal development in vertebrates (Cowan et al., 1984; Luo and O'Leary, 2005; Schuldiner and Yaron, 2014). In fact, it is estimated that humans prune half of all neural connections by the age of two and that a similar percentage of neurons are culled during mouse development (Cowan et al., 1984; Thompson and Nelson, 2001). This pruning back of exuberant connections is vital and allows for proper axonal connectivity between neurons and their ultimate targets (Luo and O'Leary, 2005; Saxena and Caroni, 2007; Vanderhaeghen and Cheng, 2010).

Pruning occurs through three primary mechanisms: axosome shedding, axon retraction, and axon degeneration (Figure 1.1). Which mechanism is operating in a given context is largely determined by the length of the process that needs to be eliminated; with shorter processes being subject to retraction or axosome shedding and longer processes being removed through degeneration (Luo and O'Leary, 2005; Neukomm and Freeman, 2014).



**Figure 1.1 Illustration of modes of axon pruning that occur during development and injury.** (A) A single neuron with two axonal branches is depicted. Axonal branch highlighted with box will be used to illustrate modes of pruning in panels B-D (B) Axon retraction involves withdrawal of the distal tip of the axon in absence of fragmentation (C) Axosome shedding involves withdrawal of the distal tip in a process that leaves behind membrane bound vesicles containing intact organelles that are later engulfed and degraded by surrounding cells (D) Axon degeneration, typically occurring in longer axonal processes, results in fragmentation and clearance of a distal portion of the axon (E) Wallerian degeneration occurs as a result of injury or chemical insult resulting in degeneration of the portion of the axon distal to the point of injury. (Figure reprinted with permission (Neukomm and Freeman, 2014)).

Axosome shedding is defined morphologically by the regression of the distal axon tip in a process that leaves behind membrane bound organelles, termed axosomes, that are later engulfed and degraded by neighboring Schwann cells in a lysosomal process (Bishop et al., 2004; Neukomm and Freeman, 2014; Song et al., 2008) (Figure 1.1C). The hallmark example of axosome shedding is refinement of axonal connections at the vertebrate neuromuscular junction (NMJ). Early in post-natal development, each NMJ is innervated by multiple motoneuron axons. These axonal arbors compete in an activity dependent manner; those that have stronger and more synchronous electrical activity are maintained, while the others retract, resulting in an NMJ innervated by a single axonal arbor from a single motoneuron (Lichtman and Colman, 2000; Luo and O'Leary, 2005). In this way approximately 90% of original NMJ synapses are eliminated without motoneuron death (Riley, 1981; Sanes and Lichtman, 1999; Tapia et al., 2012). Intriguingly, a similar process operates in the growth and retraction of axonal arbors in the *Drosophila* NMJ, indicating that this pruning mechanism is evolutionarily conserved (Fuentes-Medel et al., 2009; Liu et al., 2010; Neukomm and Freeman, 2014).

The second mechanism employed in short-distance pruning events is axon retraction. During this process, axons form a large, distal, vesicle filled retraction bulb that retracts from the area it previously innervated while remaining intact (Neukomm and Freeman, 2014)(Figure 1.1B). Retraction is mainly carried out by reorganization of axonal actin filaments in a RhoA GTPase-dependent

process that leaves the microtubule structure intact and allows distal to proximal transport of vesicles from the retracting edge of the axon (Saxena and Caroni, 2007). The most notable example of retraction is pruning of the infrapyramidal bundle (IPB), a hippocampal mossy fiber tract, in the central nervous system (CNS). Morphological studies in mouse demonstrate that Semaphorin 3A and 3F signal through their receptors Plexin-A3 and Neuropilin-2 to induce axon retraction during the first 2 months of postnatal life,(Bagri et al., 2003; Cheng et al., 2001; Luo and O'Leary, 2005; Vanderhaeghen and Cheng, 2010). Two-photon time-lapse studies have also demonstrated axon retraction in developing layer 1 cortical interneurons in mice (Portera-Cailliau et al., 2005).

The primary method employed in pruning of large-scale axonal projections, and the mechanism that this thesis will focus on, is axon degeneration (Luo and O'Leary, 2005; Neukomm and Freeman, 2014)(Figure 1.1 D). Axonal projections eliminated in this manner initially display swelling before ultimately fragmenting and being engulfed by cells in the area, including macrophages (Saxena and Caroni, 2007; Schuldiner and Yaron, 2014). This section will discuss two striking examples of this paradigm in the mammalian CNS and one in the developing fly. Degenerative pruning in the PNS, which serves as a useful experimental model, will be discussed in a later section.

Over the first postnatal week in the developing mammalian nervous system, axons of retinal ganglion cells (RGCs) initially project across the entire length of the Superior Colliculus (SC) before ultimately pruning back to an

anterior termination zone in a degenerative process (Luo and O'Leary, 2005; Saxena and Caroni, 2007; Vanderhaeghen and Cheng, 2010). Elegant studies have demonstrated that this event is orchestrated, in part, by graded expression of the repellant guidance cues Ephrins and their receptors the Ephs, in addition to coordinated waves of electrical activity, resulting in observable fragmentation of the exuberant axonal projections (Hindges et al., 2002; Hoopfer et al., 2006; McLaughlin et al., 2003a; 2003b; Yates et al., 2001). Of note, key components of the apoptotic program including Caspase-3, Caspase-6, and the p75 receptor relative DR6 are partially responsible for this pruning (Nikolaev et al., 2009; Olsen et al., 2014; Simon et al., 2012); a point that will be dealt with in greater detail in the following sections.

Pruning of layer V cortical neuron projections in mammals provides another classic example of degenerative pruning. In the adult, axons from specific layer V cortical populations project to areas in the brain as a function of their identity, with visual cortex neurons projecting to the SC and motor cortex neurons projecting to the spinal cord (Luo and O'Leary, 2005; Saxena and Caroni, 2007). However, early in development they make connections broadly, meaning that both visual and motor cortex neurons project to both the SC and spinal cord before improper connections are pruned through degeneration (Hoopfer et al., 2006). Given that only a specific portion of the complete axonal arbor is removed, one might assume that all cellular factors necessary to initiate and execute pruning would be located in the neuronal process targeted for

degeneration. However, although only a specific axonal branch is removed in this process, it depends on the activity of a transcription factor, Otx1, which moves from the cytoplasm to the nucleus during the window when degeneration is initiated (Weimann et al., 1999). Furthermore, heterotopic transplantation of visual cortex neurons to the motor cortex and vice versa results in the transplanted neurons maintaining the collateral appropriate for the new region, further implicating the cell body as a potentially critical arbiter in shaping these connections (O'Leary and Stanfield, 1989; Stanfield and O'Leary, 1985).

A transcriptional mechanism is also involved in the pruning of  $\gamma$  neuron projections in the mushroom body (MB) of developing *Drosophila* resulting in fragmentation and clearing of these axons (Watts et al., 2003). During metamorphosis, larval specific axonal projections are pruned in a mechanism acting through the hormone Ecdysone, signaling through Ecdysone Receptor-B1 expressed by  $\gamma$  neurons and upregulated in a manner dependent on TGF- $\beta$  signaling (Lee et al., 2000; Schuldiner and Yaron, 2014; Zheng et al., 2003). The transcription factor Sox14 serves as a critical target of Ecdysone Receptor signaling in the pruning of these axons. The pruning of the dendritic arbor of fly sensory dendritic arborization (da) neurons is also dependent on this transcriptional mechanism (Kirilly et al., 2009). Intriguingly, as in SC axon pruning, caspases are crucial mediators of dendrite pruning, suggesting an evolutionarily conserved mechanism for removal of some neuronal processes. However, pruning of MB axons does not seem to involve these proteases,

pointing to the existence of alternative degenerative pathways (Awasaki et al., 2006; Kuo et al., 2006; Williams et al., 2006).

### **Axon degeneration in injury and disease**

In 1850 Augustus Waller observed that the distal segment of axons that are physically severed from their cell body fragment and ultimately degenerate (Figure 1.1E). Initially thought to be a passive process, this view was upended by the discovery of the Wallerian degeneration slow (*Wld<sup>s</sup>*) mouse, which displayed drastically delayed degeneration of the axon after physical insult, indicating an active molecular mechanism was at play (Coleman and Freeman, 2010; Lunn et al., 1989). The *Wld<sup>s</sup>* gene, a result of a spontaneous dominant mutation, produces a fusion protein containing the coding region of the NAD<sup>+</sup> synthesizing enzyme NMNAT, indicating it plays a role in regulating cellular metabolism (Coleman, 2005). More recently, *Drosophila* sterile *a*/Armadillo/Toll-interleukin receptor homology domain protein (dSarm) and its mammalian ortholog Sarm1 were shown to play a critical role in axon degeneration after injury or chemical insult, highlighting an endogenous and evolutionarily conserved mechanism in both *in vitro* and *in vivo* systems (Osterloh et al., 2012; Yang et al., 2015). Further studies have shown that the *Drosophila* E3 ubiquitin ligase highwire (Hiw) and its mammalian homolog Phr1, as well as MAPK and JNK signaling, are involved in a pathway that culminates in axonal energy deficits, a rise in intracellular calcium, activation of the calcium dependent protease calpain, and

ultimately Wallerian degeneration (Babetto et al., 2013; Coleman and Freeman, 2010; Wang et al., 2012; Xiong et al., 2012; Yang et al., 2015; 2013).

In contrast with several forms of developmental axon degeneration, Wallerian degeneration is caspase independent (Finn et al., 2000; Simon et al., 2012; Vohra et al., 2010). Wallerian degeneration also does not seem to be critical for developmental axon pruning in mammals, as *Wld<sup>s</sup>* does not delay or prevent developmental pruning in the SC or layer V cortex (Hoopfer et al., 2006). Furthermore, neither *Wld<sup>s</sup>* nor *dSarm* play a role in MB axon pruning in *Drosophila* (Hoopfer et al., 2006; Osterloh et al., 2012). Finally, the physical separation of the axon from the cell body precludes involvement of somatic signaling and transcription programs from being involved in this process, a notable difference from the developmental degeneration.

Axon degeneration is a prominent feature of many neurodegenerative diseases, which often display a “dying back” of axons preceding neuronal cell body death (Höke, 2006; Luo and O’Leary, 2005). Particularly in human peripheral neuropathies, but also documented in motor neuron disease, Alzheimer’s, and Parkinson’s disease, affected neurons are characterized by degeneration beginning at their distal tip that proceeds proximally toward the cell body (Raff, 2002; Saxena and Caroni, 2007). This phenomenon has been compellingly demonstrated in several mouse models of neurodegenerative disease including Huntington’s disease, Alzheimer’s disease, and Amyotrophic Lateral Sclerosis (ALS) (Fischer et al., 2004; Li et al., 2001; Tsai et al., 2004)).

One may posit that this phenotype may be a secondary consequence of loss of nourishing factors from degenerating cell bodies. However, several mouse disease models and examples of human motor neuron diseases result from disruption of the dynein-dynactin complex, which regulates retrograde transport. In these diseases anterograde transport is preserved, indicating retrograde communication from the axon to the cell body is crucial for axon maintenance (Perlson et al., 2010). The factors involved in this signaling, and what role the cell body plays, remain to be elucidated.

The mechanisms involved in axon degeneration during neurodegenerative disease vary. For example, crossing a mouse model of progressive motor neuropathy (pmn) to *Wld<sup>s</sup>* significantly delays disease progression, but *Wld<sup>s</sup>* does not provide any protection in superoxide dismutase 1 (SOD1) transgenic mouse models of ALS (Ferri et al., 2003; Vande Velde et al., 2004). In other pathological conditions such as Huntington's disease, Alzheimer's disease, and stroke, components of the apoptotic pathway, specifically caspases, appear to play a significant role (Akpan et al., 2011; Wang et al., 2015). Recent work also shows that mice with a knockout of an anti-apoptotic Bcl-2 gene, Bcl-w, exhibit a progressive loss of epidermal nociceptive fibers without concurrent cell body loss, akin to a disease known as small fiber sensory neuropathy (Courchesne et al., 2011). The involvement of apoptotic proteins in this context mirrors the developmental pruning described in several processes above, such as pruning of SC axons, suggesting that developmental and pathological pruning may share a

common molecular mechanism, albeit one that is erroneously activated in disease. Importantly, how the neuron determines which degenerative mechanism to employ and what specific intracellular signals are engaged in that process are still undetermined.

### **Developmental pruning in the PNS and the neurotrophic hypothesis**

During the early stages of embryonic development, sensory neurons in mouse and chick experimental systems extend afferents to the periphery in excess. As development proceeds, over 50% of the extended axons and their cell bodies are culled (Oppenheim, 1991). Pioneering work done by Viktor Hamburger and Rita-Levi Montalcini investigating the biological mechanism of this phenomenon led to the development of the neurotrophic hypothesis, which states that axons of developing neurons compete for limited amounts of target derived neurotrophic factors. According to this hypothesis, those afferents that make the proper connections are preserved while those that are mis-targeted or outcompeted are fated to degenerate (Davies, 1996; Levi-Montalcini, 1987). Groundbreaking experiments led to the isolation of the first neurotrophic factor, nerve growth factor (NGF), with further studies identifying additional neurotrophins, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Huang and Reichardt, 2001).

The critical importance of NGF for pruning of sensory axons from neurons in the dorsal root ganglia (DRG) has been amply demonstrated, as reducing target derived NGF either by limb bud extirpation or treatment of developing

embryos with NGF anti-serum drastically depletes DRG neurons. Conversely, injection of NGF reduces naturally occurring neuronal death (Davies, 1996; Hamburger and Yip, 1984; Johnson et al., 1980; Levi-Montalcini, 1987). Further, genetic deletion of NGF resulted in a 70% depletion of neurons in mouse lumbar DRGs, whereas transgenic overexpression in mouse epidermis yielded enhanced DRG survival and skin innervation, providing a robust confirmation of the necessity of target derived neurotrophins in maintaining these projections (Albers et al., 1994; Crowley et al., 1994).

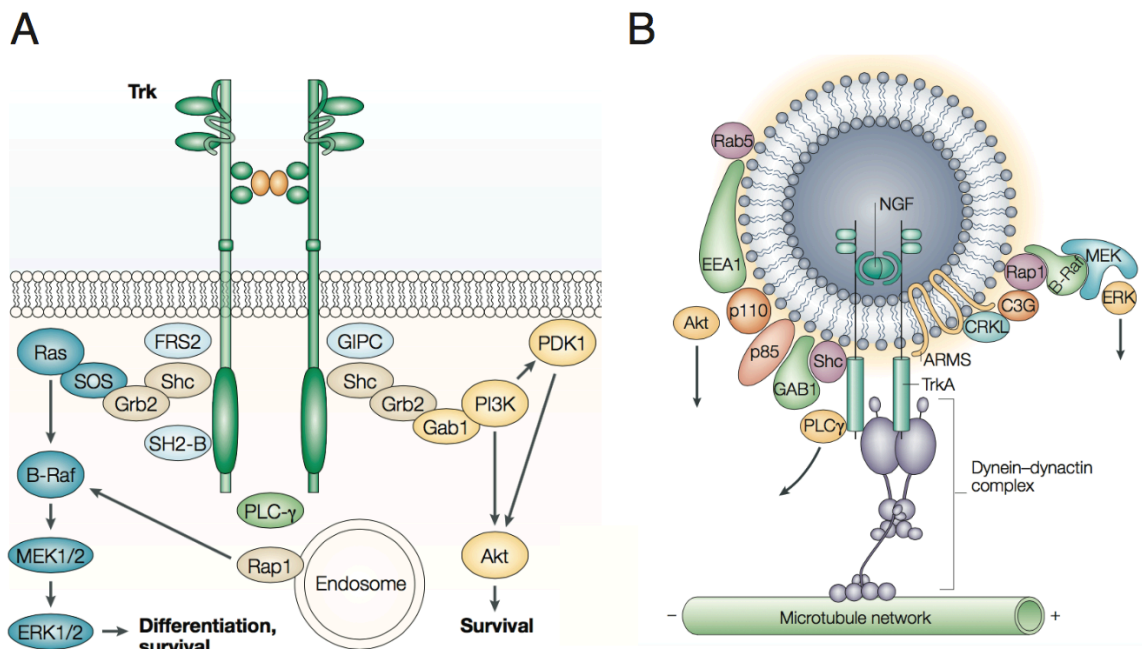
In line with developmental pruning in the SC, degeneration of NGF responsive sensory neurons is mediated by an apoptotic mechanism. Deletion of the apoptotic effector Bax drastically reduces the *in vivo* cell death seen in this paradigm and studies using light-level microscopy have shown a significant increase in small unmyelinated fibers in sciatic nerves of adult Bax knockout mice (Deckwerth et al., 1996; Sun et al., 2003; White et al., 1998). Coincident with the period of peak developmental pruning during embryogenesis, markedly increased levels of cleaved Caspase-3, a key effector protease in apoptosis, are observed in both DRG cell bodies and axons using the newly developed iDISCO whole mount immunolabeling and volume imaging protocol (Renier et al., 2014).

High affinity binding of NGF to the receptor tyrosine kinase TrkA induces its dimerization and autophosphorylation leading to recruitment and activation of a host of local and retrograde signaling cascades necessary for the survival and differentiation of neurons (Reichardt, 2006; Zweifel et al., 2005) (Figure 1.2A).

The most critical pathways activated by NGF binding to TrkA are the PI3K/Akt pathway and the MEK/ERK (also called the MEK/MAPK) pathway (Kaplan and Miller, 2000). PI3K/Akt signaling is required for neurotrophin induced neuronal growth and survival, whereas MEK/MAPK signaling is more closely involved in neuronal differentiation, only serving as a minor player in survival (Atwal et al., 2000; Kaplan and Miller, 2000; Kuruvilla et al., 2000; Mazzoni et al., 1999).

A particularly notable aspect of this system is that NGF binding to TrkA results in endocytosis of the activated receptor, which is then trafficked in a retrograde fashion back to the cell body as a signaling endosome (Zweifel et al., 2005) (Figure 1.2B). This process has been elegantly visualized *in vitro* by David Ginty and colleagues using NGF responsive sensory axons from a mouse expressing a knock-in of a FLAG-tagged TrkA (Sharma et al., 2010). When NGF responsive neurons from these mice are grown in compartmented chambers (described below) where only the distal axon is treated with NGF, TrkA signaling endosomes can be observed migrating down the axon to the cell body by immunofluorescence (Harrington et al., 2011). Signaling endosomes are transported to the cell body by dynein dependent microtubule transport and disruption of this process, such as can be achieved by overexpression of dynamin *in vitro*, leads to neuronal death (Heerssen et al., 2004). Inhibition of TrkA or PI3K signaling specifically in either cell bodies or axons of sympathetic neurons results in cell death, further pointing to the necessity of retrograde signaling for neuronal survival (Kuruvilla et al., 2000; Ye et al., 2003). Intriguingly,

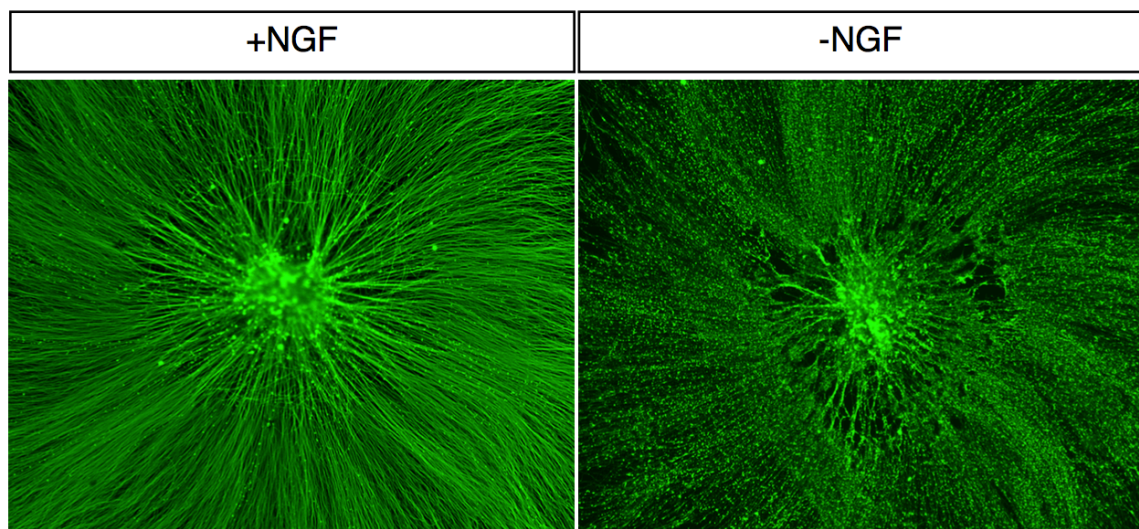
although studies have examined the effects of TrkA inhibition on neuronal cell body death, they have not examined the ability of axons to survive when TrkA signaling is specifically inhibited in cell bodies, which could yield new insights into the necessity of cell body signaling events for axonal maintenance.



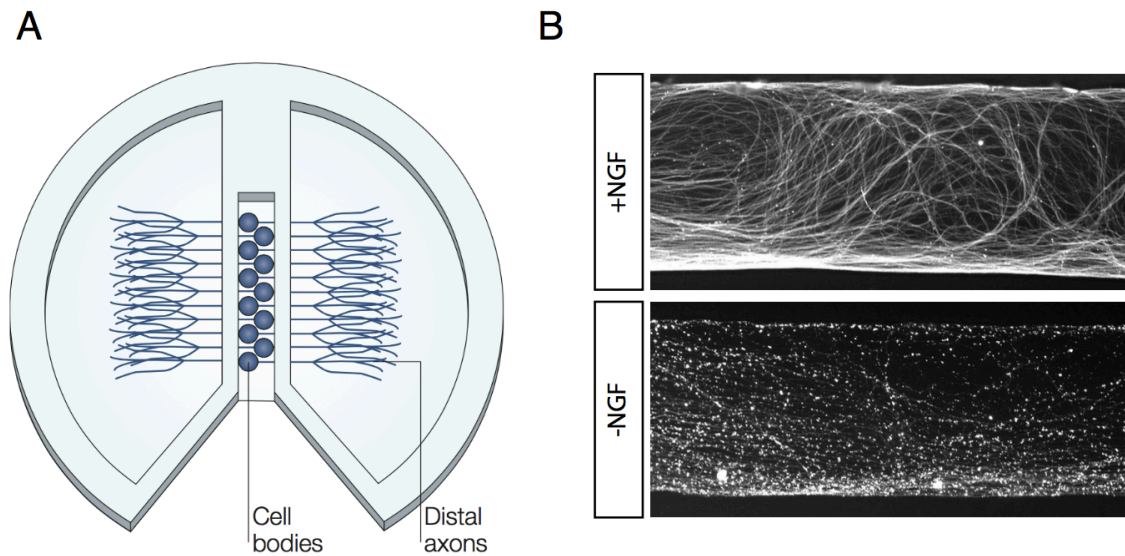
**Figure 1.2 NGF signals through the receptor tyrosine kinase TrkA** (A) NGF binding to TrkA induces dimerization and autophosphorylation. Multiple downstream signaling events occur including activation of MEK/ERK and PI3K/Akt pathways. (Reprinted with permission from (Chao, 2003)) (B) NGF-TrkA signaling complexes internalize to form signaling endosomes, which are transported by the Dynein-dynactin complex back to the cell body in a retrograde fashion. (Reprinted with permission from (Zweifel et al., 2005))

Developmental axon degeneration of sensory neurons in the PNS is particularly vital to the questions raised in this thesis due to the existence of a powerful *in vitro* system that can model many aspects of this process. Mouse DRG explants can be cultured in the presence of NGF, which induces axon outgrowth from TrkA expressing neurons. The explants can then be deprived of NGF through application of a function-blocking NGF antibody, resulting in axon degeneration and somatic apoptosis (Figure 1.3). Importantly, the segregation of cell bodies and axons seen *in vivo* can be recapitulated *in vitro* by growing neurons in compartmented chambers (also called Campenot chambers), allowing for individual treatment of either the cell body or axon compartment, which are separated by a grease barrier (Campenot, 1977; 1982; Campenot et al., 1991)(Figure 1.4). Using this system, Robert Campenot demonstrated that local trophic deprivation of distal axons induces their degeneration, even when cell bodies are still treated with NGF. Furthermore, specific NGF deprivation of cell bodies does not induce degeneration of distal axons still bathed in NGF media, highlighting a critical role for retrograde signaling from distal axons (Campenot, 1977; 1982). Several studies also claim that these chambers can be used to induce distal axon degeneration that does not result in neuronal cell body death (Campenot, 1977; Mok et al., 2009; Ghosh et al., 2011). However, it is difficult to ascertain if this is truly the case. Approximately 120,000 cells are typically plated in a Campenot chamber (see methods), whereas ~1500 axons are typically observed crossing the grease barrier in each axonal compartment, meaning a

vast majority of neurons do not project axons outside of the central cell body compartment. Without specifically labeling the neurons whose axons grew across the grease barrier, it is unclear if cell bodies are truly preserved after distal NGF withdrawal. Importantly, although there is evidence that pruning of BDNF dependent sensory axons in the mammary gland can occur without neuronal death, similar data regarding NGF responsive axons *in vivo* is lacking, making the existence of this phenomenon a key unanswered question in the field (Liu et al., 2012).



**Figure 1.3 Whole explant DRG culture system** DRGs from embryonic day 12.5 mouse can be cultured in the presence of NGF, resulting in axon outgrowth (left panel). Application of an NGF function-blocking antibody causes axons to degenerate, resulting in fragmentation the Tubulin cytoskeleton (right panel). Representative images shown using immunofluorescence to visualize  $\beta$ -III Tubulin (Tuj1).



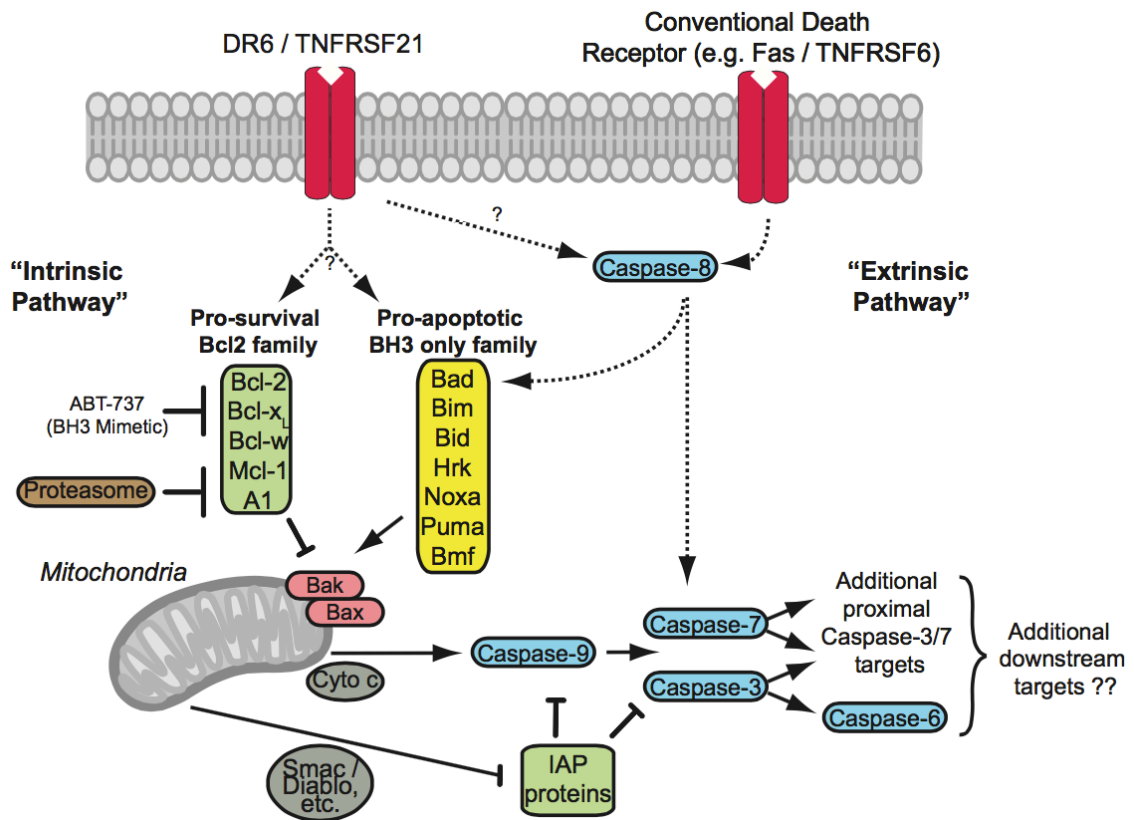
**Figure 1.4 DRG culture in compartmented Campenot chambers.** (A) Dissociated DRGs placed in the center cell compartment extend axons underneath a grease barrier, growing along lanes created by scratching the plate with a pin rake. Cell bodies and axons are fluidically isolated and can be subjected to individual treatments. (Reprinted with permission from (Zweifel et al., 2005)). (B) Degeneration of distal axons is induced adding NGF function-blocking antibody specifically to the axonal compartment. Axon degeneration visualized using  $\beta$ -III Tubulin (Tuj1) immunofluorescence.

## **Mechanisms of apoptosis**

As mentioned above, apoptotic mechanisms appear to play a fundamental role in axon degenerations in several contexts. More broadly a role for apoptotic cell death is an essential feature of animal development for species ranging from the nematode worm *C. elegans* to humans (Fuchs and Steller, 2011). The central executioners of this process are a family of evolutionarily conserved cysteine proteases, the caspases (Hengartner, 2000). Initially synthesized as proteolytically inactive zymogens, diverse apoptotic stimuli can trigger their processing into mature, active, heterotetramers (Hengartner, 2000). Apoptosis involves two distinct sets of caspases; namely the initiator caspases, Caspase-8 and Caspase-9, and the executioner caspases, Caspase-3, -6, and -7. Initiator caspases serve to cleave and activate executioner caspases, which then proceed to cleave a wide array of intracellular substrates resulting in fragmentation of the cell before cell remnants are ultimately engulfed by phagocytosis (Thornberry and Lazebnik, 1998).

Apoptosis can be triggered by either the death-receptor dependent “extrinsic” pathway or the mitochondrial “intrinsic” pathway (Figure 1.5). In the extrinsic pathway, death activating ligands bind to death receptors such as Fas and tumor necrosis factor receptor 1 (TNFR1), initiating recruitment of death effector domains to the intracellular death domains of these transmembrane receptors (Vila and Przedborski, 2003). Death effector domains recruit the central initiator caspase of the extrinsic pathway, Caspase-8, which becomes activated

as the low intrinsic protease activity of the zymogen is sufficient to induce cleavage and activation due to the drastically increased local concentration (Muzio et al., 1998). Catalytically active Caspase-8 then activates downstream effector caspases.



**Figure 1.5 Schematic of apoptotic pathway.** In the “intrinsic pathway” cellular stress signals induce Bax/Bak translocation to the mitochondria, releasing cytochrome c, which leads to activation of Caspase-9 and downstream effector caspases. This process is regulated by pro- and anti-apoptotic Bcl-2 family members. In the “extrinsic pathway”, stress signals from the TNFR family of receptors lead to activation of Caspase-8 which goes on to activate effector caspases. As indicated, the extrinsic pathway can feed into the intrinsic pathway. Speculative links (marked by a “?”) are also shown. (Illustration by David Simon).

The mitochondrial or “intrinsic” pathway integrates multiple intracellular cues leading to oligomerization of the pro-apoptotic protein Bax, resulting in mitochondrial outer membrane permeabilization (MOMP) (Figure 1.5). Permeabilized mitochondria release cytochrome c into the cytoplasm, forming the apoptosome in conjunction with Apaf-1 and leading to the cleavage and activation of the initiator caspase, Caspase-9 (Hengartner, 2000; Vila and Przedborski, 2003). As with Caspase-8 in the extrinsic pathway, catalytically active Caspase-9 cleaves downstream effector caspases, inducing their protease ability. Importantly, MOMP in mammals also releases the proteins Smac and DIABLO which can bind to and inhibit a class of proteins known as inhibitor of apoptosis proteins (IAPs), which in turn can bind to and inhibit the catalytic activity of Caspase-3 (Salvesen and Duckett, 2002).

### **Regulation of the mitochondrial apoptotic pathway by the Bcl-2 family**

The oligomerization of Bax (and the closely related protein Bak), which causes MOMP, is regulated by the Bcl-2 family of proteins, which contains both pro- and anti- apoptotic members (Adams and Cory, 1998; Youle and Strasser, 2008) (Figure 1.5). The five anti-apoptotic Bcl-2 family members – Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1 – can bind to and inhibit Bax oligomerization through interaction between the Bcl-2 homology (BH) domains contained in each protein, forming heterodimers (Adams and Cory, 1998; Ghiotto et al., 2009). The seven pro-apoptotic or “BH3 only” family members – Bid, Bim, Puma, Bad, Noxa, Hrk,

and Bmf – can inhibit the activity of the anti-apoptotic members and promote Bax oligimerization (Youle and Strasser, 2008).

Two independent mechanisms have been proposed to explain the ability of BH3 only proteins to induce apoptosis, known as the direct and indirect activation models (Adams and Cory, 2007). In the direct activation model, the seven BH3 proteins are grouped into activators and sensitizers. Activators, such as Bid, Bim, and Puma, are proposed to bind directly to Bax (or Bak), resulting in a conformational change that promotes oligimerization. In this model, the primary function of sensitizers, such as Bad and Noxa, is to bind to anti-apoptotic Bcl-2 family members, releasing activator proteins from their inhibitory binding (Adams and Cory, 2007). Evidence for this model is seen using an *in vitro* mitochondrial permeabilization assay, where Bid, Bim, and Puma are able to induce MOMP when incubated with isolated mitochondria in the presence of Bax, whereas incubation with other BH3 proteins or Bax alone does not yield cytochrome c release (Cartron et al., 2004; Letai et al., 2002). Support for this model is also seen *in vivo*, as mice with a triple genetic knockout of *Bid*, *Bim*, and *Puma* have cell death phenotypes similar to *Bax*;*Bak* double knockouts, positioning them as essential activators of these proteins that cannot be compensated for by the remaining family members (Ren et al., 2010).

The indirect activation model proposes that initiation of apoptosis by Bax oligimerization is the default state in cells, but that this activity is inhibited by binding to anti-apoptotic Bcl-2 family members such as Bcl-xL. In this paradigm,

the sole function of BH3 only proteins is to disrupt these inhibitory heterodimers by binding to anti-apoptotic proteins and releasing Bax (Adams and Cory, 2007). Bid, Bim, and Puma are positioned as the most potent effectors because they bind all anti-apoptotic Bcl-2 family members, whereas the other BH3 only proteins do not (Chen et al., 2005a). Supporting this view, mutation of the Bax binding domain of Bim and Bid does not suppress their ability to induce cell death *in vitro* (Willis et al., 2007). Furthermore, co-expression of Noxa and Bad can induce death of *Bid;Bim* double knockout cells where Puma was also knocked down, although it is possible that some residual Puma activity could account for the apoptotic response (Willis et al., 2007). Given that there is experimental evidence to support both models, it is possible that different models apply to different cell types or may be important in different contexts within the same cell (Shamas-Din et al., 2013).

Several mechanisms of transcriptional and post-translational modification regulate the activity of BH3 only proteins (Puthalakath and Strasser, 2002). The transcription factors p53, c-jun, and members of the FOXO family strictly regulate the levels of Bid, Bim, and Puma (Schuler and Green, 2005; Whitfield et al., 2001; Wong et al., 2005; Yu and Zhang, 2009; Zhang et al., 2011). Hrk is additionally under tight transcriptional control (Puthalakath and Strasser, 2002). Phosphorylation events are also critical, with Bad and Bim activity being regulated by Akt and JNK signaling respectively (Datta et al., 1997; Putcha et al., 2003). Finally, the full-length version of Bid has only weak pro-apoptotic activity,

requiring proteolytic cleavage to produce the more apoptotically active protein tBid. Interestingly, this provides a mechanism whereby the extrinsic pathway can feed back on the intrinsic pathway through Caspase-8 mediated cleavage of Bid (Li et al., 1998; Luo et al., 1998; Youle and Strasser, 2008).

### **The role of Puma in the mitochondrial apoptotic pathway**

Puma is a pro-apoptotic Bcl-2 family member classically regulated by p53 mediated transcriptional up-regulation, hence its name p53 up-regulated modulator of apoptosis (Yu and Zhang, 2009). Independently discovered by three groups in 2001 (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001), this protein, which contains a BH3 domain and a C-terminal mitochondrial localization sequence (Hikisz and Kiliańska, 2012), is now known to play a critical role in initiation of the mitochondrial apoptotic program in response to a diverse set of cellular stressors. In the most widely studied mechanism of Puma action, Puma is initially transcribed only at very low levels (if at all), but DNA damage triggers nuclear movement of p53 which then transactivates the Puma locus (Wang et al., 2007; Yu et al., 2001). This causes a dramatic increase in Puma levels within the cell, which induces Bax oligimerization and MOMP, ultimately leading to activation of downstream caspases and the death of the cell.

In addition to the classic, p53-mediated mechanism, p53-independent mechanisms of Puma induction are shown to function in several other contexts. For example, Puma is central to cell death following growth factor withdrawal, ER

stress, or ischemia/reperfusion in several *in vitro* and *in vivo* systems (Yu and Zhang, 2009). In a majority of these examples, transcriptional regulation of Puma occurs independently of p53, instead being induced by transcription factors such as Foxo3a (You et al., 2006), p73 (Ming et al., 2008), c-jun (Zhao et al., 2012), or E2F1 (Hershko and Ginsberg, 2004).

Although there is universal agreement that Puma induces cell death through the mitochondrial apoptotic pathway, whether it does this as a direct or indirect activator, is still debated. In favor of its role as a direct activator, Puma overexpression can induce a Bax conformational change and trigger MOMP in colorectal cancer cells (Yu et al., 2003) and can directly interact with Bax in *in vitro* mitochondrial permeabilization assays (Cartron et al., 2004). However, the fact that deletion of the C-terminal hydrophobic domain of Puma, which is essential for binding to Bax, did not abrogate its ability to induce apoptosis (Yee and Vousden, 2007), would argue for a more indirect role. Regardless, the ability of Puma to strongly bind to all anti-apoptotic Bcl-2 family members and disrupt their interaction with Bax (Chen et al., 2005a), positions Puma as a potent inducer of apoptosis.

Once thought to only be transcriptionally regulated, recent studies have shown that Puma is also subject to post-translational regulation. Phosphorylation of Puma on serine-10 reduces its stability and expression of a Puma mutant containing a serine to alanine mutation at this residue (S10A) which renders it unable to be phosphorylated, increases its apoptosis-inducing effect in HeLa

cells (Fricker et al., 2010). The increased apoptotic potential of S10A Puma was not a result of altered binding to anti-apoptotic Bcl-2 family members, but appears to be a result of reduced protein turnover when compared to phosphorylated Puma. Interestingly, the increased turnover of phosphorylated Puma is not mediated by caspases or macroautophagy, but is instead dependent on proteasome activity (Fricker et al., 2010). This is notable because Puma does not contain any lysine residues to which ubiquitin can be conjugated to target the protein for degradation by the proteasome. The mechanism by which this phosphorylation-induced proteasome-dependent Puma degradation occurs, has yet to be elucidated.

Puma is also unique among pro-apoptotic family members in its ability to trigger Bax activation in a mechanism involving cytosolic p53 in a non-transcriptional role. Like Bid, Bim, and Puma, p53 has the ability to induce Bax activation and MOMP in *in vitro* mitochondrial permeabilization assays (Chipuk et al., 2004). This activity of p53 is directly inhibited by binding to Bcl-xL, but in mouse embryonic fibroblasts treated with UV irradiation, nuclear p53 induces increased Puma expression which bind to Bcl-xL and frees cytosolic p53 from its inhibitory effects (Chipuk et al., 2005). While one might argue that this effect was carried out exclusively by Puma, expression of a mutant form of Bcl-xL that could bind to p53, but not any pro-apoptotic Bcl-2 family members including Bax or Puma, protected these cells from apoptosis, arguing for a direct role of p53 in Bax activation (Chipuk et al., 2005). Notably, Puma is the only pro-apoptotic Bcl-

2 family member capable of disrupting the binding between p53 and Bcl-xL (Follis et al., 2013).

### **The mitochondrial apoptotic pathway in axon degeneration**

Numerous components of the mitochondrial apoptotic pathway have been shown to play a significant role in neuronal death in several contexts. Genetic deletion of Apaf-1, a central component of the apoptosome formed after MOMP in the mitochondrial apoptotic pathway, protects neurons from apoptosis after NGF withdrawal (Wright et al., 2006). Overexpression of Bcl-2 in mice results in increased numbers of facial and RGC neurons during development and Bcl-2 overexpression in chicken DRGs protects against neurotrophin withdrawal induced apoptosis (Gagliardini et al., 1994; Martinou et al., 1994). Furthermore, genetic deletion of Bcl-xL in mice results in catastrophic death of postmitotic neurons in the brain, spinal cord, and DRGs, with neuronal specific deletion of Mcl-1 having a similar effect (Arbour et al., 2008; Motoyama et al., 1995).

Neuronal death both *in vivo* during development and *in vitro* after NGF withdrawal is Bax-dependent, and Caspase-3 activation is drastically reduced in embryonic DRGs in *Bax* knockout mice (Deckwerth et al., 1996; Renier et al., 2014; White et al., 1998). In accordance with a central role for Bax in neuronal death, several BH3 only family members have been implicated in apoptotic initiation. Specifically, roles for Bad, Bim, Puma, and Hrk have been demonstrated in neuronal apoptosis after growth factor withdrawal or DNA

damage (Ambacher et al., 2012; Datta et al., 1997; Imaizumi et al., 2004; Jean et al., 2013; Putcha et al., 2003; Wong et al., 2005; Wyttenbach and Tolkovsky, 2006).

Despite the abundant evidence that the mitochondrial apoptotic pathway induces caspase dependent neuronal death in multiple systems, its involvement in axon degeneration was largely discounted until recently. As described above, injury-induced Wallerian degeneration is not dependent on caspases or Bax/Bak activation and chemical inhibition or siRNA knockdown of canonical caspases did not protect axons from degenerating in other contexts (Finn et al., 2000; Nikolaev et al., 2009; Vohra et al., 2010; Whitmore et al., 2003). However, recent studies using knockout mice instead of knockdown have shown that the initiator caspase, Caspase-9, and the executioner caspases, Caspase- 3 and Caspase-6, play a key role in axon degeneration of PNS sensory neurons after trophic factor withdrawal (Cusack et al., 2013; Nikolaev et al., 2009; Simon et al., 2012). Recent evidence also indicates that a common mechanism for axon degeneration may be shared between the *in vitro* NGF withdrawal paradigm and developmental axon pruning *in vivo*, because developmental pruning of SC axons, a trophic factor independent process, also partially depends on Caspase-3 and Caspase-6 (Simon et al., 2012).

Bax is essential for NGF withdrawal induced axon degeneration in whole explant cultures as well as in Campenot chambers, highlighting a central role for the mitochondrial apoptotic pathway (Nikolaev et al., 2009). This connection is

further solidified by the fact that Bcl-xL overexpression blocks axon degeneration after NGF withdrawal (Vohra et al., 2010). Genetic deletion of *Bcl-w* also accelerates axon degeneration in this system *in vitro*, while *in vivo* these mice exhibit a progressive loss of epidermal nociceptive fibers without concurrent cell body loss, akin to a disease known as small fiber sensory neuropathy (Cosker et al., 2013; Courchesne et al., 2011). This could point to a role for the classical mitochondrial apoptotic pathway in axon degeneration associated with some disease models.

Several upstream factors have been identified in the molecular pathway leading to axon degeneration in the NGF withdrawal paradigm, including the JNK interactor Dual lysine kinase (DLK) and death receptor 6 (DR6) (Huntwork-Rodriguez et al., 2013; Nikolaev et al., 2009; Ghosh et al., 2011). However, the full repertoire of factors operating between the cessation of neurotrophin signaling and Bax activation remains to be described. Similarly, although application of ABT-737, an inhibitor of Bcl-2, Bcl-XL, and Bcl-w, can trigger axon degeneration, the role of BH3 proteins and the mechanism regulating their action in this system is completely unknown (Simon et al., 2012). This question is a central component of this thesis.

### **Compartmentalization of factors in axon degeneration**

In conjunction with incomplete knowledge of the full set of effectors in axon degeneration, the mechanisms leading to their activation are also unknown,

as is the site of initiation of the degenerative process. The accepted model contends that trophic deprivation of distal axons initiates a degenerative pathway contained exclusively within the axon itself, completely independent of the soma. A primary driver of this view is a study showing that while JNK signaling is an essential regulator of axon degeneration and neuronal apoptosis after NGF withdrawal, neurons containing a genetic deletion for the transcription factor c-jun, a JNK target, are only protected from apoptosis but not axon degeneration (Ghosh et al., 2011). Thus, the view emerged that local signaling events in distally deprived axons represented a process independent of cell body death. Zebrafish studies using an indirect reporter of caspase activation revealed *in vivo* caspase activation at axonal branch points, but not in the cell body, and *Drosophila* studies showing dendrite specific caspase activity in da neuron pruning were also taken as evidence for local degenerative signaling (Campbell and Okamoto, 2013; Williams et al., 2006).

Mechanisms directing the spatial restriction of degenerative signaling within the axon have also been proposed. Although neurons lacking Apaf-1 are protected from apoptosis following NGF withdrawal, a recent study found that genetic deletion of *Apaf-1* did not protect axons from local deprivation in microfluidic chambers, hinting at the existence of separate cell body and axon mechanisms of caspase activation (Cusack et al., 2013). In addition, XIAP appears to prevent caspase activation in cell bodies when distal axons are subject to trophic deprivation and local initiation of caspase activity in dendrites

using photostimulation of Mito-KillerRed did not spread back to cell bodies unless neurons were treated with IAP or proteasome inhibitors (Erturk et al., 2014; Unsain et al., 2013). Taken together, these results paint a picture of local signaling causing degenerative caspase activation that is actively restricted from the cell body by several factors.

Although these results are compelling, several lines of evidence are at odds with the prevailing view of a local pro-degenerative program contained exclusively within axons. As discussed above, developmental pruning of MB  $\gamma$  neuron projections and da neurons dendrites in *Drosophila* depends on a transcriptional cascade mediated by ecdysone receptor signaling (Lee et al., 2000; Watts et al., 2003; Zheng et al., 2003). Pruning of mammalian layer V cortical projections is similarly dependent on the transcription factor Otx1, highlighting an active role for somatic signaling in both of these processes (Weimann et al., 1999). Recent *in vitro* studies have also implicated somatic signaling as crucial for caspase-dependent axon degeneration after trophic deprivation. Axons severed from their cell bodies but protected from Wallerian degeneration by genetic deletion of *Sarm1* do not demonstrate Caspase-3 activation and subsequent axon degeneration in response to NGF withdrawal (Gerdtz et al., 2013). Further, application of a transcriptional inhibitor or inhibiting the kinase GSK3 $\beta$  specifically in the cell body compartment of Campenot chambers results in a robust protection of axons after local trophic deprivation (Chen et al., 2012). These results call into question whether the degenerative signal is contained

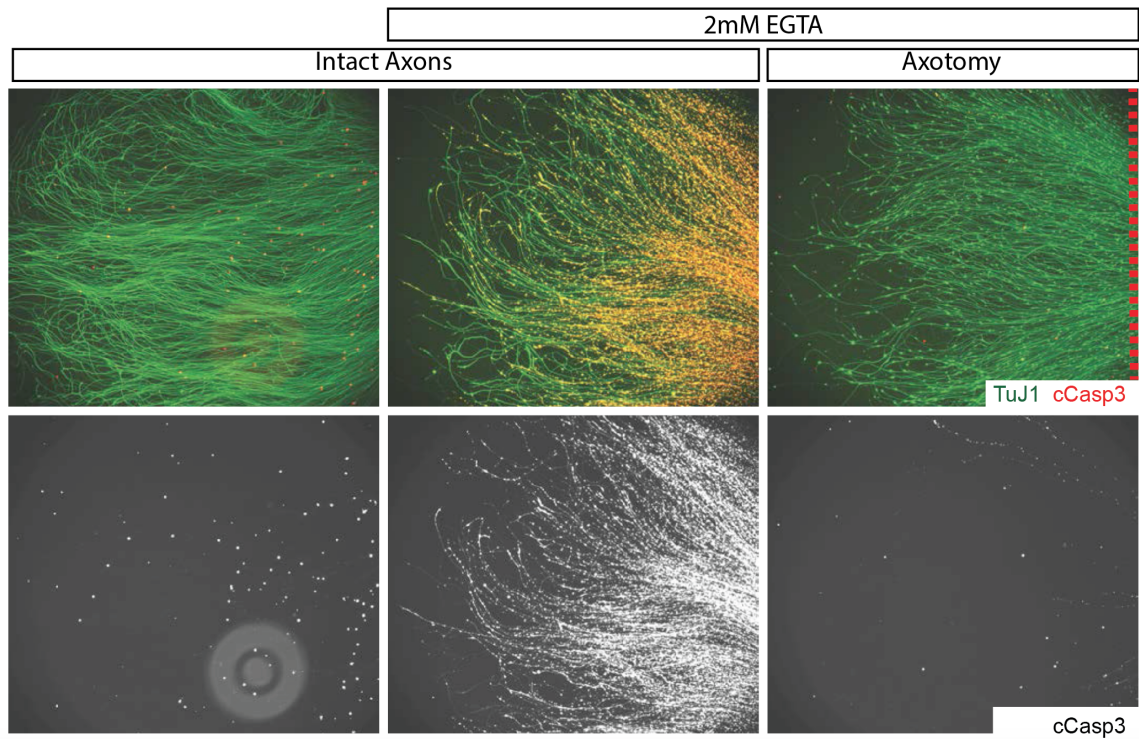
exclusively within the axon itself or whether the cell body plays an active, and essential, role in initiating axon degeneration. The effort to resolve this controversy is a central pillar of my thesis work.

### **Perspective and overview of the thesis project**

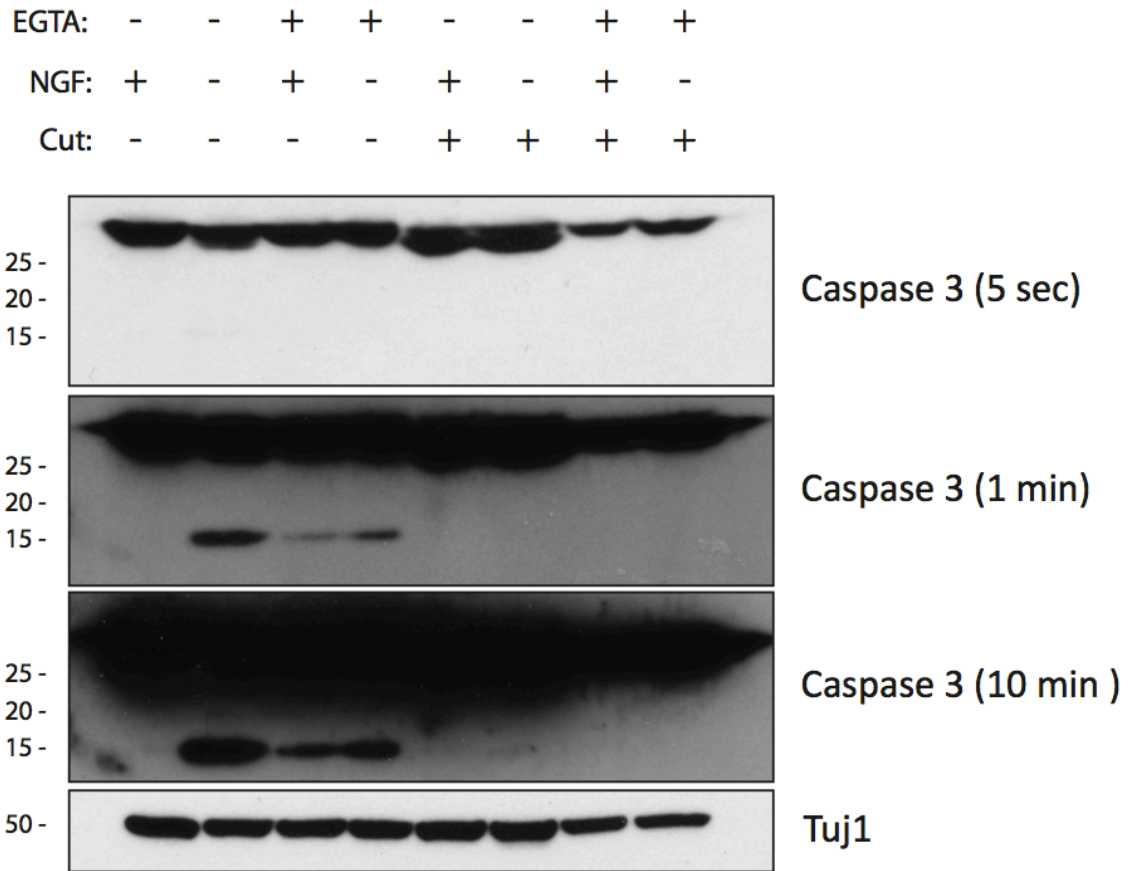
The work detailed in this thesis originally began with the goal of isolating the specific Bcl-2 family members involved in regulating Bax activation upstream of caspase-dependent axon degeneration after trophic deprivation (TD). The initial approach was focused on conducting a knockout screen looking for *in vitro* axon degeneration phenotypes in knockout mice of pro- and anti-apoptotic Bcl-2 family members. Specifically, the rationale was that when examining whole explant cultures subjected to TD, deletion of pro-apoptotic (BH3) members involved in the degenerative process would exhibit a delayed degeneration phenotype, whereas deletion of critical anti-apoptotic Bcl-2 family members would accelerate degeneration. Once these factors were identified, I sought to examine their relationship to each other and determine the mechanism by which they were regulated. The result of these experiments was the identification of Puma as the primary regulator of axon degeneration after TD in DRG neurons (detailed below), but several pieces of data inspired a change in the focus of the project.

Experiments done by Yuya Yamagishi in the Tessier-Lavigne lab showed that calcium chelation had differential effects on axon degeneration depending on

the context. As described above, Wallerian degeneration is mediated by the calpains, a family of calcium dependent proteases. As a result, addition of the calcium chelator EGTA prevents the influx of calcium that is required for the activation of calpains after cutting, delaying axon degeneration. Strikingly, the ability of EGTA to prevent calcium influx into the cell, which prevents Wallerian degeneration, also produces a stress response in DRG explant cultures that results in caspase activation in axons still connected to their cell bodies (Figure 1.6). Importantly, Caspase-3 could only be activated in axons when they were connected to the cell body, indicating an active somatic role in caspase-dependent axon degeneration. This result was mirrored by experiments discussed above whereby NGF withdrawal did not cause Caspase-3 activation in severed axons protected from Wallerian degeneration by genetic deletion of *Sarm1* (Gerdt et al., 2013). As that result was obtained using immunofluorescence, I also probed this phenomenon with Western blotting; a more sensitive assay that could identify low level Caspase-3 activity. Concordantly, I found that severed axons protected from Wallerian degeneration by EGTA addition did not show evidence of Caspase-3 activation after TD, while intact axons did (Figure 1.7). Collectively these data pointed towards active control of axon degeneration by signaling from the cell body, despite other work from the lab by David Simon indicating that all necessary apoptotic machinery is contained within the axon (see below).



**Figure 1.6 EGTA causes Caspase-3 activation in uncut axons.** DRG explants were cultured for 2 days in vitro (DIV). Axons were either left intact or physically separated from their cell bodies by cutting (indicated by dashed red line). Explants were then treated with 2mM EGTA as indicated and Caspase-3 activation was imaged with immunofluorescence (Yuya Yamagishi, Unpublished).



**Figure 1.7 Axotomy prevents Caspase-3 activation in axons after TD.** Axons from 7DIV DRG explant cultures were severed from cell bodies by manual punch-out of the center cell mass. Wallerian degeneration was prevented by addition of 2mM EGTA and cultures were subject to TD by addition of NGF function-blocking antibody as indicated. Caspase-3 activation (lower band) was assayed by immunoblot.

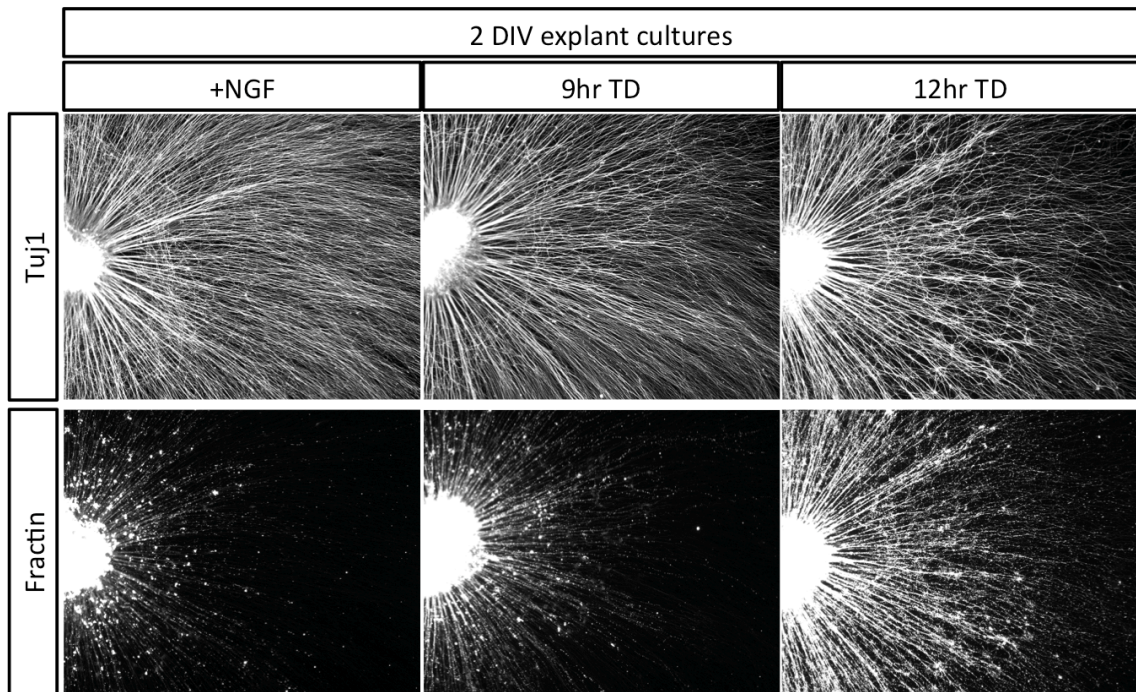
In thinking about what the critical factors in the cell body might be, I focused on a puzzling result that came out of the genetic screen I was conducting. As will be detailed below, despite significantly delaying axon degeneration after TD, Puma was only detectable within the cell body. Further, Caspase-3 activity appeared to radiate out from the cell body over a time course of TD as assayed by immunofluorescence for Fractin, an actin neoepitope formed by Caspase-3 cleavage (Sokolowski et al., 2014)(Fig 1.8). These insights fundamentally changed the direction of my thesis work leading to the identification of a cell body derived signaling program that controls axon degeneration after TD. The results, all of which were obtained in close collaboration with David Simon, are therefore presented in a format that follows the most cohesive narrative rather than reflecting the true chronology of how the project developed.

In Chapter 2, I will discuss our finding that contrary to a prior study (Ghosh et al., 2011), the apoptotic machinery is present and functional in axons, but it is not activated directly by distal TD. Rather, the initiating signal for degeneration comes from the cell body, following activation by convergent retrograde signals (loss of Akt signaling and activation of JNK signaling) from the distally deprived axon. Further, we found that the transcription factors c-jun and Foxo3a are primary targets of these convergent retrograde signals.

Chapter 3 will focus on a screen of pro-apoptotic Bcl-2 family members, which identified Puma as the key regulator of axon degeneration. We find that

Foxo3a dependent upregulation of Puma transcription is the key regulated step of this process and that Puma activity is tightly controlled by the anti-apoptotic Bcl-2 family members Bcl-w and Bcl-xL, themselves identified in a separate genetic screen. Although Puma potentially protects distal axons from degeneration following TD, we find, quite unexpectedly, Puma expression is confined to the cell body.

Chapter 4 explores results implying that rising Puma levels overcome inhibition by Bcl- xL and Bcl-w, then trigger axon degeneration by an additional somatically-derived factor(s), distinct from Puma, that moves from the cell body to the axon. I further discuss data indicating that this factor is partially JNK dependent and present p53 as a possible candidate. I conclude with a general discussion of the results and suggestions for future experiments that can build on this work.



**Figure 1.8 Caspase activity radiates from the cell body after TD.** DRG explants cultured for 2 DIV were subjected to TD for the times indicated. Immunofluorescence was used to visualize Tuj1 and the Caspase-3 cleavage product of actin, Fractin.

## **Chapter 2: A transcriptional program in the cell body is required for the initiation of axon degeneration**

### **Rationale**

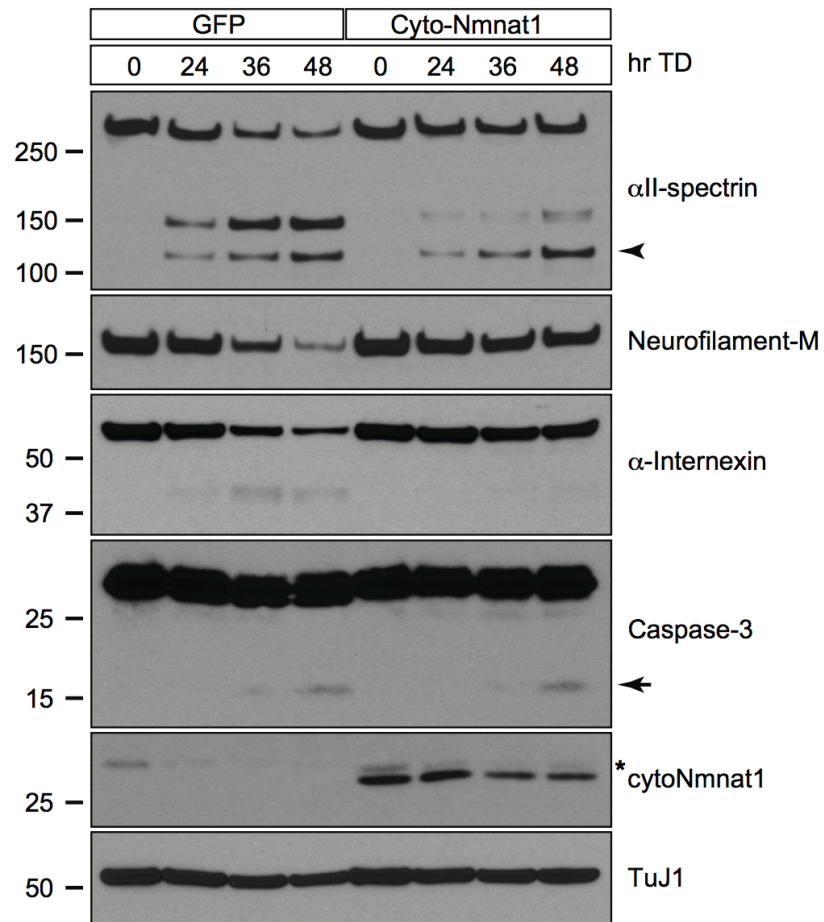
The prevailing view in the field argues that distal axon degeneration is initiated and executed by a local mechanism contained within the axon itself (Ghosh et al., 2011). This model has been called into question by a recent study showing that axons severed from their cell bodies, but protected against injury-induced Wallerian degeneration by genetic deletion of *Sarm1*, do not show cleaved Caspase-3 immunoreactivity after trophic factor withdrawal (Gerdtz et al., 2013). Further, inhibition of transcription and inhibition of GSK3 $\beta$  specifically in the cell body also prevent axon degeneration after TD, arguing against a degenerative mechanism contained exclusively within the axon (Chen et al., 2012). We sought to conclusively determine if the cell body plays an active and essential role in axon degeneration and, if so, which signaling pathways were responsible for initiating and executing the degenerative process.

### **Apoptotic machinery is present in axons but requires the cell body for activation by TD**

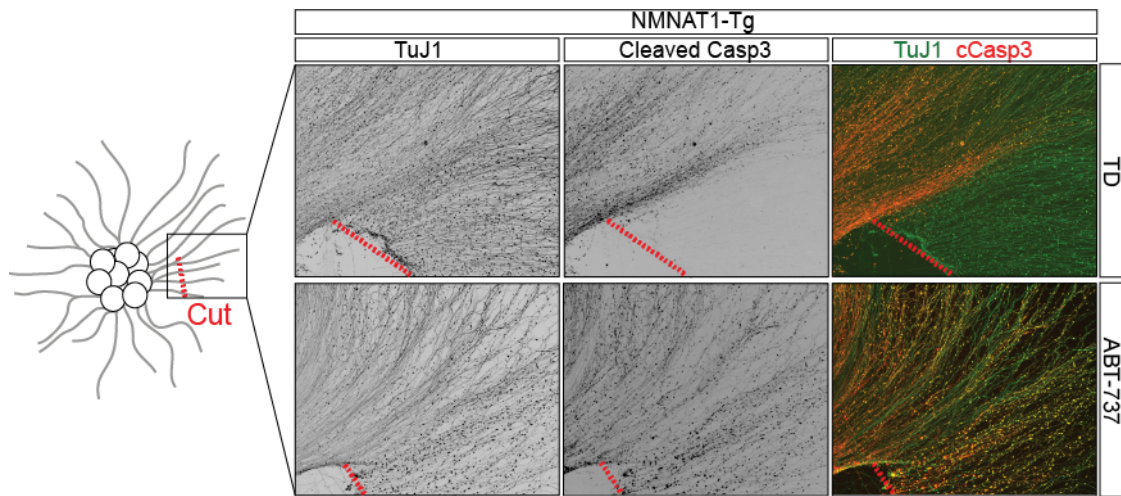
NGF-dependent sensory axons undergo Caspase-dependent degeneration when deprived of neurotrophic (NGF) support. This degeneration

is seen both when the entire neuron is deprived and when the manipulation is restricted to the axon through use of compartmented Campenot chambers that allow for independent manipulation of axons and cell bodies via fluid isolation of these compartments (Campenot, 1977).

To begin to explore the role of the cell body in regulating local degeneration, we severed axons from their cell bodies and subjected the isolated axons to TD. To exclude confounding effects of degeneration induced by the injury (Wallerian degeneration), we performed these experiments using sensory neurons from a transgenic mouse expressing cytoplasmic NMNAT1, which protects axons from Wallerian degeneration (Vohra et al., 2010) but does not interfere with cleavage and enzymatic activity of Caspase-3 in response to TD in intact neurons (Figure 2.1). Separation of axons from their cell bodies completely blocked appearance of cleaved Caspase-3 immunoreactivity 9 hrs following TD, in contrast with strong immunoreactivity in intact axons (Figure 2.2). This result confirmed that the cell body is required for caspase-dependent degeneration in response to TD (Gerdtz et al., 2013).

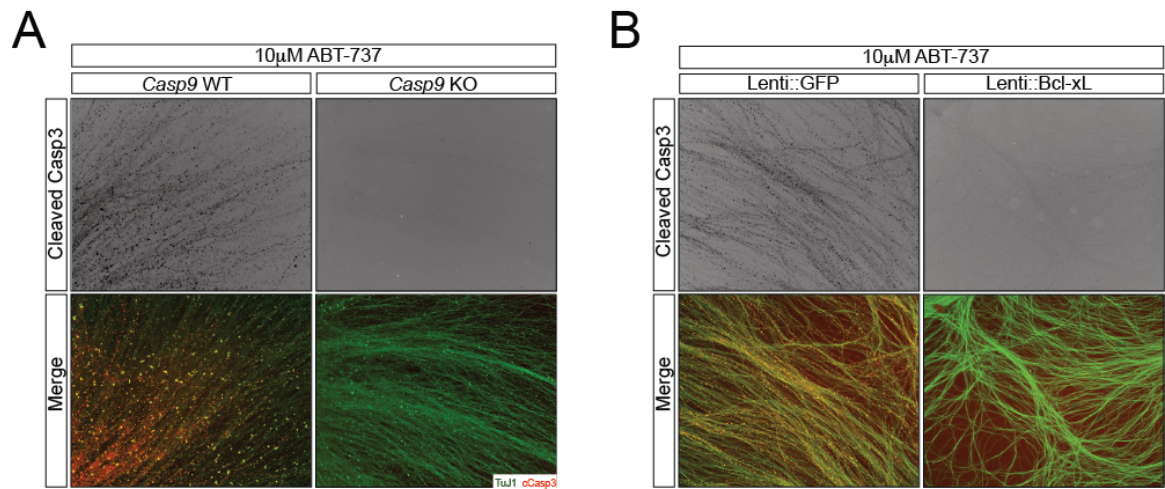


**Figure 2.1 Cytoplasmic NMNAT1 protects axons downstream of Caspase-3 activation.** WT embryonic DRG cultures were transduced with lentivirus expressing cyto-NMNAT1 or GFP and grown for 12 days. Lysates were prepared at indicated time points after TD. Axon degeneration and the activation of Caspase-3 and downstream calpains was assayed by immunoblot.



**Figure 2.2 Caspase-3 cleavage after TD requires the cell body, but Caspase-3 can be induced exogenously in isolated axons.** Following 2 days in culture, a portion of the axons in explants from cytoNMNAT1-Tg mice were physically severed from their cell bodies (indicated with a dashed red line) followed immediately by TD (top row) or the addition of 10 $\mu$ M ABT-737 (bottom row) for the indicated times. See illustration. Cleaved Caspase-3 was visualized by immunostaining.

A possible explanation for cell body dependence would be if the cell body is continually required to replenish components of the apoptotic pathway. To test whether the apoptotic machinery is present and functional in axons that have been disconnected from their cell bodies, we exposed isolated axons to ABT-737, a selective antagonist of the three pro-survival factors Bcl-2, Bcl-xL and Bcl-w (Oltersdorf et al., 2005), which causes *Bax*- and *Caspase-3*-dependent axon degeneration when applied directly to axons in Campenot chambers, even in the presence of NGF (Simon et al., 2012). We found that the response to ABT-737 is also *Caspase-9*-dependent and can be blocked by Bcl-xL overexpression (Figure 2.3A, B), further indicating its specificity. 10  $\mu$ M ABT-737 triggered robust Caspase-3 cleavage in isolated NMNAT1-Tg axons, as assessed by immunohistochemistry (Figure 2.2). To quantify this effect, we lysed isolated NMNAT1-Tg axons in a buffer containing a luciferase based probe for Caspase-3/7-like enzymatic activity (hereafter referred to as 'DEVDase' activity, as the Caspase target sequence in the probe is DEVD). Consistent with the results of immunohistochemistry, application of 10  $\mu$ M ABT-737 increased DEVDase activity in isolated axons but TD did not (Figure 2.4A). TD also did not increase Caspase activity triggered by ABT-737 in these isolated axons, as assessed by measuring DEVDase activity (Figure 2.4A) and cleaved Caspase-3 immunoreactivity (Figure 2.4B).

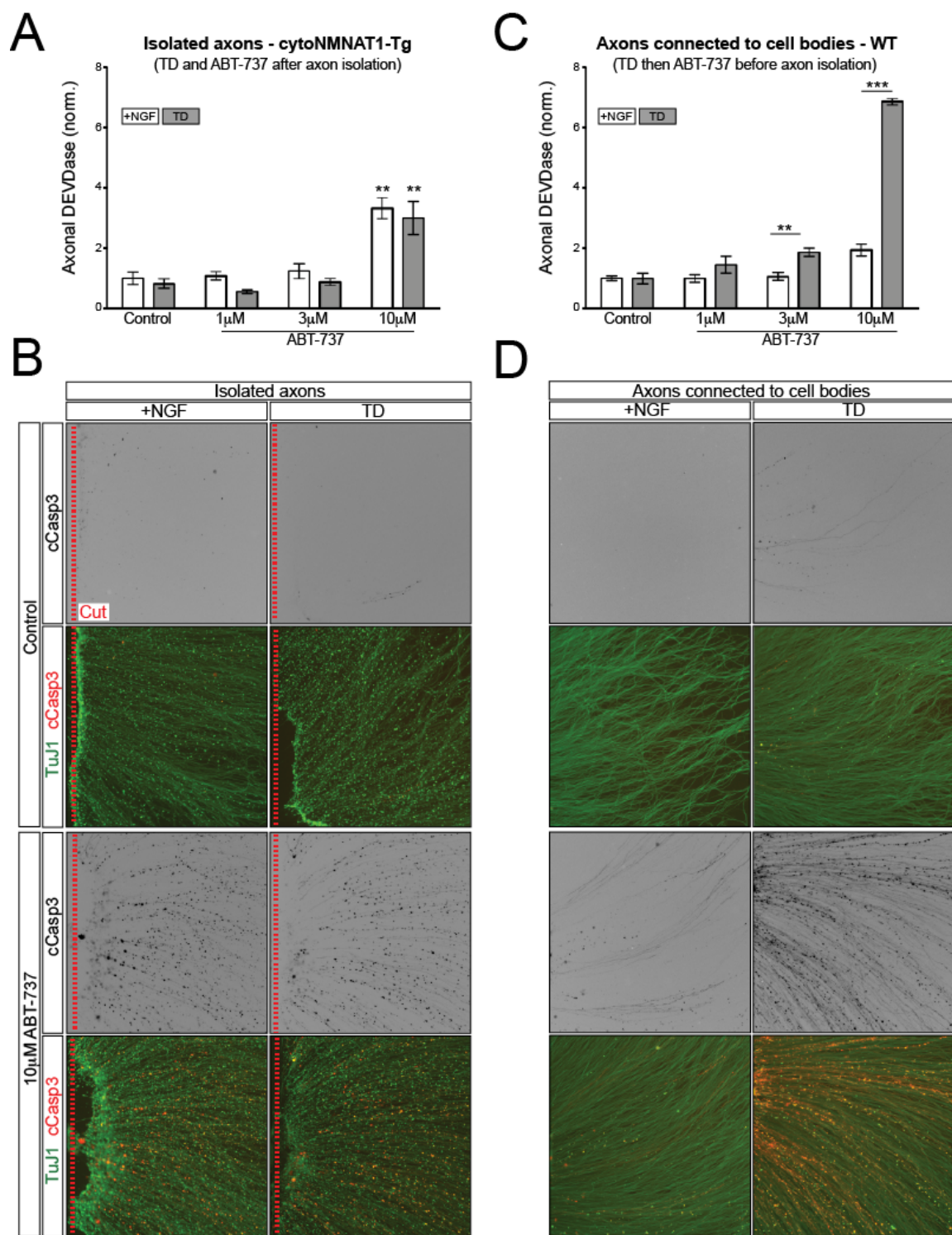


**Figure 2.3 Genetic deletion of Caspase-9 or overexpression of Bcl-xL is protective against ABT-737.** (A) Axon degeneration induced by ABT-737 is Caspase-9 dependent. 7DIV WT or *Caspase-9* KO embryonic DRG explants were treated with 10μM ABT-737 for 12hr. Caspase-3 cleavage is visualized by immunostaining. (B) Caspase-3 cleavage following ABT-737 application is blocked by Bcl-xL overexpression. Embryonic DRG cultures were transduced with lentivirus expressing Bcl-xL or GFP and treated with 10μM ABT-737 for 12hr. Caspase-3 cleavage in axons was visualized with immunostaining.

**Figure 2.4 Effect of TD on ABT-737 treatment in cut and uncut axons.**

(A and B) Isolated axons are not sensitized to ABT-737 treatment after TD. Axons from 2DIV cytoNMNAT1-Tg dissociated and reaggregated DRG explants were severed from their cell bodies (indicated with a dashed red line) and treated with ABT-737 for 4hr in the presence of NGF or following 2hr TD (for a total of 6hr TD) as indicated. DEVDase activity in these isolated axons was subsequently measured by Caspase-Glo-3/7 assay (A) or Cleaved Caspase-3 was visualized by immunostaining (B). n=4 for all conditions except 1 $\mu$ M and 10 $\mu$ M ABT-737 plus TD where n=3. Values are normalized to the average value of the +NGF control condition. Statistical significance for each 10 $\mu$ M dose is determined relative its respective Control condition.

(C and D) TD sensitizes axons to ABT-737 treatment. Embryonic DRG cultures were treated with ABT-737 in the presence of NGF or following 2hr TD (for a total of 6hr) as indicated. Following treatment, either cell bodies were removed and axonal DEVDase activity was measured with the Caspase-Glo-3/7 reagent (C) or Cleaved Caspase-3 was visualized by immunostaining (D). n=4 for all conditions except 10 $\mu$ M ABT-737 plus TD where n=3. Values are normalized to the average value of the +NGF control condition. Values are presented as mean  $\pm$  SEM; \*\*p<.01; \*\*\*p<.001 by ANOVA with Bonferroni tests.



We also applied ABT-737 to intact neurons, and observed a similar degree of DEVDase activation at 6hrs in their axons (which were isolated for DEVDase measurement at that time) as we had seen in isolated axons that were treated with ABT-737 only after being severed from their cell bodies (compare Figure 2.4A and C). Interestingly, in these intact neurons, a brief period of TD that was itself insufficient to cause a detectable increase in DEVDase activity dramatically potentiated the response of their axons to ABT-737 (Figure 2.4 C,D). Thus, TD triggers an early cell body-dependent increase in the pro-apoptotic state of axons.

Collectively, our results indicate that axons contain all necessary apoptotic machinery required to initiate Caspase-3 activation, but local TD is incapable of activating this machinery directly in isolated axons. Instead, the triggering of axonal apoptosis by local TD requires connection of the axon to its cell body and is presaged by an early increase in the sensitivity of the axon to direct activation of the apoptotic pathway. However, these results still do not establish whether the cell body is simply required in a permissive way for the effects of TD, or whether the cell body is a more active participant.

### **Akt signaling controls axon survival and requires the cell body**

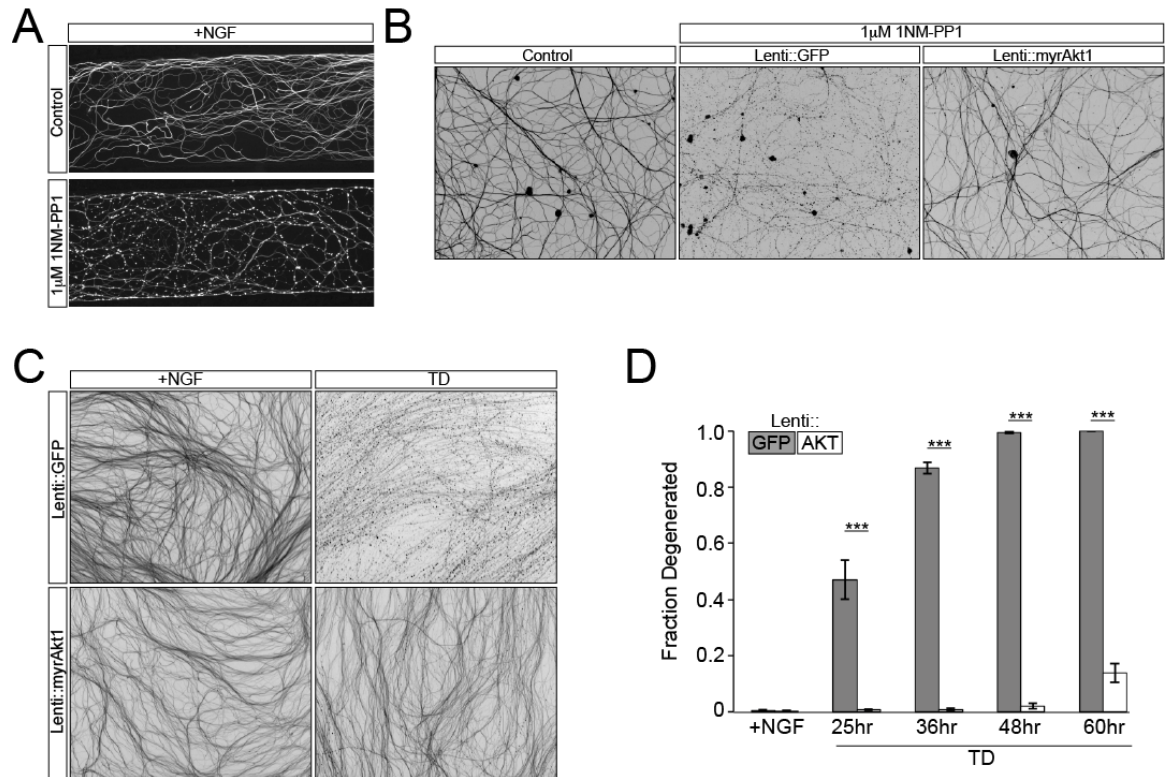
To elucidate the role of the cell body, we set out to map the pathway of degeneration. For this, we first examined how local removal of NGF initiates the degenerative response. One possibility is that the process is initiated by loss of

pro-survival signaling mediated by the kinase activity of TrkA, the NGF receptor. It has, however, been proposed alternatively that TrkA is a “dependence receptor”, which, when unliganded, sends a proapoptotic signal that is independent of its kinase activity (Nikoletopoulou et al., 2010). To distinguish these possibilities, we interfered specifically with kinase-dependent signaling using sensory neurons from the TrkA F592A knock-in mouse in which TrkA is engineered to be sensitive to acute inhibition of its kinase activity by the small molecule 1NMPP1, which does not inhibit wild-type kinases (Chen et al., 2005b). Application of 1NMPP1 specifically to axons induced axon degeneration (Figure 2.5A), consistent with loss of kinase activity being the driver of degeneration, rather than gain of a proapoptotic kinase-independent signal.

A major pro-survival pathway downstream of TrkA kinase is Akt signaling, so we next explored involvement of Akt in the degeneration induced by TrkA inhibition by 1NMPP1. The degeneration was blocked by lentiviral expression of a constitutively active, myristoylated form of Akt1 (myrAkt1) (Figure 2.5B). MyrAkt1 also potently protected axons from degeneration following TD (Figure 2.5C,D). Phosphoinositide PIP3-dependent activation of Akt is antagonized by the tumor suppressor phosphatase and tensin homolog (PTEN), so genetic deletion of *PTEN* represents an alternative strategy to activate Akt signaling. Sensory neuron cultures were therefore established from a conditional mutant of *PTEN* and infected with lentivirus expressing either GFP or Cre recombinase. Deletion of *PTEN* similarly protected axons from degeneration induced by TD,

though the duration of protection was shorter than for myrAkt1 expression (Figure 2.6A, B), presumably because it is less effective at activating downstream Akt signaling.

That Akt signaling regulates axon survival downstream of TrkA suggested acute pharmacological inhibition of Akt as a useful tool to probe the role of the cell body during TD. Acute pharmacological inhibition of Akt in the axonal compartment of Campenot chambers using the selective pan-Akt inhibitor GDC-0068 induces axon degeneration (Figure 2.6C). Activation of axonal Caspase-3 induced by Akt inhibition was eliminated in *Caspase-9* knockout cultures, indicating a specific activation of the apoptotic pathway by this treatment (Figure 2.6D). Further mirroring the dependence on cell body seen with local TD (Figure 2.2), we found axonal Caspase-3 activation by GDC-0068 was prevented by axotomy in cultures derived from the NMNAT1 transgenic or by co-incubation with the transcriptional inhibitor Actinomycin D (Figure 2.6E). These results further support a requirement for ongoing somatic transcriptional activity in mediating axonal Caspase-3 activation.



**Figure 2.5 Constitutive Akt signaling protects against TrkA inhibition and TD.** (A) Local inhibition of TrkA signaling induces axon degeneration. WT or TrkA F592A knock-in DRG sensory neurons were cultured in Campenot chambers and the axonal compartment was treated with vehicle or 1μM 1NM-PP1 for 24hr. Degeneration was visualized by immunostaining for TuJ1. (B) Constitutive Akt1 signaling promotes axon survival after TrkA inhibition. TrkA F592A dissociated DRG cultures were transduced with lentivirus expressing myristoylated-Akt1, Bcl-xL, or GFP and treated with 1μM 1NM-PP1 for 24hr. Degeneration was visualized by immunostaining for TuJ1. Lentiviral Bcl-xL overexpression also provided similar axonal protection (data not shown). (C and D) Constitutive Akt1 signaling promotes axon survival after TD. Embryonic DRG cultures were transduced with lentivirus expressing myristoylated-Akt1 or GFP and subjected to TD after 7 days in culture. Axon degeneration was visualized by TuJ1 immunostaining (C) and quantified over time (D). n=3 for each condition. Values are presented as mean ± SEM; \*\*\*p<.001 by ANOVA with Bonferroni tests.

## **Figure 2.6 Akt signaling regulates axon degeneration via the cell body**

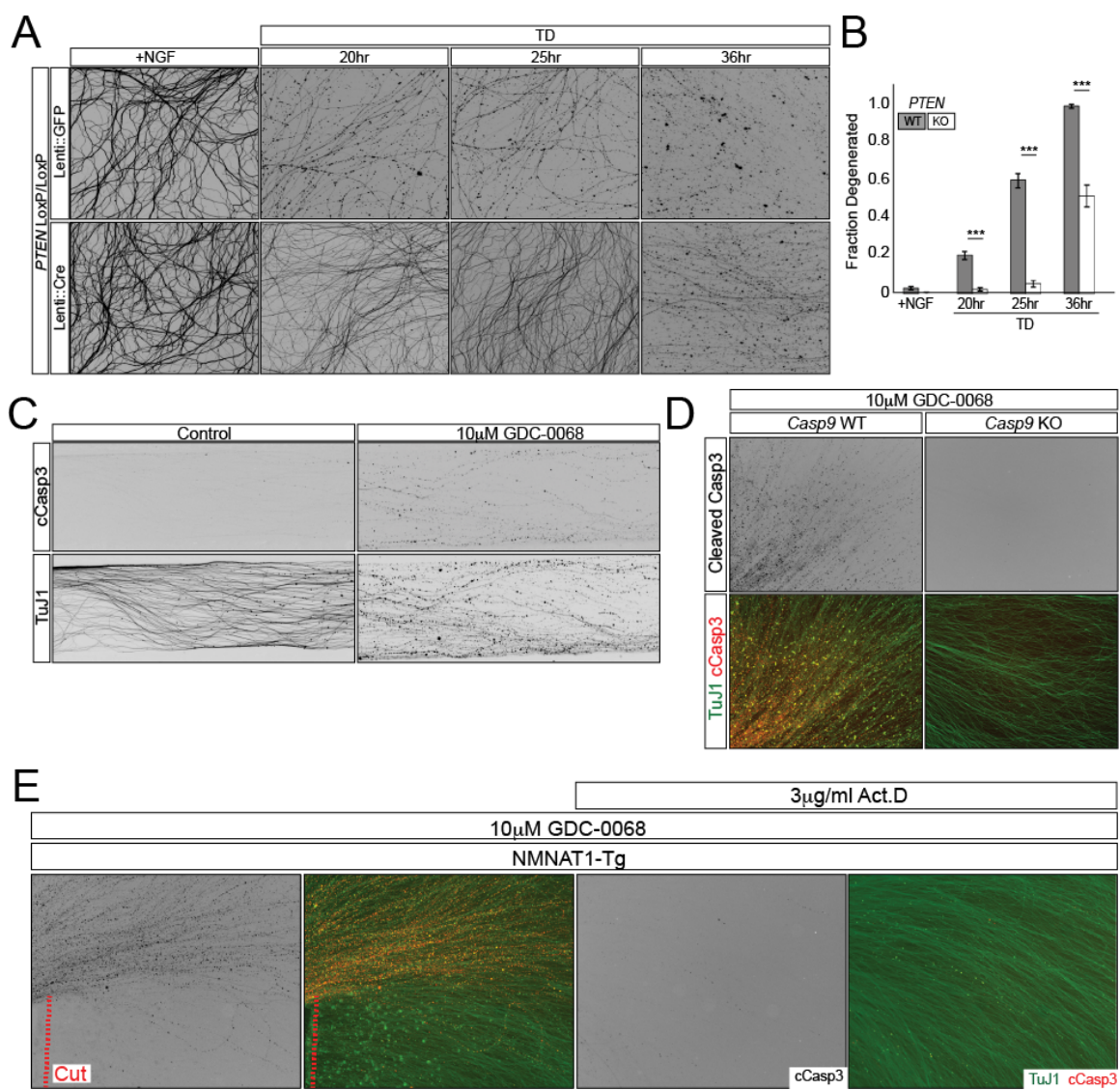
(A and B) Conditional deletion of PTEN delays axon degeneration after TD. *PTEN*<sup>loxP/loxP</sup> dissociated and reaggregated DRG cultures were transduced with lentivirus expressing CRE or GFP and axon degeneration following TD was visualized by TuJ1 immunostaining (A) and quantified over time (B). Values are presented as mean  $\pm$  SEM. n=5 for Lenti::GFP conditions and n=6 for Lenti::Cre conditions except 25hr TD where n=7

(C) Local AKT inhibition induces axon degeneration. WT embryonic DRGs were cultured in Campenot chambers and 10 $\mu$ M of the Akt inhibitor GDC-0068 or vehicle was added to the axonal compartment for 18hr. Axon degeneration was visualized with immunostaining for TuJ1 and cleaved Caspase-3.

(D) Axon degeneration induced by Akt inhibition is Caspase-9 dependent. WT or *Caspase-9* KO embryonic DRG explants were cultured for 7 days and treated with the indicated compounds for 12hr. Caspase-3 cleavage was visualized by immunostaining.

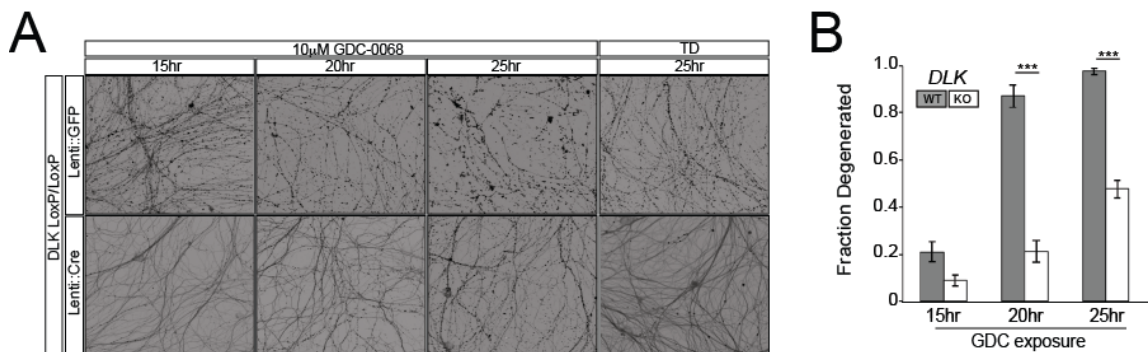
(E) Axon degeneration following Akt inhibition requires transcription and communication with the cell body. Axons from cytoNMNAT1-Tg explant cultures were severed from their cell bodies (red line) or pre-treated with 3 $\mu$ g/ml Actinomycin D for 1hr prior to addition of 10 $\mu$ M GDC-0068 for 12hrs. Axon degeneration and cleavage of Caspase-3 were visualized by immunostaining.

Values are presented as mean  $\pm$  SEM; \*\*p<.01; \*\*\*p<.001 by ANOVA with Bonferroni tests.



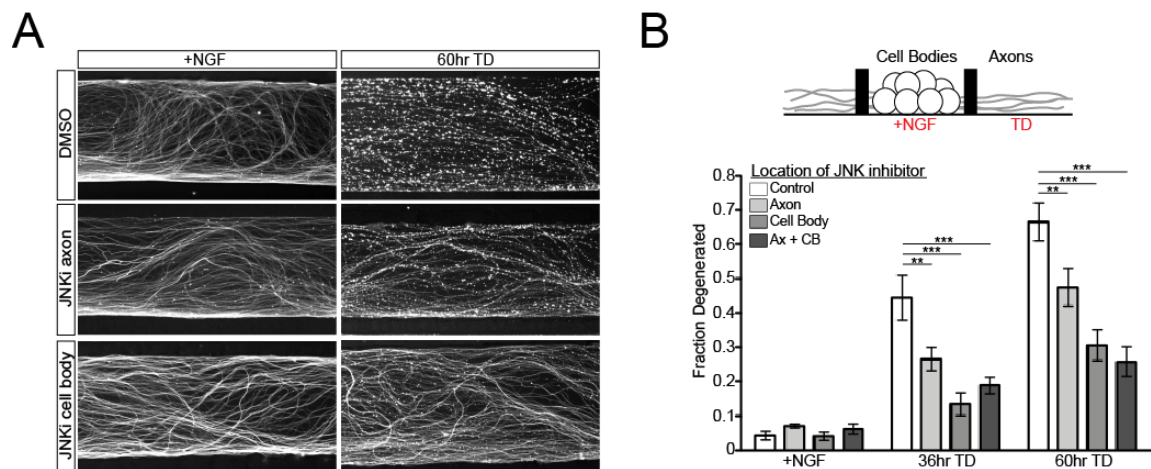
## Pro-degenerative JNK signaling is activated by loss of Akt signaling and requires the cell body

Previous studies have implicated the MAPKKK DLK as a JNK pathway activator in the regulation of axon degeneration in response to TD (Huntwork-Rodriguez et al., 2013; Ghosh et al., 2011). We therefore used the Akt inhibitor to examine a possible connection between loss of Akt signaling and the JNK pathway. Degeneration caused by acute Akt inhibition was significantly blunted in cultures of neurons in which *DLK* was genetically deleted (Figure 2.7A, B), placing DLK activation downstream of TD-associated loss of Akt signaling. Akt can directly phosphorylate DLK and inhibit its kinase activity (Wu et al., 2015), suggesting that DLK activation in response to local TD may reflect a disinhibition by removal of Akt signaling, although this remains to be established in our system.



**Figure 2.7 Conditional deletion of *DLK* delays axon degeneration induced by Akt inhibition.** *DLK*<sup>loxP/loxP</sup> DRG cultures were transduced with lentivirus expressing CRE or GFP and subjected to treatment with 10μM GDC-0068 for indicated time points. Axon degeneration was visualized by TuJ1 immunostaining (A) and quantified over time (B). For Lenti::GFP n=5 for 15hr, n=7 for 20hr, and n=4 for 25hr. For Lenti::Cre n=8 for all conditions. Representative images are presented in Figure S2E. Values are presented as mean ± SEM; \*\*\*p<.001 by ANOVA with Bonferroni tests.

It has been suggested that DLK directly promotes degeneration through local activation of the apoptotic pathway in axons (Ghosh et al., 2011). We cultured wild-type sensory neurons in Campenot chambers and subjected their axons to TD in the presence of a small molecule JNK inhibitor in either the axonal or cell body compartment. We found that axonal inhibition of JNK signaling provides modest protection of axons, consistent with previous findings (Chen et al., 2012; Ghosh et al., 2011), but also found that inhibition of JNK activity exclusively in the cell body compartment (which had not previously been tested) protected axons to a much greater degree (Figure 2.8A, B). This result was surprising because a previous study discounted the JNK target c-Jun as a significant regulator of axon degeneration (as opposed to cell body degeneration) (Ghosh et al., 2011). Here we revisited those findings and observed instead that, in addition to protecting against somatic activation of Caspase-3, genetic deletion of *c-Jun* also promotes significant axon survival in dissociated sensory neuron cultures and explant cultures (Figure 2.9A,B,D,E). We believe the discrepancy in findings is likely due to inefficient excision of *c-Jun* in the prior study, which used a transgenic line in which Cre recombinase is driven by the Nestin promoter. Indeed, we found that lentiviral delivery of Cre to *c-Jun* conditional knockout cultures, as done here, provides a much more complete deletion of the mature c-Jun protein (Figure 2.9C). Thus, our results argue that activation of DLK regulates cell body JNK/c-Jun signaling that is essential for axon degeneration, but is not sufficient to directly activate degeneration locally in axons.



**Figure 2.8 Axon degeneration after TD requires somatic JNK signaling.** Sensory neurons were cultured in Campenot chambers where cell bodies and axons are separated by a grease barrier that allows for fluidic isolation (See illustration). Cultures were treated with 10uM JNK inhibitor VIII or vehicle in either or both the cell body and axonal compartments, followed by TD as indicated. Degeneration was visualized by immunostaining for Tuj1 (A) and quantified (B). n=4 for each condition. Values are presented as mean  $\pm$  SEM; \*\*p<.01; \*\*\*p<.001 by ANOVA with Bonferroni tests.

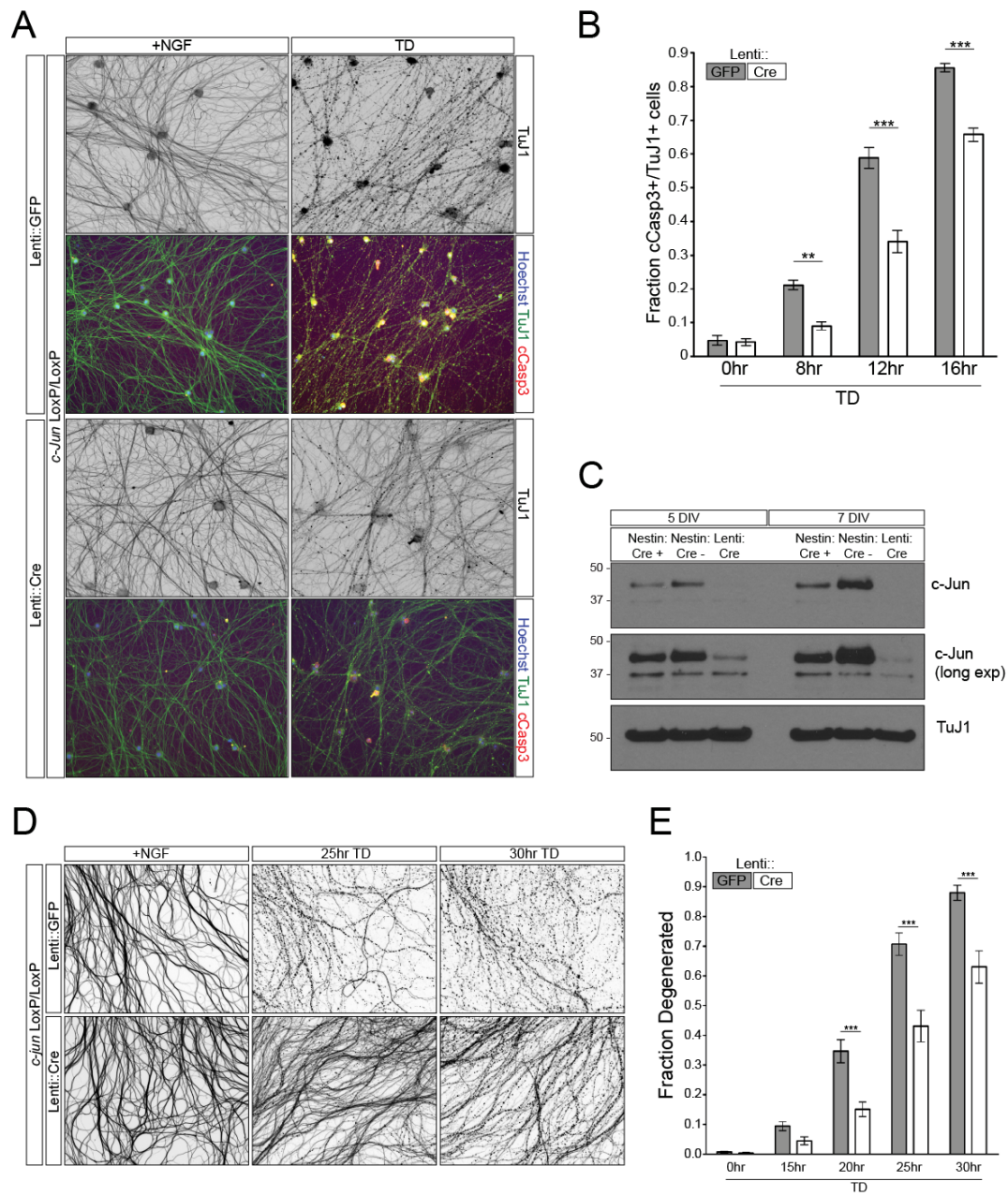
**Figure 2.9 The role of *c-jun* in axon degeneration.**

(A and B) Genetic deletion of *c-Jun* delays somatic caspase-3 activation and axon degeneration after TD. Dissociated embryonic *c-Jun*<sup>loxP/loxP</sup> DRG cultures were transduced with lentivirus expressing Cre or GFP and subjected to TD as indicated. Somatic Caspase-3 activation was visualized by immunostaining for Tuj1 and cleaved Caspase-3 (A) and quantified as the average fraction of cleaved Caspase-3+/Tuj1+ cells across 3 non-overlapping fields in each well (B). n=4 for each condition.

(C) Viral Cre expression is necessary for full excision of *c-Jun* in DRGs. *c-Jun*<sup>loxP/loxP</sup>; Nestin::Cre or *c-Jun*<sup>loxP/loxP</sup> embryonic DRGs were grown as dissociated reaggregated cultures and transduced with lentivirus expressing Cre where indicated. Cultures were lysed at indicated times and *c-Jun* expression was assayed by immunoblot.

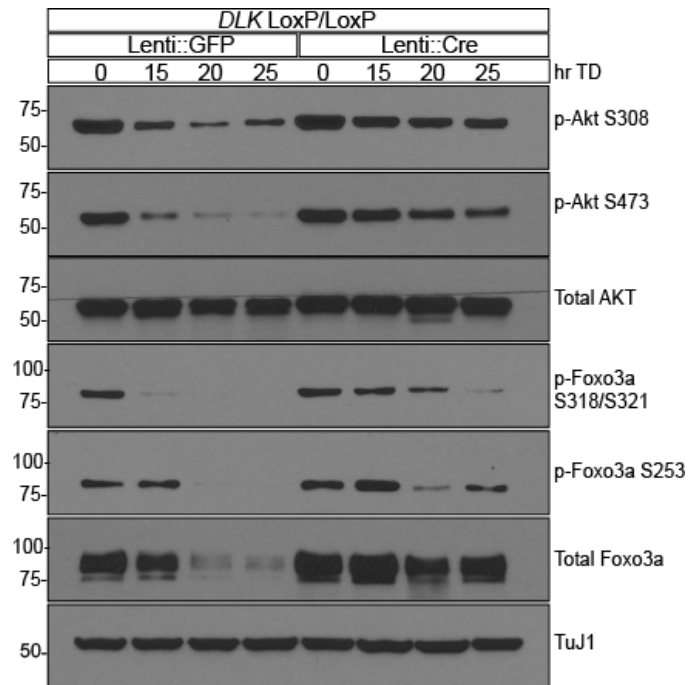
(D and E) Conditional deletion of *c-jun* delays axon degeneration after TD. *c-jun*<sup>loxP/loxP</sup> dissociated and reaggregated DRG cultures were transduced with lentivirus expressing CRE or GFP and axon degeneration following TD was visualized by Tuj1 immunostaining (D) and quantified over time (E). Values are presented as mean ± SEM. n=4 for all conditions except for 25hr and 30hr TD where n=6.

Values are presented as mean ± SEM; \*\*p<.01; \*\*\*p<.001 by ANOVA with Bonferroni tests.

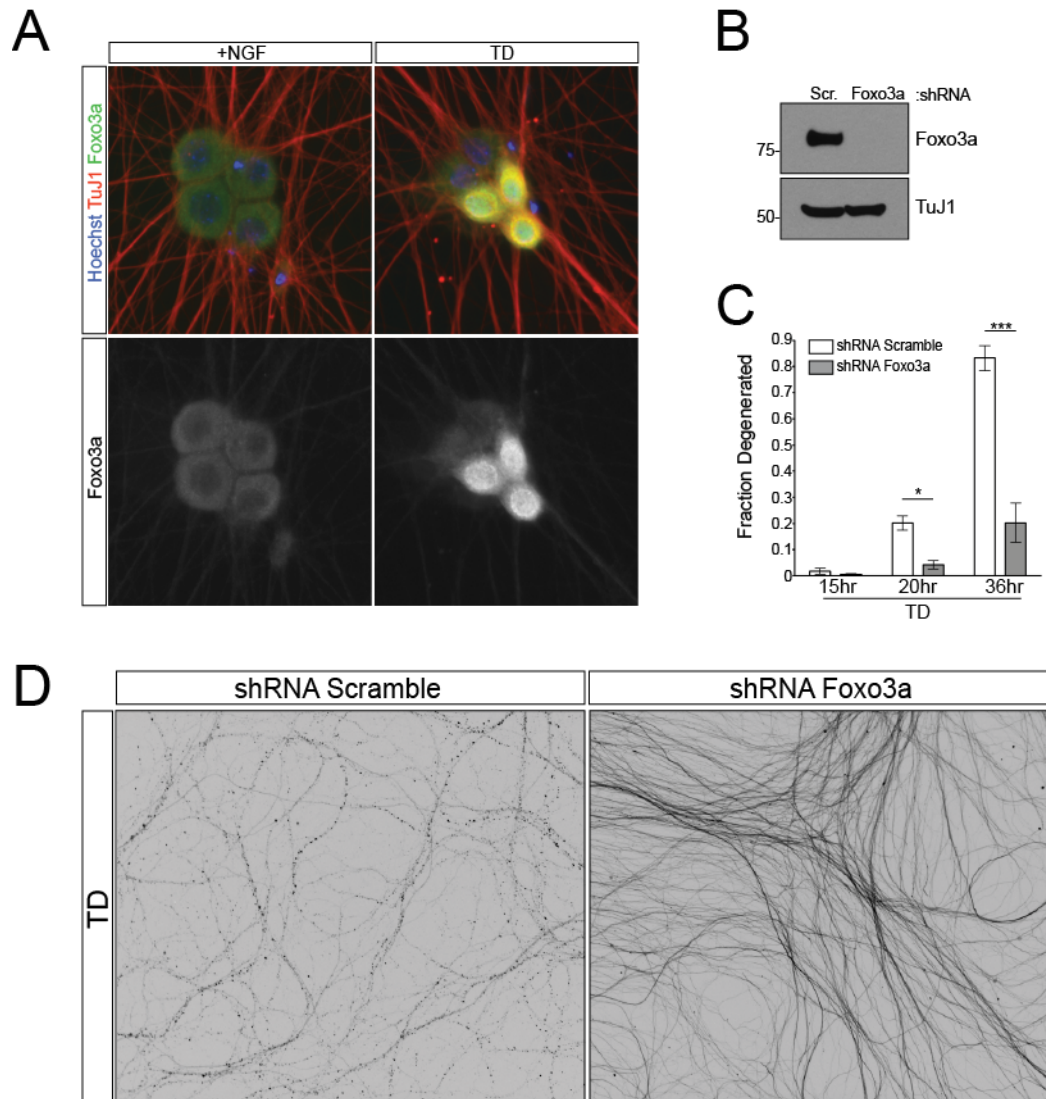


### **TD activates a Foxo3a-dependent pro-degenerative program**

The observations that axon degeneration requires somatic JNK/c-Jun activity (Figure 2.8A,B) and axon degeneration following Akt inhibition requires transcription (Figure 2.6E) point to a pro-degenerative role of the cell body. We took a candidate approach and searched for transcription factors known to be downstream of Akt and JNK. Specifically, we focused on the transcription factor Foxo3a, whose pro-apoptotic activity is inhibited by Akt-mediated phosphorylation (Brunet et al., 1999). Following TD, we observed concurrent decreases in phosphorylation of Akt and of Foxo3a (Figure 2.10), consistent with transcriptional activation of Foxo3a. Interestingly, in *DLK* knockout cultures the decreases in Akt phosphorylation and Foxo3a phosphorylation following TD occurred significantly more slowly, suggesting that DLK might participate in a feedback loop that enhances Akt inactivation following TD. Nuclear import to enable transcriptional activation normally follows de-phosphorylation of Foxo3a, and indeed we observed a nuclear migration of Foxo3a within several hours of TD (Figure 2.11A). Importantly, shRNA-mediated knockdown of Foxo3a strongly protected axons from degeneration following TD (Figure 2.11B-D), thus identifying Foxo3a as a key regulator of axon degeneration.



**Figure 2.10 Dephosphorylation of Akt and Foxo3a during TD is suppressed by conditional deletion of *DLK*.** *DLK*<sup>loxP/loxP</sup> embryonic DRG cultures were transduced with lentivirus expressing CRE or GFP and lysed at indicated time points after TD. Phosphorylation of Akt and Foxo3a were assessed by immunoblot using phospho-specific antibodies.



**Figure 2.11 Foxo3a is a key regulator of axon degeneration after TD** (A) TD induces nuclear import of Foxo3a. WT dissociated DRGs were cultured for 7DIV and subjected to TD for 9hr. Foxo3a localization was visualized by immunostaining for TuJ1 and Foxo3a. (B) Knockdown of Foxo3a was confirmed by immunoblotting. (C and D) Foxo3a knockdown protects against axon degeneration after TD. WT embryonic DRGs were grown as dissociated reaggregated cultures and subjected to lentiviral-shRNA knockdown of Foxo3a or Scrambled control. Axon degeneration after TD was visualized by immunostaining for TuJ1 (D) and quantified (C). Values are presented as mean  $\pm$  SEM; \* $p < .05$ ; \*\*\* $p < .001$  by ANOVA with Bonferroni tests.

## Conclusions

Here we demonstrate that although the apoptotic machinery is present in axons, it is not activated directly by TD and, instead, requires the cell body. Distal TD abrogates TrkA kinase signaling, resulting in concurrent loss of retrograde pro-survival Akt signaling and gain of retrograde pro-degenerative DLK signaling. This leads to the activation of two transcriptional programs in the cell body, JNK/c-jun and Foxo3a. Inhibition of JNK signaling specifically in the cell body compartment of Campenot chambers protects axons from degenerating after distal TD. Similarly, conditional deletion of *c-jun* or knockdown of *Foxo3a* significantly delays axon degeneration in explants. Taken together, we have definitively shown that the cell body is required for axon degeneration after TD and identified the central transcriptional pathways required to initiate the pro-degenerative signal.

## **Chapter 3: The pro-apoptotic protein Puma functions in the cell body to regulate axon degeneration**

### **Rationale**

Having identified JNK/c-jun and Foxo3a as key mediators of axon degeneration, we sought to elucidate the pro-degenerative targets of these transcriptional programs. Bax is required for axon degeneration after TD (Nikolaev et al., 2009) and Bax activity is regulated by members of the Bcl-2 family of proteins, several of which are regulated transcriptionally (Puthalakath and Strasser, 2002). As a result, we conducted a genetic screen to identify pro-apoptotic Bcl-2 family members that play a role in distal axon degeneration after TD.

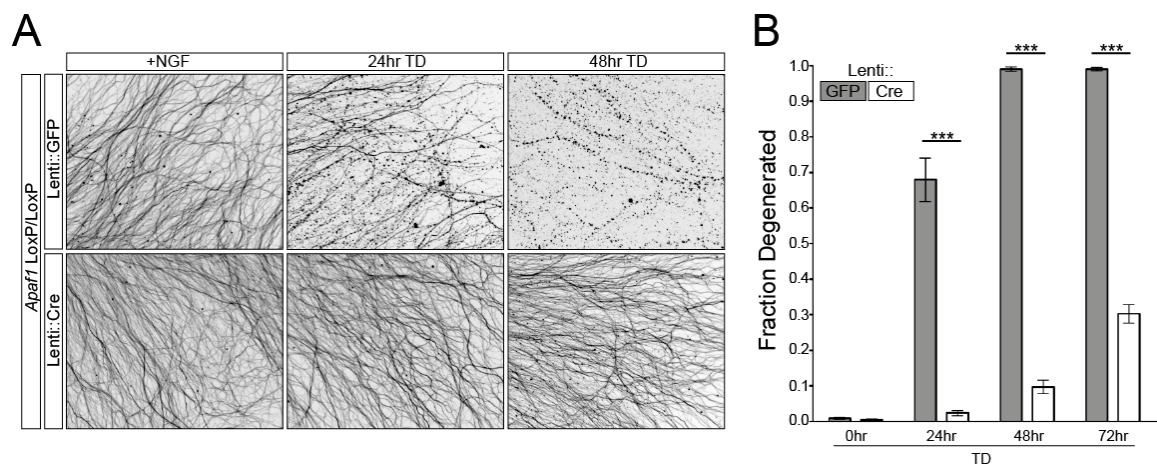
### **Pro-apoptotic Puma regulates axon degeneration**

Axon degeneration after TD is Bax dependent (Nikolaev et al., 2009), but a recent study showed that although *Apaf1* was required for neuronal apoptosis after NGF withdrawal, it was not required for distal axon degeneration after local TD, implying that a non-canonical mitochondrial apoptotic program operates in axons independent of cell bodies (Cusack et al., 2013). However, we found that conditional deletion of *Apaf1* in sensory neurons resulted in robust protection of axons after TD, disputing the earlier finding and establishing that the canonical mitochondrial apoptotic program is executed in degenerating axons (Figure 3.1). It is possible that this discrepancy results from the earlier study examining axon pruning in microfluidic chambers, where axons retract to the divider without signs of fragmentation, instead of in whole explant or Campenot cultures, although this

remains to be determined. Given this finding we reasoned that upstream processes may converge on the classical regulators of Bax activity, the pro-apoptotic 'BH3-only' family of Bcl-2 proteins, which includes 7 members (Figure 3.2A). We therefore examined the expression of the 7 pro-apoptotic Bcl-2 family members in axons, cell bodies, and whole DRGs. While Hrk, Bmf, and Noxa were unable to be detected with multiple antibodies, Bid, Bim, Puma, and Bad were found in cell bodies, but only Bid was expressed in axons (Figure 3.2B). Given that pro-apoptotic Bcl-2 family members are regulated by several transcriptional and post translational mechanisms (Puthalakath and Strasser, 2002), we examined their expression in cell bodies and axons over a time course of TD (Figure 3.2C,D). Puma and Bim showed noticeable increases in the cell body while a Bid cleavage product, corresponding to the molecular weight of the more apoptotically active caspase cleavage product tBid (Gross et al., 1999), was seen at later time points (Figure 3.2C). Bid cleavage was also seen in axons, initially highlighting this as the primary target for a subsequent screen (Figure 3.2D).

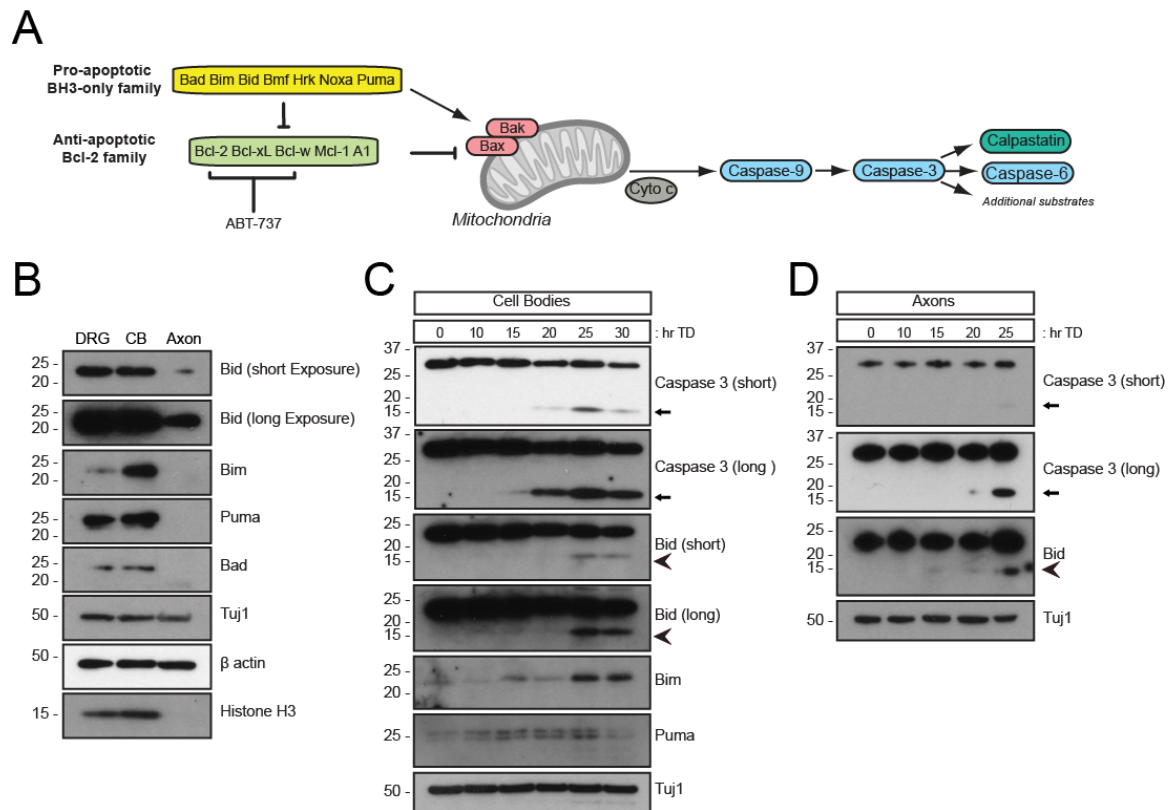
Based on the expression analysis, we screened a panel of knockout mice deficient in members of this family for axonal protection in sensory neuron explant cultures after TD. Mice with a genetic deletion for *Hrk* were included, despite not seeing evidence of expression, due to the known role of Hrk in neuronal apoptosis after TD (Imaizumi et al., 2004). Axons were not protected in *Bad* or *Hrk* single mutants or in *Bid*;*Bim* double mutants (Figure 3.3A-D, data not

shown). Furthermore, shRNA-mediated knockdown of Noxa provided no obvious axonal protection (data not shown). Notably however, we observed potent axonal protection after genetic deletion of *Puma* (Figure 3.4 A,B). Loss of Puma also completely blocked the appearance of axonal cleaved Caspase-3 in response to acute Akt inhibition (Figure 3.4C). We also cultured *Puma* knockout neurons in Campenot chambers and similarly observed potent axonal protection following TD in the axonal compartment (Figure 3.4D,E). Near complete protection was seen at early time points in *Puma* knockout cultures (e.g. only 10% of axons had degenerated by 60 hr after TD in Campenot chambers, Figure 3.4D,E), indicating that Puma is a key regulator of degeneration. It should be noted, however, that this protection is slightly less complete than seen in *Bax* and *Caspase-3* and *-9* knockout cultures (Nikolaev et al., 2009; Simon et al., 2012), with *Puma* knockout 2DIV cultures degenerating by 40hr after TD (Figure 3.4A right panel). This suggests the presence of an additional Bax-activating factor(s), which remains to be defined, that functions in parallel to Puma and provides the residual pro-apoptotic activity.

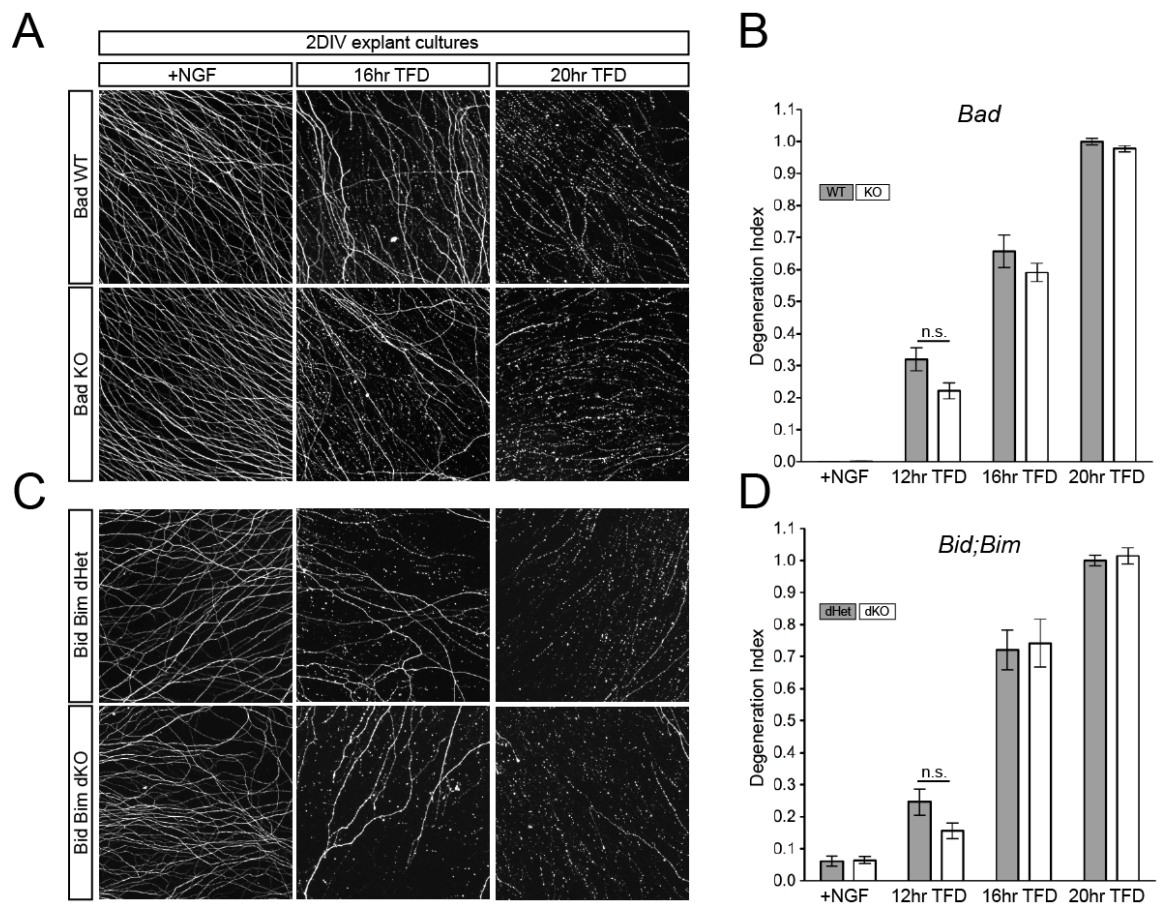


**Figure 3.1 Conditional deletion of *Apaf1* delays axon degeneration after TD.**

*Apaf1*<sup>loxP/loxP</sup> dissociated and reaggregated DRG cultures were transduced with lentivirus expressing CRE or GFP and axon degeneration following TD was visualized by TuJ1 immunostaining (A) and quantified over time (B). Values are presented as mean  $\pm$  SEM. n=4 for all conditions. Values are presented as mean  $\pm$  SEM; \*\*\*p<.001 by ANOVA with Bonferroni tests.



**Figure 3.2 Several members of the pro-apoptotic Bcl-2 family are expressed in DRGs.** (A) The mitochondrial apoptotic pathway is regulated by pro- and anti-apoptotic members of the Bcl-2 family. The balance of these factors regulates Bax/Bak oligomerization and activation of downstream Caspases. (B) Several anti-apoptotic Bcl-2 family members are expressed in DRGs. Separate cell body and axon preparations were harvested from WT embryonic DRG cultures for immunoblot analysis. (C and D) Levels of pro-apoptotic Bcl-2 family members in cell bodies and axons after TD. WT DRGs cultured for 7DIV and subjected to TD for the indicated times. Separate cell body (C) and axon (D) preparations were made and analyzed by immunoblot. Arrow indicates cleaved Caspase-3 and arrowhead indicates cleaved Bid (tBid).



**Figure 3.3 *Bad* KO and *Bid;Bim* dKO do not protect against axon degeneration after TD.** Embryonic DRG cultures from the indicated genotypes were grown for 2 days and subjected to TD for indicated times. Axon degeneration was visualized by immunostaining for TuJ1(A and C) and quantified (B and D). Representative images shown.  $n=4$  for *Bad* KO and WT.  $n=3$  for *Bid;Bim* dKO and dHet.

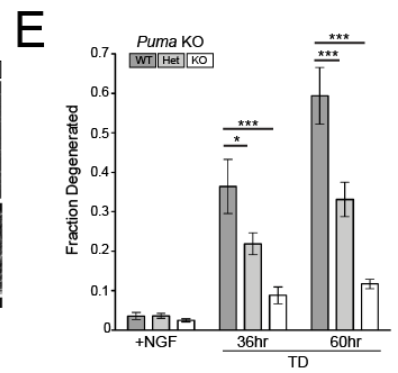
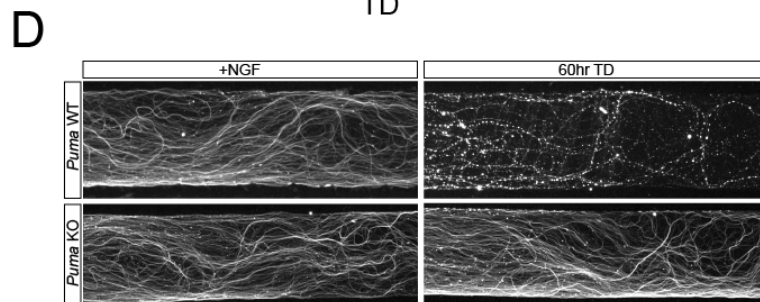
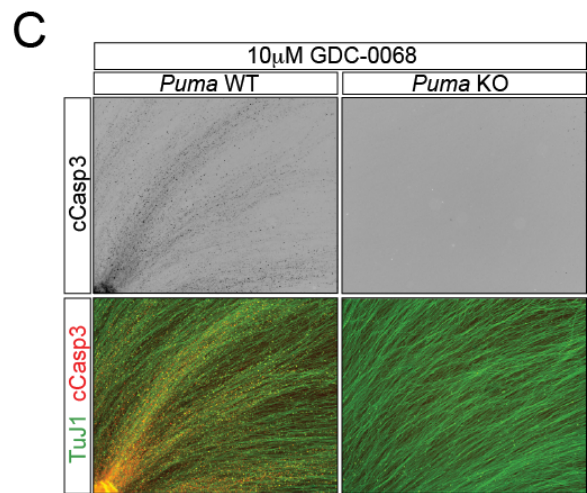
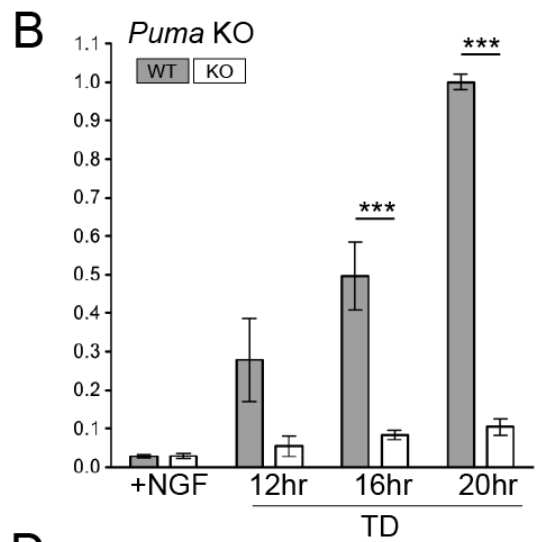
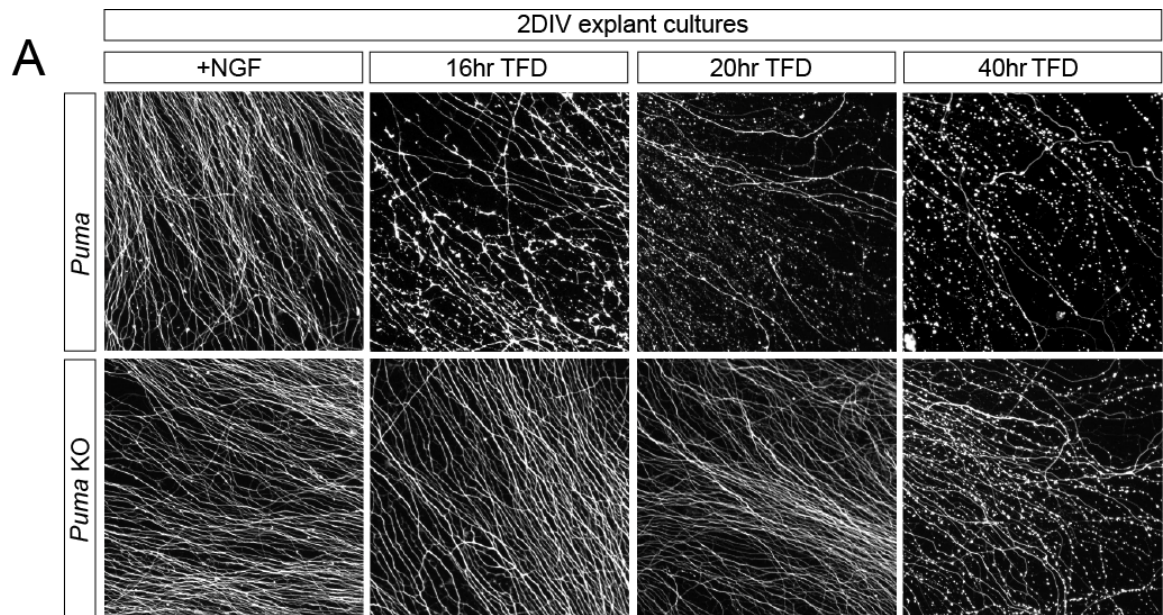
**Figure 3.4 Puma is required for caspase dependent axon degeneration.**

(A and B) *Puma* KO protects against axon degeneration in whole explant culture after TD. Embryonic DRG cultures from the indicated genotypes were grown for 2 days and subjected to TD for indicated times. Axon degeneration was visualized by immunostaining for TuJ1(A) and quantified (B). Representative images shown. n=4 KO and 7 WT for *Puma*.

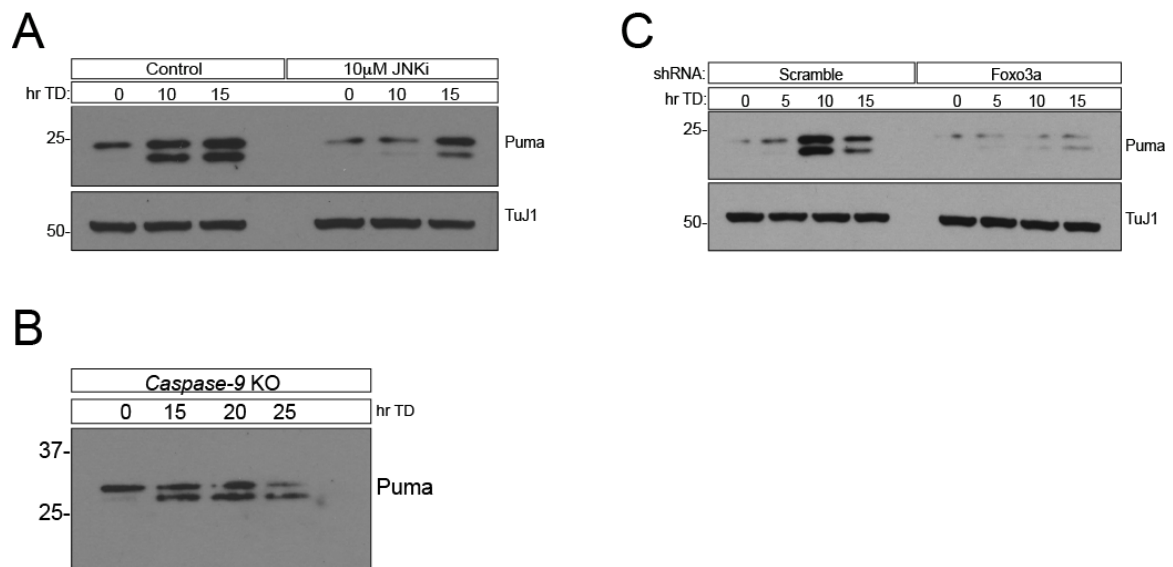
(C) Caspase-3 cleavage following Akt inhibition requires Puma. *Puma* WT or KO embryonic explants were cultured for 7 days and treated with 10 $\mu$ M of the Akt inhibitor GDC-0068 for 12hr. Caspase-3 cleavage was visualized by immunostaining.

(D and E) Axon degeneration following TD requires Puma. *Puma* WT, Het, or KO embryonic DRGs were cultured in Campenot chambers and the axonal compartment was subject to TD for the indicated times. Degeneration was visualized by immunostaining for TuJ1 (D) and quantified (E). n=6 +NGF, n=6 36hr TD, and n=5 60hr TD for WT. n=6 +NGF, n=6 36hr TD, and n=3 60hr TD for Het. n=8 TD, n=7 36hr TD, and n=3 60hr TD for KO.

Values are presented as mean  $\pm$  SEM; \*p<.05; \*\*\*p<.001 by ANOVA with Bonferroni tests.



Puma is classically described as a transcriptionally regulated gene induced by cellular stress (Yu and Zhang, 2009). Interestingly, we found that Puma is basally expressed in sensory neuron cultures in the presence of NGF, and its levels are potentiated in response to TD. The rise in Puma is also accompanied by the *de novo* appearance of a second Puma band at a slightly lower molecular weight (Figure 3.5A), whose molecular identity and physiological significance remain to be determined. The increase in Puma expression and appearance of the second band occurred to a similar extent in *Caspase-9* knockout cultures (Figure 3.5B), which are protected from degeneration (Simon et al., 2012), indicating that both the rise of overall Puma levels and the appearance of the second band are not simply a secondary consequence of the degenerative process. Pharmacological inhibition of JNK prevented this TD-induced rise in Puma (Figure 3.5A), consistent with a known role of c-Jun in promoting Puma expression (Wong et al., 2005) and providing a mechanism for the somatic requirement for JNK signaling that we observed (Figure 2.8). Puma has also been identified as a transcriptional target of Foxo3a (You et al., 2006), and accordingly we found that shRNA-mediated knockdown of Foxo3a in sensory neurons largely blocked the rise in Puma levels that accompanies TD (Figure 3.5C). Thus, activation of Puma appears to be a point of convergent action of Akt/Foxo3a and Jnk/c-Jun signaling that is required for axon degeneration following distal TD.



**Figure 3.5 Factors regulating Puma expression.** (A) Puma expression after TD is regulated by JNK. WT embryonic DRG neurons were treated with 10 $\mu$ M JNK inhibitor VIII or vehicle and protein was harvested at indicated time points after TD. Puma expression was assayed by immunoblot.

(B) Appearance of Puma doublet is not Caspase-9 dependent. 7 DIV *Caspase-9* KO DRG cultures were harvested after TD. Puma expression was assayed by immunoblot.

(C) Foxo3a regulates Puma expression during TD. Dissociated and reaggregated cultures from WT embryonic DRG neurons were subjected to lentiviral-shRNA knockdown of Foxo3a or Scrambled control. Protein was harvested at indicated time points after TD. Puma expression was assayed by immunoblot.

### **Puma functions in the cell body to promote axon degeneration**

Puma can directly regulate Bax/Caspase activation, and since Caspase activation is observed in axons and deletion of *Puma* protects axons in Campenot chambers, we expected Puma to be present in axons as well. We were therefore very surprised not to detect any Puma protein in axons. Sensory neuron cultures were subjected to a time course of TD followed by clear separation and specific lysis of axons and cell bodies using methods that we have previously established (Yang et al., 2013). TD was accompanied by a rise in Puma protein levels in cell bodies, but there was no detectable signal in axons as assessed by immunoblotting (Figure 3.6A). To confirm this finding, we used mass spectrometry. We identified a number of stable Puma peptides, and used these as a basis to specifically probe for Puma in extracts of axons and cell bodies before and during TD. As an internal control for similar protein content between samples, we found that two separate proteins with similar mass to Puma (Capzb and 14-3-3G) are present at equivalent levels in cell body and axon samples (Figure 3.6B-D). Using this approach we confirmed the presence of Puma in the cell body, but again did not detect Puma in the axon under any condition (Figure 3.6B-D). We cannot of course exclude the presence of very low, undetectable levels of Puma in axons, however such expression would not be physiologically relevant as the strong expression of the Puma antagonists Bcl-xL and Bcl-w that we observe in axons (see below) would be expected to inhibit the activity of any Puma that is present.

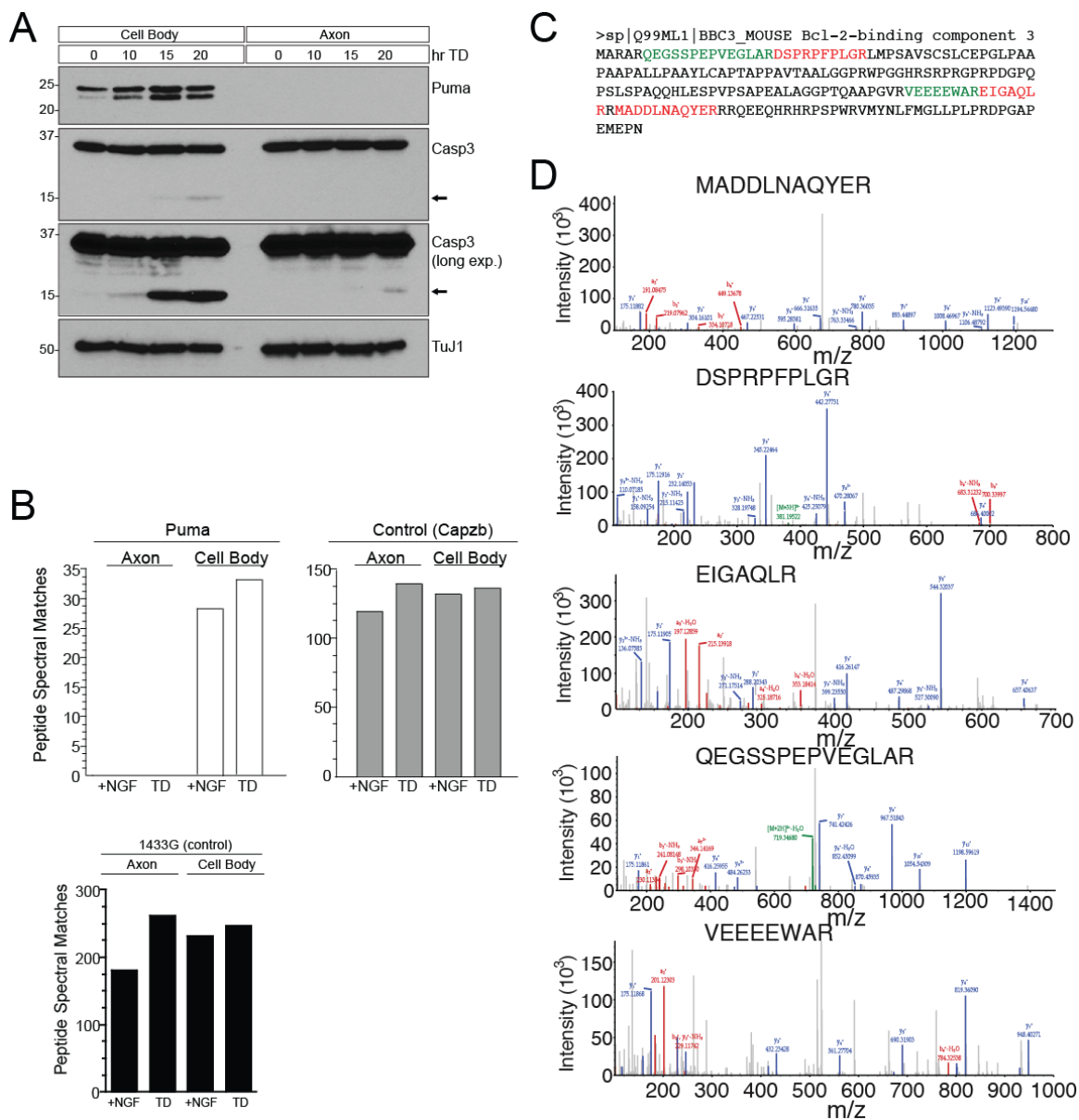
**Figure 3.6 Puma expression is confined to the cell body.**

(A) Puma is not detected in axons. Separate cell body and axonal preparations were collected from WT embryonic DRG cultures at indicated time points after TD and subjected to immunoblot analysis. Arrow indicates cleaved Caspase-3.

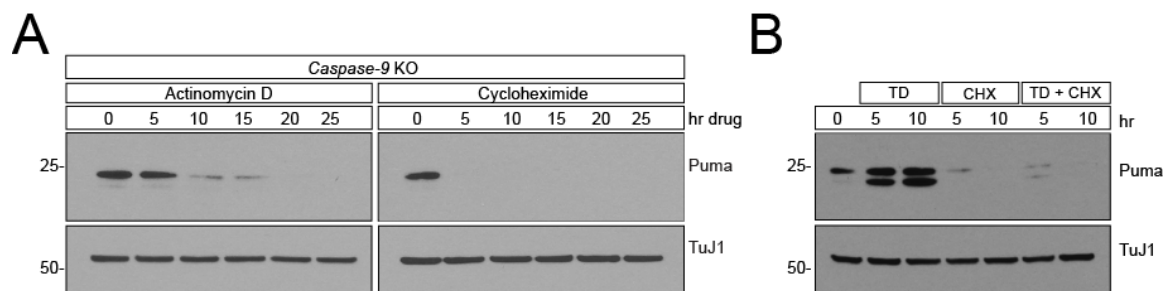
(B) Puma peptides are exclusively identified in the cell body. At 12DIV Large-scale cultures made from WT embryonic DRGs were subjected to TD for 24hr. Separate cell body and axonal samples were collected and analyzed by tandem mass spectrometry. The number of peptide spectral matches are shown for each sample for either Bbc3 (Puma) or the loading control Capzb (capping protein actin filament z-line) and 14-3-3 protein gamma (1433G). Peptides corresponding to Puma were found only in cell body samples, while peptides corresponding to Capzb and 1433G were found in all samples.

(C) Identified peptides are mapped on to the sequence of Puma. Peptides identified in triplicate are shown in red, those identified in the initial identification experiment are shown in green.

(D) Annotated spectra for all 5 identified Puma peptides.



To explore the molecular basis of this somatic restriction we exposed sensory neuron cultures to either the transcriptional inhibitor Actinomycin D or the translation inhibitor cycloheximide. *Caspase-9* knockout cultures were used to prevent an apoptotic response due to drug-induced cellular stress; as mentioned, Puma levels change to the same extent following TD in these cultures as in wild-type cultures (Figure 3.5B). Interestingly, following 20hr of Actinomycin D or 5hr of cycloheximide treatment, Puma protein became undetectable (Figure 3.7A), suggesting that Puma has an extremely short half-life in healthy cells and is subject to an active gene expression program. Puma levels are similarly sensitive to cycloheximide during TD (Figure 3.7B), indicating that *de novo* expression rather than stabilization of existing Puma protein underlies its rise during TD. This rapid turnover might in principle contribute to Puma's exclusion from sensory axons, though it is possible that there are active mechanisms that exclude it as well. Importantly, the dependence of axon degeneration on Puma, together with the exclusion of Puma from the axon, imply the existence of a somatically-derived triggering factor distinct from Puma that moves to the axon after TD as a consequence of Puma's somatic activity.



**Figure 3.7 Critical role of transcription and translation in maintaining basal Puma levels.** (A) Maintenance of basal Puma levels requires transcription and translation. *Caspase-9* KO embryonic DRG cultures in the presence of NGF were treated with ActinomycinD or cycloheximide, harvested at the indicated time points, and subjected to immunoblot analysis. (B) TD does not maintain Puma levels in the absence of translation. WT embryonic DRG cultures were subjected to TD, cycloheximide treatment, or TD following 1hr cycloheximide pre-incubation as indicated. Puma levels were analyzed by immunoblot.

### **Bcl-xL and Bcl-w regulate the survival of sensory axons**

Puma's proapoptotic activity is antagonized by its binding to each of the known five anti-apoptotic Bcl-2 family members, three of which are the targets of ABT-737 (Figure 3.2A). We therefore examined the expression of the five anti-apoptotic factors in axons, cell bodies, and whole DRGs by immunoblotting (Figure 3.8A). We were unable to detect A1 protein in either axons or cell bodies; each of the four additional family members, Bcl-2, Bcl-w, Bcl-xL, and Mcl-1 were expressed in sensory cell bodies, but only Bcl-xL and Bcl-w were detected in axons (Figure 3.8A). Similarly to our approach with the pro-apoptotic Bcl-2 family proteins, we sought to determine the effect of TD on the levels of anti-apoptotic members, revealing two notable findings. First, Bcl-w appears to increase in the DRG cell body after TD, possibly due to a cessation of NGF stimulation which a recent study observed to halt mRNA and protein movement to axons (Cosker et al., 2013) (Figure 3.8B). Secondly, although levels of Bcl-xL do not show a significant change in cell bodies or axons, a lower molecular weight band appears in both axons and cell bodies coincident with Caspase-3 activation (Figure 3.8B,C). This band corresponds to the Bcl-xL Caspase-3 cleavage product Bcl-xS, which actually acts as a potent pro-apoptotic protein in neurons (Ofengeim et al., 2012). Further, Bcl-xL localization within axons appears to shift from a continuous distribution along the axon to a noticeably punctate appearance before signs of axon swelling and fragmentation after TD (Figure 3.8D). This highlighted Bcl-xL, which had not previously been examined

**Figure 3.8 Localization and dynamics of anti-apoptotic Bcl-2 family members in DRGs.**

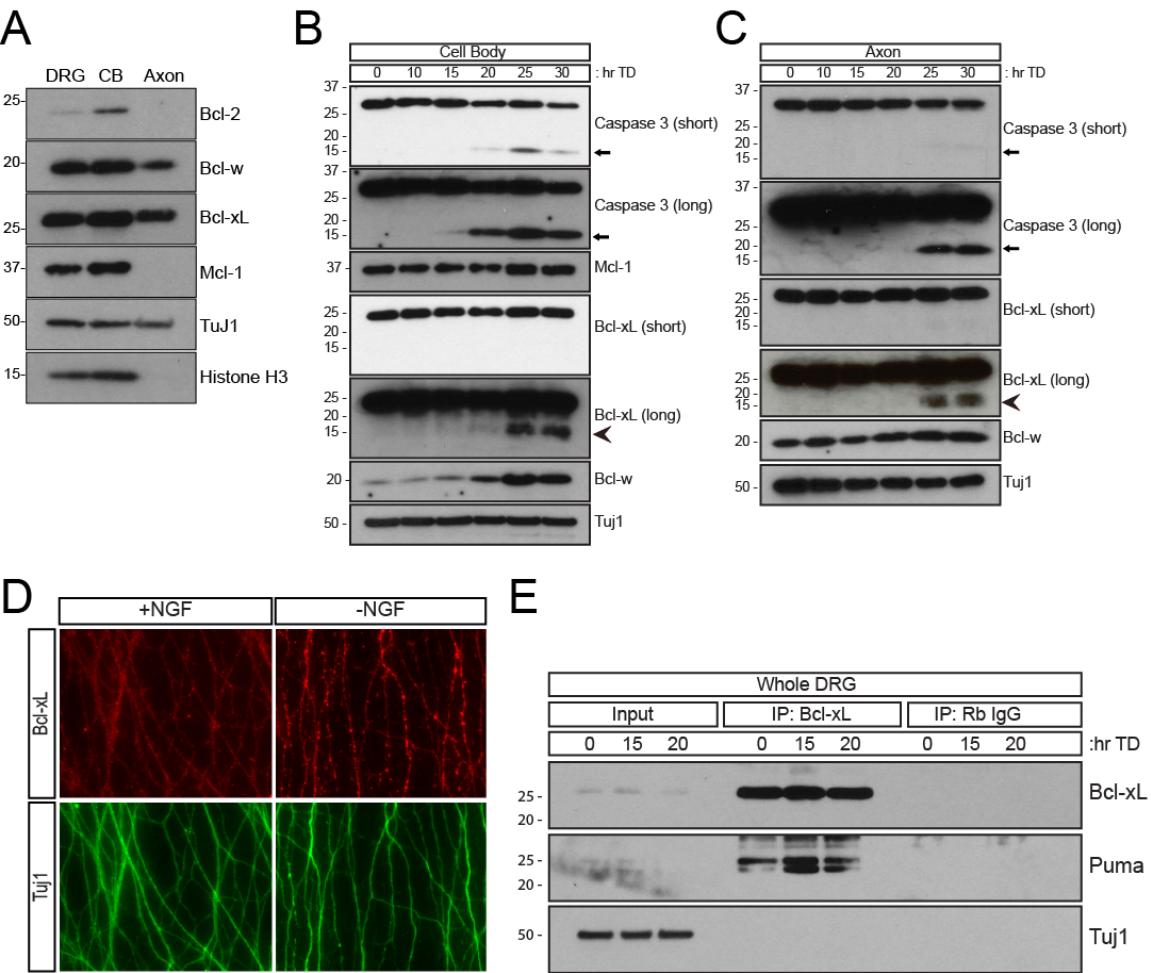
(A) Several anti-apoptotic Bcl-2 family members are expressed in DRGs. Separate cell body and axon preparations were harvested from WT embryonic DRG cultures for immunoblot analysis.

(B and C) Levels of anti-apoptotic Bcl-2 family members in cell bodies and axons after TD. WT DRGs cultured for 7DIV and subjected to TD for the indicated times. Separate cell body (B) and axon (C) preparations were made and analyzed by immunoblot. Arrow indicates cleaved Caspase-3 and arrowhead indicates cleaved Bcl-xL (Bcl-xS).

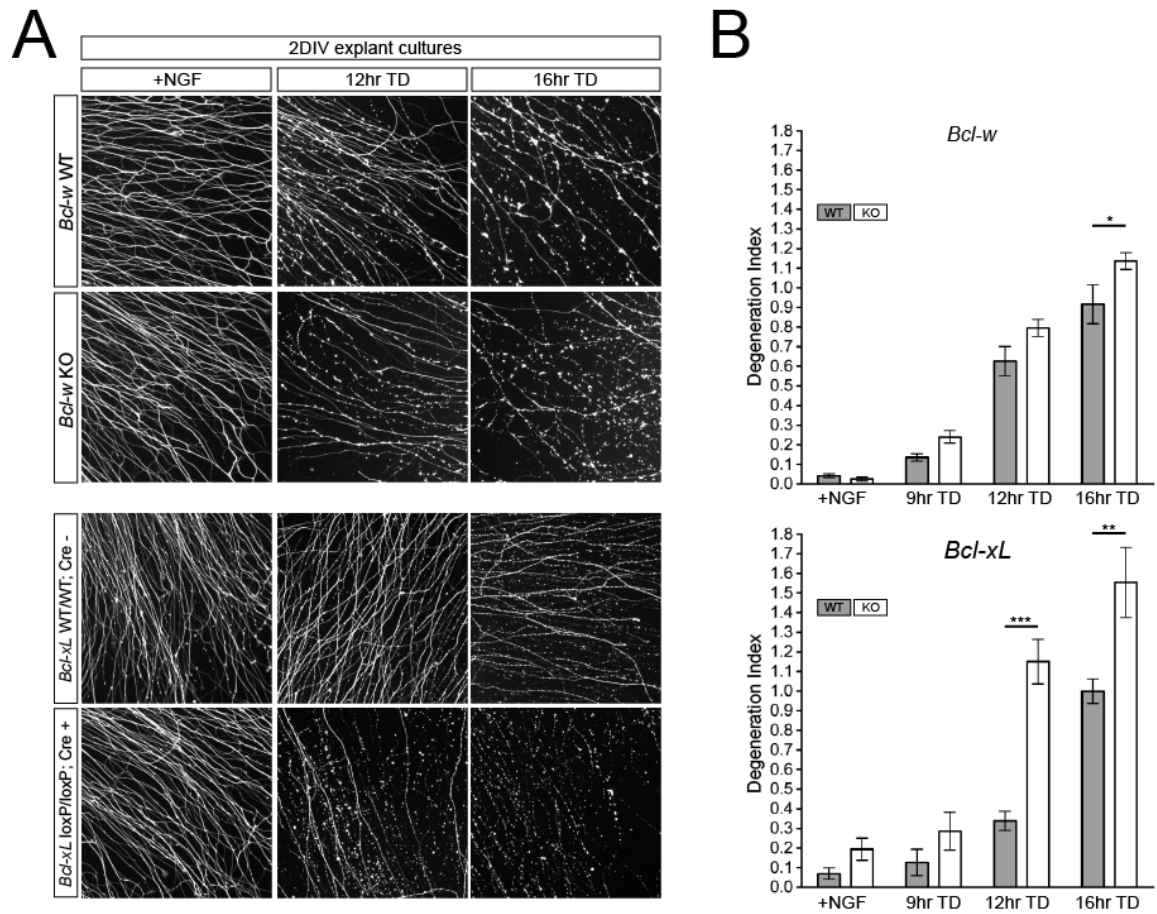
(D) Localization of Bcl-xL in axons changes after TD. 2DIV explants were subject to 9hr TD and Bcl-xL was visualized with immunostaining.

(E) Puma shows increased binding to Bcl-xL after TD. 7DIV WT DRG explants were lysed after TD for indicated times. Lysates were precleared with Protein A/G beads and normalized to protein concentration using BCA. Immunoprecipitation performed with antibody to Bcl-xL or isotype control and analyzed by immunoblot.

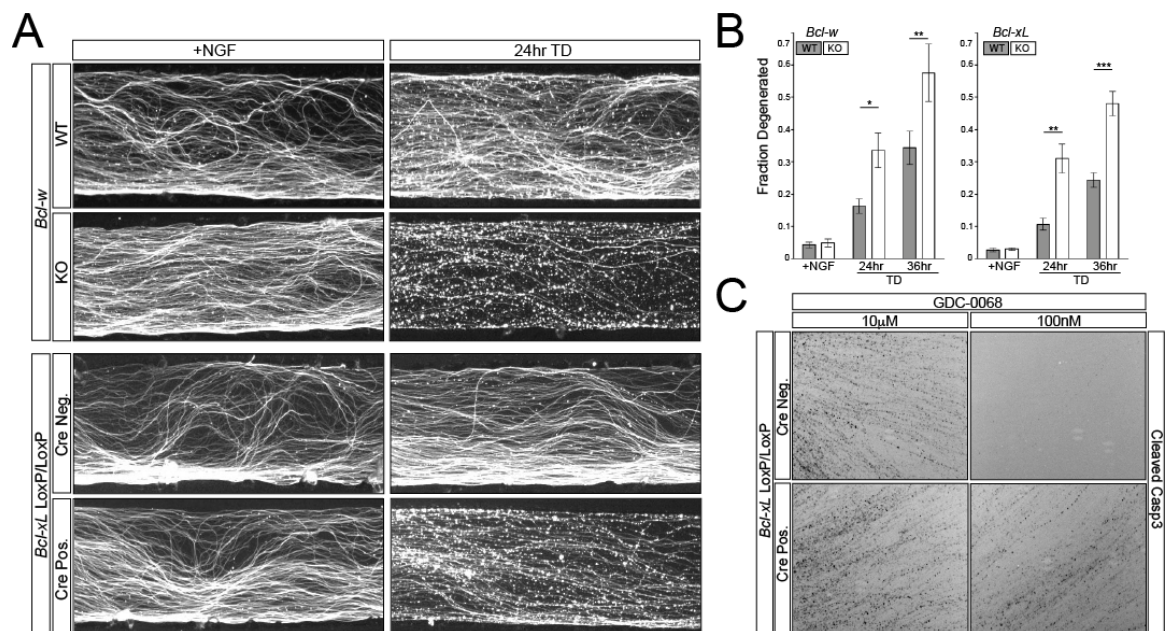
outside of overexpression experiments in this context, as a compelling candidate to regulate Puma activity. This was supported by immunoprecipitation experiments showing increased Bcl-xL and Puma binding after TD (Figure 3.8E), possibly as a result of increasing Puma levels (Figure 3.6A).



We decided to focus our analysis on Bcl-xL and Bcl-w in light of the results above and for three additional reasons: they are the only ones detected in axons; Mcl-1 is not targeted by ABT-737; and the Bcl-2-specific inhibitor ABT-199 (Souers et al., 2013) did not induce axon degeneration in our assays (data not shown). Loss of *Bcl-w* accelerated axon degeneration in response to TD in both whole sensory explant cultures and in Campenot chambers (Figure 3.9 A,B, 3.10A,B), consistent with previous findings (Courchesne et al., 2011). Surprisingly, loss of *Bcl-xL* resulted in a comparable phenotype, suggesting a shared role in the regulation of axon survival (Figure 3.9A,B, 3.10A,B). As expected from these results, genetic loss of *Bcl-xL* similarly sensitized axons to Akt inhibition (Figure 3.10C), as did genetic loss of *Bcl-w* (data not shown). Thus, Bcl-xL and Bcl-w each individually antagonize proapoptotic signaling that drives induced axon degeneration.



**Figure 3.9 Genetic deletion of *Bcl-w* or *Bcl-xL* enhances axon degeneration in explant cultures after TD.** Embryonic DRG cultures from the indicated genotypes were subjected to TD. Cultures were visualized by immunostaining for TuJ1 (A) and quantified (B).  $n=3$  *Bcl-xL*<sup>loxP/loxP</sup>; Nestin::Cre and 4 for *Bcl-xL*<sup>+/+</sup> Cre positive and Cre negative embryos.  $n=3$  WT and 4 KO embryos for *Bcl-w*. Values are presented as mean  $\pm$  SEM; \* $p<.05$ ; \*\* $p<.01$ ; \*\*\* $p<.001$  by ANOVA with Bonferroni tests.



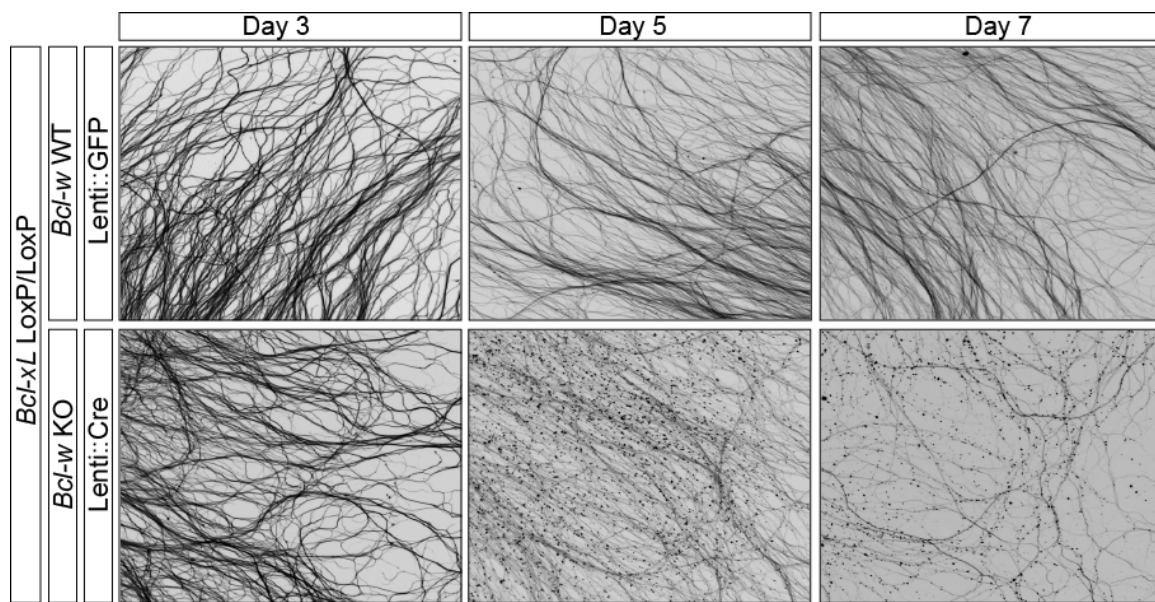
**Figure 3.10 Bcl-xL and Bcl-w regulate axon survival.**

(A and B) Axon degeneration following TD is accelerated by genetic deletion of *Bcl-xL* or *Bcl-w*. Embryonic DRGs from the indicated genotypes were cultured in Campenot chambers and the axonal compartment was subjected to TD for the indicated time points. Axon degeneration was visualized by TuJ1 immunostaining (A) and quantified (B).  $n=3$  +NGF,  $n=3$  24hr TD, and  $n=3$  36hr TD for *Bcl-xL*<sup>loxP/loxP</sup>.  $n=6$  +NGF,  $n=5$  24hr TD, and  $n=6$  36hr TD for *Bcl-xL*<sup>loxP/loxP</sup>; Nestin::Cre.  $n=9$  +NGF,  $n=8$  24hr TD, and  $n=9$  36hr TD for *Bcl-w* WT.  $n=9$  +NGF,  $n=8$  24hr TD, and  $n=9$  36hr TD for *Bcl-w* WT.  $n=6$  for all time points for *Bcl-w* KO. (C) Conditional deletion of *Bcl-xL* sensitizes axons to Akt inhibition. 7 DIV Embryonic DRG cultures from *Bcl-xL*<sup>loxP/loxP</sup> and *Bcl-xL*<sup>loxP/loxP</sup>; Nestin::Cre embryos were treated with the indicated concentrations of Akt inhibitor for 12hr. Caspase-3 cleavage was visualized by immunostaining. Values are presented as mean  $\pm$  SEM; \* $p<.05$ ; \*\* $p<.01$ ; \*\*\* $p<.001$  by ANOVA with Bonferroni tests.

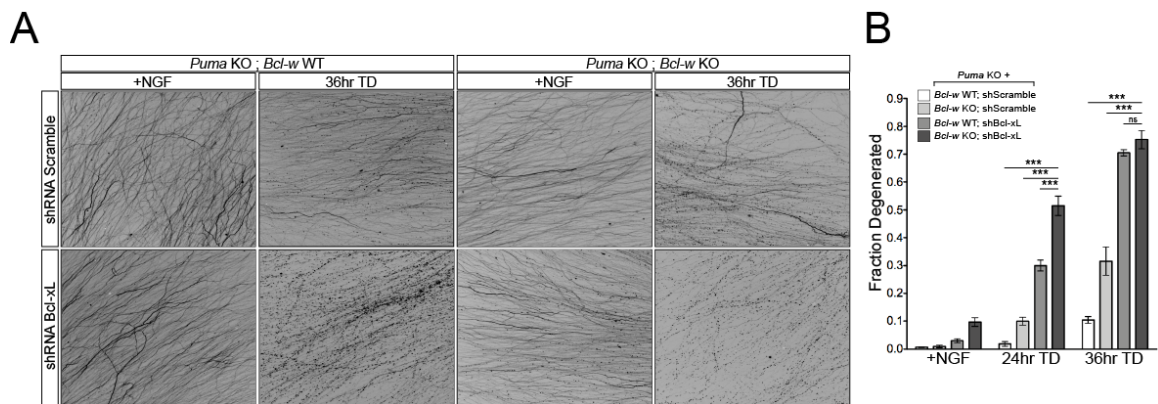
We next examined the effect of removing both *Bcl-xL* and *Bcl-w*. Strikingly, combined genetic deletion of the two caused gradual axon degeneration even in the presence of NGF (Figure 3.11), such that after 7 days in culture axons no longer survive. What causes this degeneration? The basal expression of Puma in sensory neurons in the presence of NGF distinguishes these neurons from many other cell types where Puma is absent until it is induced by cellular stress (Yu and Zhang, 2009). We therefore tested whether Puma was responsible for the degeneration. Consistent with this possibility, we found that the progressive degeneration observed in the presence of NGF following combined loss of *Bcl-xL* and *Bcl-w* is suppressed by further genetic deletion of *Puma* (Figure 3.12) (in this experiment, *Puma* and *Bcl-w* were deleted genetically, and *Bcl-xL* was knocked down by shRNA, because of the difficulty of obtaining triple knockout mice). That *Puma* is epistatic to *Bcl-xL* and *Bcl-w* in the presence of NGF indicates both that basally expressed Puma is functional, and that a physiological role of the anti-apoptotic proteins in this context is to suppress basal Puma activity. This finding also supports a role for Puma as an apoptotic activator even in the presence of NGF (albeit one that is normally inhibited by Bcl-xL/w).

Finally, we examined the effect of TD on neurons with combined loss of *Bcl-xL*, *Bcl-w* and *Puma* (we could not study the effect of TD on *Bcl-xL*;*Bcl-w* double knockouts because they degenerated spontaneously: Figure 3.11). Interestingly, degeneration was still observed after TD (Figure 3.12). A likely

possibility is that this residual degeneration reflects the action of the parallel Bax-activating factor that provides residual pro-degenerative activity in the absence of Puma (see above), and which we therefore infer is also inhibited by Bcl-w and Bcl-xL (since it is revealed in their absence).



**Figure 3.11 Combined deletion of *Bcl-w* and *Bcl-xL* initiates axon degeneration in the presence of NGF.** Embryonic DRGs from indicated genotypes were grown as dissociated reaggregated cultures and transduced with lentivirus expressing CRE or GFP. Axon degeneration was visualized by immunostaining for TuJ1 at 3, 5, and 7 days post- infection.



**Figure 3.12 Combined elimination of *Bcl-w* and *Bcl-xL* is epistatic to *Puma* deletion after TD.** *Puma* KO or *Puma* KO; *Bcl-w* KO dissociated reaggregated DRG cultures were subjected to lentiviral-shRNA knockdown of *Bcl-xL* or Scrambled control. Following TD, axon degeneration was visualized by immunostaining for TuJ1 (A) and quantified (B).  $n=2$  for all *Puma* KO conditions and  $n=4$  for all *Puma* KO; *Bcl-w* KO conditions.

Values are presented as mean  $\pm$  SEM; \*\*\* $p < .001$  by ANOVA with Bonferroni tests.

## Conclusions

Here we identify the pro-apoptotic Bcl-2 family member Puma as the central mediator of axon degeneration after TD. Foxo3a and JNK/c-jun dependent upregulation of this protein is the key regulated step of this process. Surprisingly, Puma is localized exclusively to the cell body where its pro-degenerative capacity is inhibited by the anti-apoptotic Bcl-2 proteins Bcl-xL and Bcl-w, themselves expressed in both the cell body and axon. The somatic restriction of Puma, combined with the fact that axons with combined loss of *Bcl-xL*, *Bcl-w* and *Puma* still degenerate after TD, implies the existence of an additional somatically derived pro-degenerative factor, distinct from Puma, that moves in an anterograde fashion down the axon after TD.

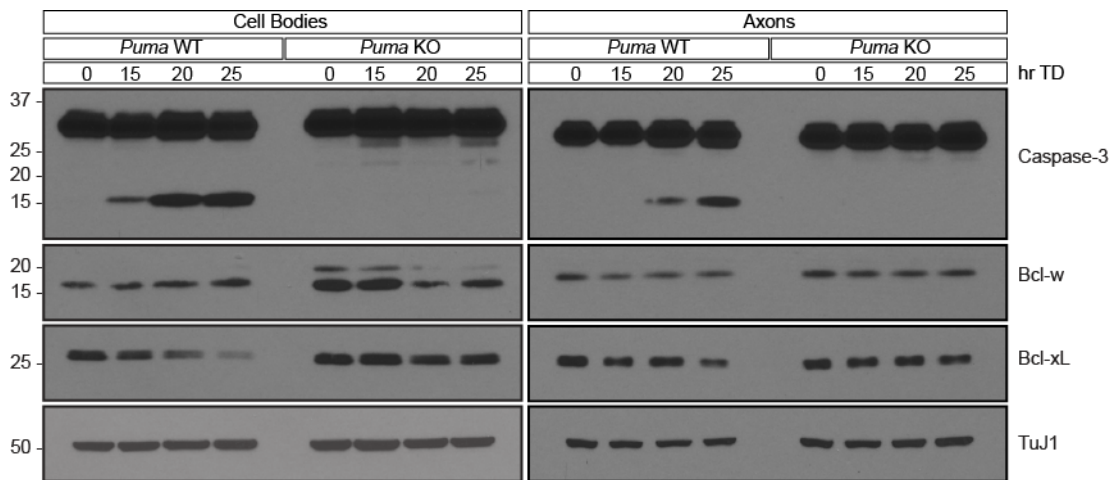
## **Chapter 4: A somatically-derived pro-degenerative signal initiates caspase-dependent axon degeneration**

### **Puma activates a somatically-derived pro-degenerative signal**

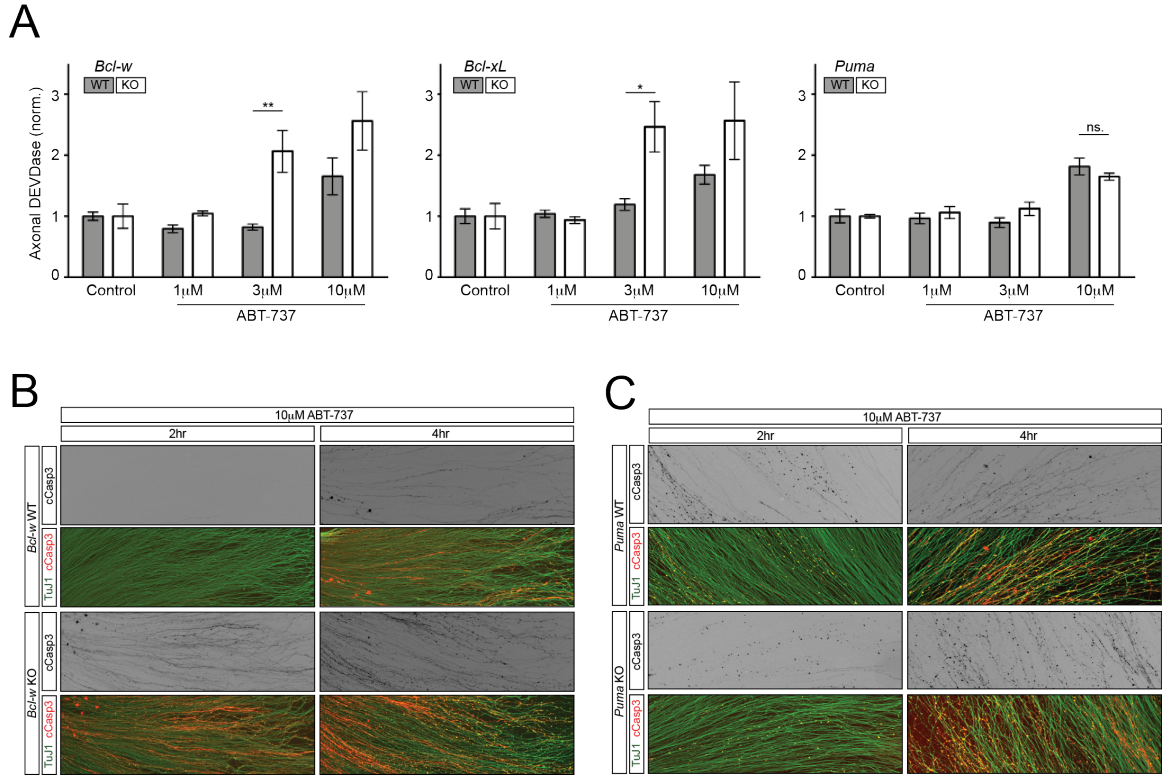
The picture that emerges from these studies is that basal Puma activity in the cell body is antagonized by Bcl-xL and Bcl-w, and that TD leads to a rise in Puma (and appearance of a second band) that overcomes this inhibition to activate an anterograde pro-degenerative signal that travels down the axon. We argued above that this signal was not likely to be Puma itself because it is not detectable in axons (Figure 3.6A,B). Several additional lines of evidence reinforced this conclusion.

First, axons express abundant Bcl-xL and Bcl-w that could inhibit any low levels of Puma that might be present (Figure 3.8A), and although this expression declined after TD, the decrease was very modest, an effect that was not altered by *Puma* deletion (Figure 4.1), so that even after TD both remain abundant and presumably able to block any low level of Puma in axons. (Note: The decline in Bcl-xL in the cell body observed here is larger than that seen in Figure 3.8B possibly because dissociated and reaggregated cultures allow better elimination of support cells which can act as a confounding factor.) Second, we used ABT-737 as a probe of the balance of pro- and anti-apoptotic Bcl-2 family members in the axon. In these experiments, we monitored short-term responses to ABT-737 (applied for 4 hrs). If Bcl-xL and Bcl-w function to inhibit the apoptotic pathway

within axons, we would predict that genetic deletion of either factor should sensitize the axons to activation of this pathway by ABT-737. Likewise, if Puma functions to promote the apoptotic pathway within axons, we would predict that loss of *Puma* would make the axons less sensitive to ABT-737. We found that axons lacking either *Bcl-xL* or *Bcl-w* were indeed more sensitive to Caspase activation by ABT-737 across a range of doses, as assessed by DEVDase activity, whereas deletion of *Puma* did not alter axonal sensitivity (Figure 4.2A). Accordingly, axons lacking *Bcl-xL* or *Bcl-w* displayed earlier Caspase activation in response to a single dose of ABT-737, whereas those lacking *Puma* did not display a delay (Figure 4.2B,C). These results thus support a direct role for Bcl-xL and Bcl-w within axons, but also support a lack of direct role for Puma in axons.



**Figure 4.1 Behavior of Bcl-xL and Bcl-w proteins in *Puma* KO.** *Puma* WT and KO DRGs were grown as dissociated and reaggregated cultures for 7 days and subjected to TD for the indicated times. Axons and cell bodies were harvested independently and the behavior of Bcl-w and Bcl-xL proteins were analyzed by immunoblot.

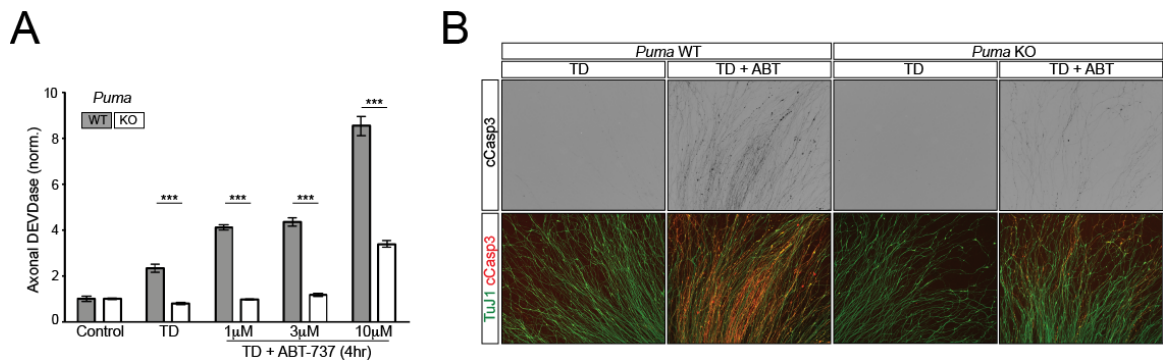


**Figure 4.2 Bcl-2 family members affect axonal response to ABT-737.**

(A) Genetic deletion of *Bcl-xL* or *Bcl-w* enhances caspase-like activity in axons treated with ABT-737, but deletion of *Puma* does not. Embryonic DRGs cultures from the indicated genotypes were treated with a range of ABT-737 concentrations. Cell bodies were removed at the end of the treatment and axonal DEVDase activity was measured using the Caspase-Glo-3/7 assay.  $n=4$  for all conditions except 10  $\mu$ M in *Bcl-xL* WT, 3  $\mu$ M in *Bcl-xL* KO, and 10  $\mu$ M in *Bcl-w* WT where  $n=3$ . Values are normalized to the average value of the control condition for each respective genotype. (B) Genetic deletion of *Bcl-w* accelerates Caspase activation after ABT-737 application. *Bcl-w* KO DRG cultures in the presence of NGF were treated with 10  $\mu$ M ABT-737 for the times indicated and Caspase-3 activation was visualized by immunofluorescence. (C) *Puma* does affect Caspase activation after ABT-737 application. *Puma* KO DRG cultures in the presence of NGF were treated with 10  $\mu$ M ABT-737 for the times indicated and Caspase-3 activation was visualized by immunofluorescence. Values are presented as mean  $\pm$  SEM; \* $p<.05$ ; \*\* $p<.01$  by ANOVA with Bonferroni tests.

Next, we returned to potentiation of the axonal response to ABT-737 by short-term TD (Figure 2.4C,D). Since this potentiation is dependent on the cell body, we predict that it should require Puma activity. Indeed, genetic deletion of *Puma* abolished the potentiating effect of TD on the axonal response to ABT-737 (Figure 4.3). This finding reinforces that Puma is capable of regulating axonal sensitivity but appears to do so at the level of the cell body.

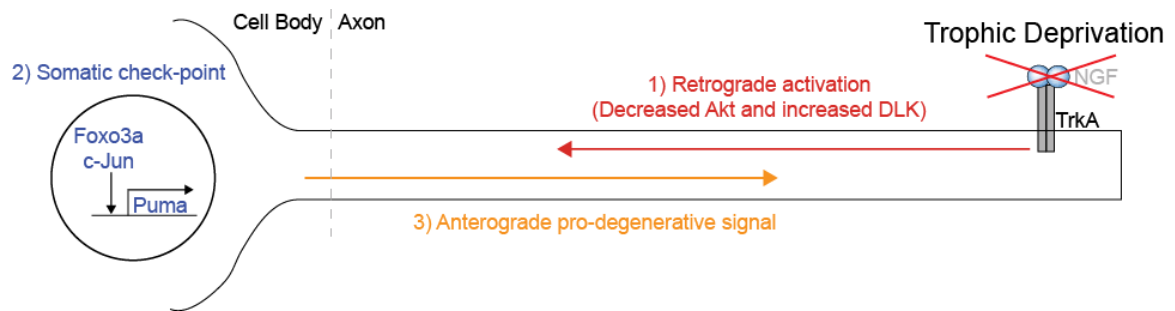
Collectively, these results further support the view that Puma functions in the cell body to activate an anterograde pro-degenerative signal that is distinct from Puma itself (Figure 4.4).



**Figure 4.3 Axonal sensitization to ABT-737 after TD requires Puma.**

Embryonic DRGs cultured from *Puma* WT and KO embryos were treated with ABT-737 for 4hr in the following 2hr TD (for a total of 6hr TD) as indicated. Following treatment, either cell bodies were removed and axonal DEVDase activity was measured with the Caspase-Glo- 3/7 reagent (A) or Caspase-3 activation was visualized by immunostaining (B).  $n=4$ . Values are normalized to the average value of the control condition for each respective genotype.

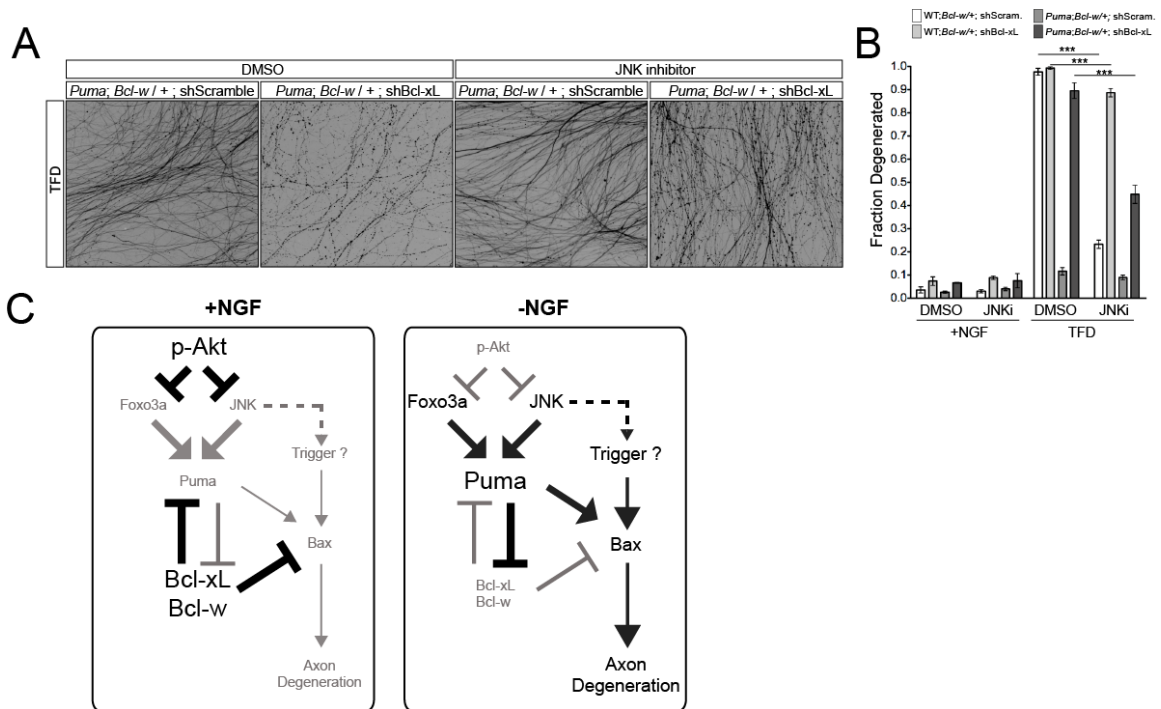
Values are presented as mean  $\pm$  SEM; \*\*\* $p<.001$  by ANOVA with Bonferroni tests.



**Figure 4.4 Model for the role of the cell body in the initiation of axon degeneration.**

### **The somatically-derived pro-degenerative signal is JNK dependent**

To gain mechanistic insight into the nature of the the somatically derived pro-degenerative signal we returned to the result that axons with combined loss of *Bcl-w*, *Bcl-xL*, and *Puma* are healthy in the presence of NGF but still degenerate after TD (Figure 3.12). As mentioned above, this supports a model whereby an additional pro-degenerative factor, normally regulated by *Bcl-xL* and *Bcl-w* and de-repressed by *Puma*, is activated by TD. Since JNK signaling is critical to Puma upregulation seen after TD (Figure 3.5A), we sought to examine if the additional pro-degenerative is dependent on this pathway. Pharmacological inhibition of the JNK suppressed the degeneration observed (Figure 4.5A.B), suggesting that indeed, the JNK signaling pathway regulates the activity of this pro-degenerative triggering factor. This further fills in the emerging picture of the molecular pathway involved in regulating axon degeneration (Figure 4.5C).



**Figure 4.5 Activity of the anterograde pro-degenerative signal is regulated by JNK.**

(A and B) *Puma*<sup>-/-</sup>; *Bcl-w*<sup>+/-</sup> embryonic DRG dissociated reaggregated cultures were subjected to lentiviral-shRNA knockdown of Bcl-xL or Scrambled control. Cultures were then subjected to TFD in the presence of 10μM JNK inhibitor VIII or DMSO control. Axon degeneration was visualized by immunostaining for TuJ1 (A) and quantified (B).. n=4 for all conditions.

(C) Model of the molecular pathway involved in axon degeneration after TD. In the presence of NGF, Akt inhibits Foxo3a and JNK/c-jun activity, which keeps Puma levels low and ensures that Bcl-xL and Bcl-w can inhibit Bax and prevent degeneration. After TD, Akt inhibition of Foxo3a and JNK/c-jun is abolished, leading to increased Puma levels that inhibit Bcl-xL and Bcl-w in the cell body while also potentially activating Bax. Simultaneously, JNK signaling regulates the activity of the anterograde signal, which initiates axon degeneration in a Puma dependent manner.

### **P53 regulates axon degeneration independent of Puma transcription**

Expression of several critical mediators of the mitochondrial apoptotic program are regulated by the transcription factor p53, including *Bax*, *Apaf1*, and *Puma* (Beckerman and Prives, 2010). Given this, we sought to determine the function of *p53* in axon degeneration after TD and found that deletion of this gene delayed axon degeneration both in whole explant culture and after TD of distal axons in Campenot chambers (Figure 4.6A, B). Surprisingly, this robust protection was not due to reducing *Puma* expression after TD, as both WT and KO cultures showed similar expression (Figure 4.6C). *Bax* can also be directly activated by p53, acting in a non-transcriptional role highly reminiscent of pro-apoptotic Bcl-2 family members (Chipuk et al., 2004). Of note, the pro-apoptotic function of p53 in this context is known to be tightly regulated by Bcl-xL binding, which itself can be disrupted by *Puma* (Chipuk et al., 2005; Follis et al., 2013; Xu et al., 2006). As both *Puma* and *Bcl-xL* are essential components of the pathway outlined in this study, we reason that the protection observed by genetic deletion of *p53* may be due to its non-transcriptional role and, further, it may in fact be the anterograde pro-degenerative factor described above. In accordance with this, p53 KO explants did not show a noticeable reduction in Caspase-3 activation in cell bodies after TD but did show a striking reduction in axonal Caspase-3 activity and Bid cleavage (Figure 4.6C,D). We cannot completely rule out a transcriptional role for *p53* in this process, however the phenotype observed is unlikely to be a result of decreased *Bax* expression because axons of DRG

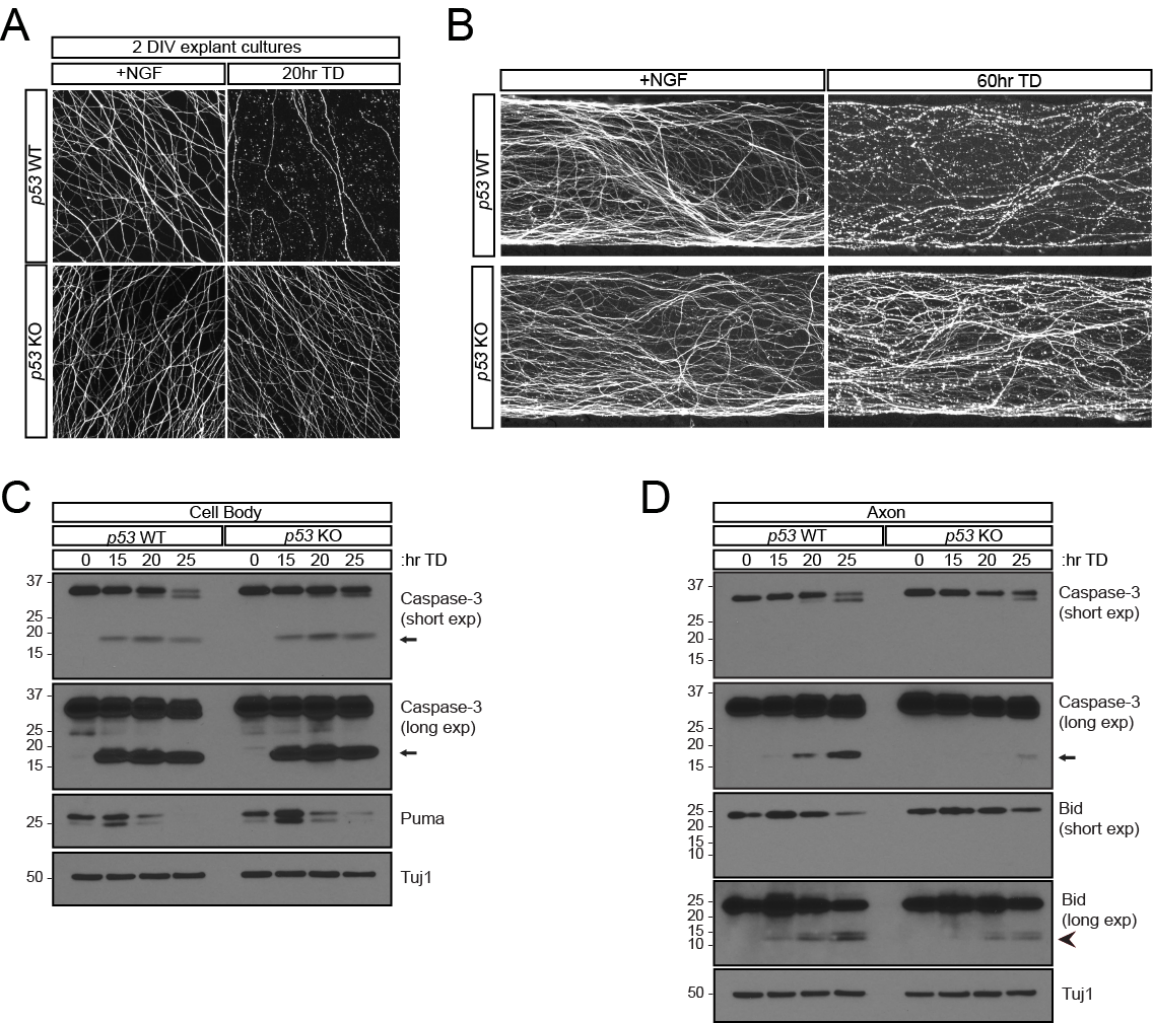
**Figure 4.6 Genetic deletion of *p53* protects axons after TD**

(A) *p53* KO protects against axon degeneration in whole explant culture after TD. Embryonic DRG cultures from the indicated genotypes were grown for 2 days and subjected to TD for indicated times. Axon degeneration was visualized by immunostaining for TuJ1.

(B) Axon degeneration following TD requires Puma. *P53* WT or KO embryonic DRGs were cultured in Campenot chambers and the axonal compartment was subject to TD for the indicated times. Degeneration was visualized by immunostaining for TuJ1.

(C and D) *p53* KO reduces Caspase-3 activation in axons after TD, but does not affect *Puma* expression. *p53* WT and KO DRGs were grown as dissociated and reaggregated cultures for 7 days and subjected to TD for the indicated times. cell Cell bodies (C) and Axons (D) were harvested independently and analyzed by immunoblot.

explant cultures heterozygous for *Bax* are not protected from TD (data not shown), but the effect may still be caused by reduced expression of other proapoptotic proteins such as *Apaf1*. Nonetheless this result does position *p53* as a compelling candidate for the as yet unidentified factor downstream of *Puma*.



## Conclusions

We demonstrate that rising Puma levels in response to TD overcome inhibition from Bcl-xL and Bcl-w to activate an anterograde pro-degenerative signal that travels down the axon. Genetic deletion of *Puma* does not make axons less sensitive to ABT-737, further arguing against it having a direct pro-apoptotic role within the axon. However, Puma is required for the potentiation of axonal ABT-737 response that results from short-term TD, demonstrating a role for Puma in regulating axonal sensitivity at the level of the cell body. The additional anterograde factor implied by these results is partially JNK dependent. We show that p53 regulates TD induced distal axon degeneration, but not by reducing Puma expression. As p53 is known to have a non-transcriptional role as a direct Bax activator and genetic deletion of *p53* appears to reduce TD-induced Caspase-3 activation specifically in the axon, it represents a promising candidate for the additional factor.

## Chapter 5: Discussion and Future Directions

A prevailing view in the field has been that axon degeneration is a local event confined to the specific axon that loses neurotrophic support (Luo and O'Leary, 2005; Neukomm et al., 2014; Pease and Segal, 2014; Schuldiner and Yaron, 2015). This view is supported indirectly by observations in the clinical literature that axon degeneration precedes overt cell loss in a range of neurodegenerative conditions. Here we challenge this view and show that, during developmental sensory axon death in response to distal trophic deprivation, signaling via the cell body is an integral component of the molecular pathway that initiates axon degeneration. We show that the apoptotic machinery is present in the axon but that its activity is gated by an active process in the cell body which can be broken down into three discrete steps following distal TD: (1) retrograde signaling to the cell body (2) the key regulated step of Foxo3a-mediated upregulation of Puma in the cell body, enabling it to overcome inhibition by Bcl-xL and Bcl-w, and initiate the degenerative program, and (3) the ultimate triggering of axon degeneration by an as yet unidentified somatically-derived factor distinct from Puma that moves down the axon (Figure 4.4). By integrating distinct signaling pathways that originate within the axon, the cell body functions as a key check-point to initiate axon degeneration.

## **Retrograde signaling converges to increase Puma expression**

In chapter 2, we first confirmed previous studies by showing that in axons separated from their cell bodies, TD cannot induce Caspase-3 activation (Gerdt et al., 2013). However, through use of the Bcl-2 antagonist ABT-737, we established that axons do in fact contain a full complement of the apoptotic machinery, as it can be activated exogenously. Furthermore, TD can potentiate the effect of Bcl-2 inhibition, but only in axons retaining their cell body connection, positioning the cell body as a key regulator of the axonal apoptotic pathway.

The cessation of TrkA kinase activity after TD results in a retrograde pro-degenerative signal communicated to the cell body that is coordinated by loss of axonal Akt signaling. Constitutive activation of Akt1 or deletion of its negative regulator PTEN strongly protect axons from TD-induced degeneration. Akt is a multifunctional kinase with a number of well-documented roles in the survival and proliferation of various tissues. This study demonstrates that it suppresses the activity of two central pro-degenerative signaling pathways; namely the DLK/JNK/c-Jun pathway and the Foxo3a-dependent transcriptional program which, as we show in chapter 3, upregulates pro-apoptotic *Puma* expression.

Loss of Akt signaling activates the DLK/JNK/c-Jun pathway culminating in pro-apoptotic MAPK signaling and activation of c-Jun. Interestingly, the loss of Akt activity after TD is slowed in the *DLK* knockout, suggesting a feedback loop back from DLK to Akt that accelerates inactivation of Akt in wild-type axons (Figure 2.10). A similar feedback loop exists in neuronal apoptosis induced by

arsenite (Wong et al., 2005) suggesting this could be a general phenomenon. Whether DLK inhibition and Akt are directly linked is unknown, but DLK does contain a number of Akt consensus sites that are inhibitory (Wu et al. 2015). Future studies aimed at introducing DLK mutations into those sites could establish direct phosphorylation of DLK by Akt as a central regulatory node in axon degeneration.

As mentioned above, the previous view in the field was that local JNK signaling, independent of the cell body, was responsible for initiating TD-induced axon degeneration. This model was supported by a result showing that genetic deletion of *c-Jun* in neurons protected cell bodies but not axons (Ghosh et al., 2011). We demonstrate that finding is incorrect, as more efficient excision of *c-Jun* in fact protects both axons and cell bodies. Further, specifically inhibiting JNK in the cell body provides even more robust protection against TD-induced degeneration than axonal JNK inhibition (Fig 2.8). Thus, we prove that pro-degenerative DLK/JNK signaling functions as a retrograde signal to the cell body with the ultimate role of activating the *c-Jun* transcriptional pathway. The genes that are regulated by this process remain to be elucidated although there is reason to believe Puma is one of the primary targets, as c-Jun is a known Puma transcriptional activator (Zhao et al., 2012). JNK/c-Jun dependent regulation of Puma has also been demonstrated in models of apoptosis after potassium withdrawal of cerebellar granule neurons or  $\beta$ -amyloid treatment of cortical neurons (Akhter et al., 2015; Ambacher et al., 2012). Although we do show JNK

inhibition decreases the Puma response after TD (Fig 3.5A), future experiments examining this in *c-Jun* mutant neurons are necessary. In addition, although we demonstrate that the previous cell body independent model is incorrect, we have not completely ruled out a role for local JNK signaling in the axon. Indeed, axonal JNK inhibition provides a modest axonal protection (Fig 2.8), but whether this is because it interferes with the retrograde signal moving back to the cell body or because it suppresses a local pro-degenerative signaling program in the axon remains to be determined.

The second function of Akt signaling is to suppress Foxo3a-dependent Puma upregulation. We show that Foxo3a is dephosphorylated upon distal TD and migrates to the nucleus where it is required to increase Puma expression (Figure 2.10, 2.11, 3.5C). A similar role for Akt/Foxo3a pathways in Puma induction has been described in the context of apoptotic cell body death of cerebellar granule neurons deprived of extracellular potassium (Ambacher et al., 2012). Interestingly, somatic GSK3 $\beta$ , a known Akt target (Cross et al., 1995), has also been implicated in promoting Puma expression in cerebellar neurons (Ambacher et al., 2012) and in sensory axon degeneration following TD (Wu et al., 2015), so it might contribute to the Puma induction we describe here. Future experiments could fully examine the role of these pathways by combining deletion/chemical inhibition of all three pathways and determining if this fully abolishes Puma expression in this system.

## **Puma drives a pro-degenerative cascade in the cell body**

In chapter 3 we show that upregulation of Puma defines the key regulated step in the initiation of axon degeneration. Genetic deletion of *Puma* potentially protects axons after TD (Figure 3.4) highlighting its central function in the degenerative cascade. Surprisingly, Puma is expressed in healthy neurons even in the presence of trophic support (Figure 3.5A) (unlike in many other cell types where it is absent until induced by stress), but its pro-degenerative activity is blocked by pro-survival Bcl-xL and Bcl-w. The most notable characteristic of the rise in *Puma* after TD is the appearance of a second lower-molecular weight band, whose identity is currently unknown. A Puma doublet is not observed in the vast majority of studies, but it is upregulated in sympathetic neurons after DNA damage (Wytenbach and Tolkovsky, 2006), suggesting it could potentially be a neuronal specific isoform. To gain insight into this peptide, RNAseq could be utilized to identify unique *Puma* transcripts that increase after TD with a predicted lower molecular weight. Once identified, this Puma transcript could be cloned to examine its Bcl-xL and Bcl-w binding affinity relative to that of the heavier Puma isoform. Further, its ability to induce Bax activation could be tested using *in vitro* mitochondrial permeabilization assays as has been done in other systems (Cartron et al., 2004; Letai et al., 2002).

Puma is known to be post-translationally regulated by phosphorylation of serine-10. This event reduces Puma stability and Puma containing a serine to alanine mutation in this residue has increased apoptotic potential (Fricker et al.,

2010). We show that only the upper Puma band is expressed in DRGs in the presence of NGF, which would fit with it being phosphorylated. Further, the short half-life of Puma in DRGs (Figure 3.7), makes the regulation of its stability by phosphorylation an intriguing possibility. Treatment of sensory neurons lysates with phosphatase could determine if the upper Puma band is this unstable, phosphorylated form, as western blots would show the distinct upper and lower bands collapsing to one lower band. In this scenario, studies uncovering the kinase responsible for the phosphorylation and how this leads to inhibition could uncover another striking level of regulation in this process.

### **The identity of the anterograde pro-degenerative factor**

Following TD, the rise in Puma levels overcomes the inhibition of Bcl-w and Bcl-xL, leading to the activation of an anterograde pro-degenerative signal that travels down the axon. Our results imply that this signal is distinct from Puma itself, as Puma is only detectable in the cell body, even after TD, as assessed using two independent techniques (Figure 3.6). While it remains possible that a small amount of Puma (below the threshold of detection) is present in axons, it would be neutralized by the abundant Bcl-xL and Bcl-w present there, so any Puma within axons is unlikely to account for the large degenerative phenotypes documented here. The identity of the anterograde pro-degenerative signal remains to be determined. Our results also imply that to activate the Caspase pathway this signal must overcome the antagonistic effects of Bcl-xL and Bcl-w

within the axons. Further, its activity is regulated by JNK signaling, showing that it shares a common molecular pathway with TD-induced Puma expression.

In thinking of the nature of this factor, several distinct possibilities exist, which are outlined below:

*Model 1: Activated Caspase-3 is the triggering factor*

The first possibility is that the anterograde triggering factor is activated Caspase-3 itself, normally produced by somatic activation of the mitochondrial apoptotic pathway in a Puma dependent manner. In support of this, Caspase-3 activation in the cell body precedes axonal Caspase-3 activation in explants subjected to TD (Figure 3.6). Caspases are increasingly recognized for their non-apoptotic roles in shaping cell physiology and morphology (Hyman and Yuan, 2012; Kuranaga and Miura, 2007), suggesting that sub-apoptotic Caspase-3 activity may be able to induce neuronal pruning. This model requires the existence of an additional pro-apoptotic factor operating in the cell body in a redundant manner to Puma, because genetic deletion of *Puma* does not provide equivalent protection to *Bax* knockouts and neurons lacking Puma, Bcl-w, and Bcl-xL still degenerate after TD (Figure 3.12). It is possible that the Puma-redundant factor is one of the other pro-apoptotic Bcl-2 family members that is expressed in the cell body (i.e. Bim or Bid), possibly kept in check by Mcl-1 or Bcl-2. Lentiviral knockdown of these additional pro-apoptotic Bcl-2 family members on top of cultures depleted of Puma, Bcl-w, and Bcl-xL could serve to identify this factor.

To test whether Caspase-3 is the triggering factor, two crucial experiments could be performed. The first would be to examine if low-level Caspase-3 activity is observed in the cell bodies of neurons whose distal axons are subjected to TD in Campenot chambers, instead of depriving whole explants, which also results in apoptosis. Although studies have claimed that an increase in cell death is not seen after distal axon deprivation (Campenot, 1982; Campenot et al., 1991; Mok et al., 2009), Caspase-3 activity has never been assessed. Because only a minority of the neurons plated in the center cell body compartment will be connected to axons that successfully cross the grease barrier into the axonal compartment, Alexafluor conjugated Cholera toxin subunit B (CTB) could be added to the axon compartment to retrogradely label the cell bodies connected to those axons. Axons could then be subjected to distal TD and Caspase-3 activation in the CTB labeled cell bodies could be assessed by immunofluorescence. Increased cleaved Caspase-3 immunoreactivity would support a model whereby Caspase activity originating in the cell body flows out to the axon, resulting in degeneration. Nuclear morphology of these neurons could also be assessed to ensure that axon degeneration in this context is not merely a consequence of somatic death.

Caspase-3 activity appears to radiate out from cell bodies in explants subject to TD (Figure 1.8), but to truly argue that this represents the movement of active Caspase-3, one would need to exclusively track the movement of cleaved Caspase-3 and not the zymogen. In an ideal scenario, a photoconvertible

fluorescent tag such as mEOS (McKinney et al., 2009) could be attached specifically to cleaved Caspase-3. The tag could be activated in cell bodies of neurons in Campenot chambers subject to axonal TD and one could use live imaging to determine if the radial pattern of Caspase-3 activity is the result of movement of cleaved Caspase-3 from the cell body to the axon. However, this method cannot be used because there is currently no way to tag cleaved Caspase-3 without tagging the zymogen as well, making it impossible to conclude that movement of the fluorescent tag truly represents the movement of proteolytically active Caspase-3. A similar issue would plague the use of fluorescent reporters used for live imaging of Caspase-3 activity (Bardet et al., 2008; Zhang et al., 2013). In this case, live imaging of DRGs in Campenot chamber subject to axonal TD may reveal Caspase activity, as assessed by Caspase induced fluorescence, moving from the cell body distally down the axon. However, whether this represents the movement of proteolytically active Caspase-3 or some other factor that is inducing Caspase-3 cleavage as it moves distally down the axon would be impossible to determine, making this model extremely difficult to test with the tools currently available.

#### *Model 2: Cytochrome c is the triggering factor*

Cytochrome c, a central component in the mitochondrial apoptotic program, is a second possible candidate for the anterograde pro-degenerative signal. Waves of cytochrome c have been observed to propagate across other

cell types to activate the apoptotic program (Garcia-Perez et al., 2012; Lartigue et al., 2008), raising the possibility that as in the Caspase-3 model proposed above, a cytochrome c wave – one unable to induce cellular death – may be flowing out into the axons and nucleating the apoptosome. This model similarly requires the existence of a Puma-redundant factor and would predict that cytochrome c concentration in axons would increase after TD, which could be observed by Western blot of isolated axons or through immunofluorescence using a fine time course. Employing the mEOS strategy detailed above (tagging cytochrome c in this case) would also enable live imaging of cytochrome c movement in axons subject to TD.

*Model 3: An additional Bax activator is the triggering factor*

Finally, there is a distinct possibility that the anterograde signal is a Bax activating factor distinct from Puma, but dependent, at least in part, on its upregulation. In this model, the TD-induced Puma increase releases this factor from Bcl-xL and Bcl-w inhibition. It is possible that this protein is basally expressed or newly synthesized in response to TD, with its activity being partially JNK dependent in either scenario (Figure 4.5A, B). This model would posit several key characteristics that potential candidates should possess: (1) identity as a Bax activator; (2) the ability to bind to and be inhibited by Bcl-xL and/or Bcl-w; (3) observable movement from the cell body to the axon in response to TD; and 4) a protective phenotype when genetically deleted.

Future studies could potentially identify this factor utilizing a candidate knockdown approach on top of a *Puma* knockout, which should, in theory, produce a Bax-like protective phenotype. The classical apoptotic regulator p53 is a particularly promising candidate, as we have shown it delays axon degeneration after TD by a mechanism independent of transcriptional activation of *Puma* (Figure 4.6). Furthermore, it can act as a direct Bax activator in a manner inhibited by binding Bcl-xL (Chipuk et al., 2004; Xu et al., 2006). This inhibitory binding of p53 to Bcl-xL is specifically disrupted by Puma with other pro-apoptotic Bcl-2 family members being much less effective (Chipuk et al., 2005; Follis et al., 2013). Intriguingly, loss of p53 does not seem to affect Caspase-3 activation in cell bodies of explants subject to TD, but it does have a noticeable effect on axonal Caspase-3 activation (Figure 4.6C,D). It remains possible that p53 could still be influencing axon degeneration by transcriptional regulation of other targets. RNAseq experiments could potentially address this possibility. Additionally, if p53 protein moves out to the axon following TD, it would argue for a more direct role.

Bid represents another compelling target for an anterograde signal within this model. First, it is the only pro-apoptotic Bcl-2 family member that can be observed in axons (Figure 3.2B). Secondly, cleavage of Bid to form the more apoptotically active isoform tBid is seen in both cell bodies and axons after TD (Figure 3.2C,D). Bid cleavage can be mediated by Caspase-3 in a non-canonical process (Gross et al., 1999), so a mechanism can be envisioned whereby Puma

triggers sub-apoptotic somatic Caspase-3 activity, resulting in the formation of tBid that then flows out to the axon. The fact that *Bid* knockouts do not have a protective phenotype in whole explant culture seems to argue against its role as a triggering factor, but it remains possible that *Bid* deletion could have a phenotype in Campenot chambers where a program more specific to axon degeneration, rather than whole cell apoptosis, occurs. Furthermore, a *Bid;Puma* double knockout may have a more Bax-like phenotype than *Puma* knockout alone, which would also lend credence to this possibility.

### **Why is axon degeneration controlled by the cell body?**

Beyond defining the molecular steps leading from distal trophic deprivation to axon degeneration, our findings highlight the pivotal, active role of the cell body, which functions as a locus of control in axon degeneration in response to distal trophic factor deprivation. At a cellular/biochemical level, this central role of the cell body seems remarkable. Indeed, we find that distal trophic deprivation deactivates pro-survival Akt and activates pro-degenerative DLK signaling locally within axons (Figure 2.10) and the axons contain a fully functional apoptotic machinery (Figure 2.4). However, the upstream Akt/DLK signals are incapable of biochemically connecting to and activating the axonal caspase cascade directly within the axon, instead requiring retrograde activation of the somatic pro-degenerative program, with Puma as an unexpected somatically-restricted gating factor. These findings highlight a remarkable biochemical compartmentalization

of the upstream activator and downstream effector pathways for control of degeneration within the axon.

Why would such a mechanism exist in the neuron? At a functional level, this compartmentalization and stringent control of distal axon degeneration by retrograde activation of a somatic cell death program may provide an essential buffer, ensuring that transient local changes in trophic support do not trigger irreversible local degeneration events, and instead allowing the cell body to integrate pro-degenerative and pro-survival signals to execute axon degeneration in a global and a coordinated fashion. Indeed, as can be observed in the pruning of mammalian layer V cortical neurons, a transcriptional program is required to mediate branch specific pruning *in vivo* (Luo and O'Leary, 2005; Weimann et al., 1999). The necessity of this program is inherent in the fact that the cortical region that the neuronal cell body is located in determines which branch will be pruned, with neurons in the visual cortex pruning a different branch than those in the motor cortex (Luo and O'Leary, 2005; Vanderhaeghen and Cheng, 2010). This indicates that during development the axon's environment is not inherently degenerative, otherwise projections from both motor and visual cortical neurons in the same area would both degenerate. In this respect, having the cell body as the central arbiter ensures removal of the proper branch based on integration of all available signals.

Within systems where there is evidence of branch specific pruning by an apoptotic mechanism, such as that seen in the Superior Colliculus, how exactly a

specific branch is targeted for degeneration without initiating Caspase activation in all branches remains an open question. An intriguing option would be a mechanism whereby the cell body, after integrating pro-degenerative cues, releases an anterograde pro-apoptotic signal that is repressed in all branches except those destined to degenerate. A recent study demonstrated that this could potentially be accomplished by altering levels of anti-apoptotic proteins, such as XIAP, in response to local axonal signals (Cusack et al., 2013). In addressing these questions, future studies will not only delineate the mechanisms of developmental axon pruning, but more broadly elucidate how cells can achieve effective biochemical compartmentalization.

## Chapter 6: Materials and Methods

### Mice

Animals were bred and used according to IACUC protocols at The Rockefeller University. Embryos were harvested from pregnant dams at stage E12.5. Wild-type cultures were generated from CD1 mice. For mutant strains, comparisons between wild-type and mutant embryos derive from the same pregnant female.

The following knockout strains were used for this study:

**Table 6.1 Knockout mouse strains used in this study**

Null Allele	Reference
Bad	(Ranger et al., 2003)
Bcl-w	(Ross et al., 1998)
Bid	(Yin et al., 1999)
Bim	(Bouillet et al., 1999)
Caspase-3	(Kuida et al., 1996)
Caspase-9	(Simon et al., 2012)
Hrk KO	(Coultas et al., 2007)
Puma	(Villunger et al., 2003)

*Bid;Bim* and *Puma;Bcl-w* double knockout strains were generated by intercrossing doubly heterozygous mice.

The following conditional knockout mice were used for this study:

**Table 6.2 Conditional knockout mouse strains used in this study**

Conditional Allele	Reference
Apaf1	unpublished, generated by Dr. Zhigang He, deposited at Jackson Laboratory
Bcl-2	(Thorp et al., 2009)
Bcl-Xl	(Rucker et al., 2000)
c-Jun	(Behrens et al., 2002)
DLK	(Miller et al., 2009)
Mcl-1	(Vikstrom et al., 2010)
PTEN	(Groszer et al., 2001)

As indicated within the text, conditional knockout strains were either crossed to a Nestin-Cre driver (Tronche et al., 1999) or maintained in isolation and subsequent cultures were infected with lentiviral Cre. The following transgenic and knock-in strains were used:

**Table 6.3 Transgenic mouse strains used in this study**

Allele	Reference
Cytoplasmic-NMNAT1 transgenic	(Sasaki et al., 2009)
TrkA F592A knock-in	(Chen et al., 2005b)

### Cell culture

Sensory neuron cultures were harvested from E12.5 embryos and grown in Neurobasal media containing 2% (v/v) B27 (Life Technologies), 0.45% (v/v) Glucose, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and supplemented with 50ng/mL NGF (Promega). Unless otherwise indicated, all cultures were grown on plastic plates coated with poly-D-lysine (PDL; 100 µg/ml) and mouse natural laminin (10µg/ml, Life Technologies). Cells were treated with mitotic inhibitor (5µM 5-fluorouracil and 5 µM uridine) the morning after plating. In cases where protein was harvested, sensory neurons were dissociated (0.05% Trypsin-EDTA) and re-suspended in culture media at  $2 \times 10^4$  cells/µl. Cells were spotted (1µl) at the center of PDL-Laminin-coated 24 well plates that were air-dried. Culture media was added following a 12-15min period in a humidified incubator to allow the cells to settle. Campenot chambers were generated as previously described (Nikolaev et al., 2009). Briefly, sensory neurons from E12.5 embryos were dissociated and resuspended at a concentration of  $2.5 \times 10^6$

cells/mL in Neurobasal/B27 media containing 0.35% methylcellulose (m/v). The axonal compartment of the Campenot chambers was filled with 500 $\mu$ L methylcellulose-containing media. A grease barrier was established at the bottom on the center compartment and 60 $\mu$ L of cells were loaded into the center compartment and allowed to settle overnight before 2.5 mL of media was added to the outer dish the following morning. The following day, mitotic inhibitor was added to the outer dish only and half media changes were performed every 2 days without adding fresh mitotic inhibitor. Local degeneration assays were performed by rinsing the outer compartment of the chamber with Neurobasal media and replacing it with NB/B27 media containing NGF function-blocking antibody (50 $\mu$ g/mL).

### **Measurement of axonal DEVDase activity**

Sensory neurons were cultured both for 2 days as DRG explants in 48 well plates or for 7 days as dissociated and re-aggregated spots ( $2 \times 10^4$  cells / spot) in 24 well plates. Following treatment, the cell body region was removed with a manual punch-out for explants as previously described (Yang et al., 2013) or using a small scalpel for re- aggregated spots. Immediately after removal of the cell bodies, half of the media was removed and replaced with an equal volume of Caspase-Glo 3/7 reagent (Promega). Following a one-hour lysis at room temperature, the contents of each well was transferred to an opaque 96-well plate and luminescence was determined using a plate reader. For axon specific

measurements in neurons expressing cytoplasmic NMNAT1, axons were severed then subjected to TD, followed by lysis.

### **Lentiviral production**

Lentiviral constructs and the associated packaging plasmids psPAX2 and pMD2.G were precipitated into 293FT using calcium phosphate. After 16 hours the media was switched to serum free viral production media: Ultraculture (Lonza) supplemented with 1% (v/v) Penn-Strep/L-glututamine, 1% (v/v) 100mM Sodium Pyruvate, 1% (v/v) 7.5% sodium bicarbonate, and 5 mM sodium butyrate. 293FT supernatants were harvested at the 46-hour time point, neutralized with 1M Tris-HCl, pH 7.5 to 50mM final concentration, and filtered through 0.45 µm polyethersulfone syringe filters. Lentiviral titer was determined using an ELISA-based quantification of p24 viral coat protein according to the manufacturer's instruction (Clontech). Upon determining titer the virus was concentrated using Lenti-X concentrator (Clontech) and re-suspended in Neurobasal/B27 DRG media containing 50ng/mL NGF. Lentivirus was added to DRG cultures 1-2 days after plating at a multiplicity of infection (MOI) of 10. Lentiviral shRNA constructs in the pLKO.1 backbone were chosen from the Broad/TRC collection. For each gene of interest, 4-5 shRNA constructs were tested for efficient target knockdown by western blot. The following shRNA clones were selected for use: Bcl-xL (TRCN0000010905), Foxo3a (TRCN0000071614). Lentiviral shRNAs were produced as above without further

concentration. shRNA viruses were added at 2 days post-plating at 5-10 MOI. In all cases where lentivirus is used, cultures were harvested 7 days post-infection. Lentiviral GFP was generated using pLenti-CMV-GFP (Addgene #17445). cDNAs encoding Cre-IRES-GFP, cytoplasmic Nmnat1-(R125A/R127A), and myrAkt1 were cloned in place of GFP using standard techniques.

### **Protein harvest and Western blotting**

Axons and cell bodies were independently harvested from sensory neurons cultured as re-aggregated spots using either a biopsy punch needle or scalpel (Fine Science Tools) as previously described (Yang et al., 2013). To collect protein, cultures were first rinsed with Hank's balanced salt solution followed by direct solubilization of axons and cell bodies in an SDS/Urea-based lysis buffer containing: 50 mM Tris-HCl (pH 6.8), 8M Urea, 10 % (w/v) SDS, 10mM sodium EDTA, 50 mM DTT, supplemented with Brilliant Blue G. Axon or cell body material from 5-10 spots were pooled for each condition. Proteins were resolved on 4-15% Tris-HCl gradient gels (Bio-Rad) and blotted using standard techniques. Primary antibodies used at 1:500 except Tuj1, which was used at 1:2000. HRP conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:10,000. Proteins were detected using enhanced chemilumescence (Pierce).

## **Immunohistochemistry**

Cultures were fixed in 4% Paraformaldehyde containing 10% sucrose for 20 minutes at room temperature and either stored in PBS or immediately blocked for at least one hour at room temperature in 3-6% donkey serum in PBS containing 0.1% Triton-X- 100 (PBS-X). Slides were incubated in primary antibody overnight in 3% donkey serum in PBS-X at room temperature in a humidified chamber. All primary antibodies were used at a dilution of 1:500 except Tuj1, which was used at 1:1000. Secondary antibodies coupled to Alexa-based dyes (Life Technologies) were added at 1:500 in 3% donkey serum in PBS-X. Slides were imaged on a Nikon Eclipse 90i wide-field fluorescence microscope.

## **Bcl-xL Immunoprecipitation**

Cultures were lysed in 150 mM NaCl, 10 mM HEPES, pH 7.4, 0.3% CHAPS for 1 hour on ice. Cellular debris was eliminated by spinning lysates at 20,000xg for 5 minutes and cell were pre-cleared with protein A/G agarose beads (Santa Cruz). Protein was quantified and normalized by BCA (Pierce) and lysates were split into two equal volumes. For each condition Bcl-xL (54H6) was added at 1:250 with equivalent concentration isotype control being added to the other tube. Immunoprecipitation carried out at 4°C overnight with rotation. The following morning 20µL of protein A/G beads were added to each sample, and then samples were rotated at 4°C for 1hr. Beads were spun down at 1000xg and washed 5x in CHAPS buffer before protein was dissociated in SDS/Urea-based lysis buffer at 37°C for 30 minutes.

## Antibodies and inhibitors

**Table 6.4 Antibodies used in this study**

<b>Antibody</b>	<b>Source</b>
Bcl-w (31H4 mAb, #2724)	Cell Signaling Technology
Bcl-xL (54H6 mAb, #2764)	Cell Signaling Technology
Bcl-2 (D17C4 mAb, #3498)	Cell Signaling Technology
Puma (pAb, #7467)	Cell Signaling Technology
total Caspase-3 (8G10 mAb, #9665)	Cell Signaling Technology
cleaved Caspase-3 D175 (pAb, #9661)	Cell Signaling Technology
Phospho-Foxo3a S318/S321 (pAb, #9465)	Cell Signaling Technology
Phospho-Foxo3a S254 (pAb, #9466)	Cell Signaling Technology
total Foxo3a (75D8 mAb, #2497)	Cell Signaling Technology
Phospho-Akt T308 (C31E5E mAb, #2965)	Cell Signaling Technology
Phospho-Akt S473 (D9E mAb, #4060)	Cell Signaling Technology
total Akt (40D4 mAb, #2920)	Cell Signaling Technology
c-Jun (60A8 mAb, 9165)	Cell Signaling Technology
Foxo3a (#07-1719)	Millipore
Mcl-1 (600-401-394S)	Rockland Immunochemicals
TuJ1	Covance
Fractin (AB3150)	EMD-Millipore
TrkA (AF1056)	R&D

Function-blocking NGF antibody was a kind gift from Genentech, Inc. The following inhibitors were used: GDC-0068, ABT-737 (Selleck Chemicals), JNK inhibitor VIII, 1NM-PP1 (EMD-Millipore), Lactacystin (Calbiochem).

## Mass Spectrometry

To recover sufficient axonal material, DRGs were harvested at E12.5 to E13.5 from 4 CD1 litters, dissociated as above, and  $2.5 \times 10^6$  to  $3 \times 10^6$  neurons were plated in 200  $\mu$ l modified silicon dividers on a poly-D-lysine / laminin-coated 10cm

dishes. After three hours, Neurobasal / B27 media supplemented with 50ng/ml NGF was added and the silicone dividers were removed. At DIV 12, the plates were rinsed with Neurobasal/B27 without NGF and subjected to TD for 24hr using an anti-NGF function-blocking antibody. Axon and cell body samples were harvested by washing twice with Hank's balanced salt solution and physical separation of axons and cell bodies using a razor as described (Yang et al., 2013). Axon and cell body samples were homogenized in a triton- based buffer [100mM Bicine pH 8.0, 150mM NaCl, 270mM Sucrose, 1% Triton-X100], protein was quantified by BCA, and equal amounts were analyzed by 1-D SDS PAGE (4-20% gradient TGX gels Bio-Rad). Proteins were separated by SDS-PAGE and visualized using Colloidal Coomassie stain (Bio-Rad). Gel bands were excised between 17 and 36 kDa, based on molecular weight marker (MW), corresponding to the MW of Puma (confirmed by Western Blot). Protein bands were trypsinized as described previously (Shevchenko et al., 1996) and extracted peptides analyzed by LC-MS/MS (Ultimate 3000 nano-HPLC system coupled to a Q-Exactive Plus mass spectrometer, Thermo Scientific). Peptides were separated on a C18 column (12cm/75  $\mu$ m, 3  $\mu$ m beads, Nikkyo Technologies) at 200 nl/min with a gradient increasing from 5% Buffer B/95% buffer A to 45% buffer B/55% Buffer A in 82 min (buffer A: 0.1% formic acid, buffer B: 0.1% formic acid in acetonitrile). LC-MS/MS experiments were performed as either data dependent or parallel reaction monitoring (PRM). For PRM experiments 15 peptides from 3 proteins were targeted (Puma: DSPRPFPLGR, EIGAQLR, VEEEEWAR,

MoxADDLNAQYER, QEGSSPEPVEGLAR; 14-3-3 protein gamma:  
 YLAEVATGEK, NCSETQYESK, NVTELNEPLSNEER, YLAEVATGEKR,  
 TAFDDAIAELDTLNEDSYK; Capzb: NDLVEALKR, DYLLCDYNR,  
 SGSGTMNLGGSLTR, LTSTVMLWLQTNK, LEVEANNAFDQYR). Data were  
 recorded in profile mode. Precursor mass spectra were recorded between  $m/z$   
 300-1100 at 35,000 resolution. Tandem MS spectra were recorded at 17,500  
 resolution with  $m/z$  100 as lowest mass. Normalized collision energy was set at  
 27, with AGC target and maximum injection time being  $5e5$ , and was 100ms,  
 respectively. Tandem MS matching: data were extracted using Proteome  
 Discoverer 1.4 (Thermo Scientific) and searched against Uniprot complete  
 Mouse proteome databases (January 2013) concatenated with common  
 contaminants (Bunkenborg et al., 2010) using Mascot 2.4 (Matrix Science). N-  
 terminal glutamate to pyroglutamate conversion, oxidation of methionine, and  
 protein N-terminal acetylation were allowed as variable modifications. Matched  
 peptides were filtered using either Fixed Value peptide spectrum match (PSM) or  
 Percolator (Spivak et al., 2009). PRM data were analyzed using Xcalibur Quan  
 Browser 3.0.63 (Thermo Scientific). For Puma, intensities of the three (EIGAQLR)  
 and four (MoxADDLNAQYER) most intense fragment ions with  $m/z$  greater than  
 the precursor were extracted using a window of 5ppm.

## **Quantification of axon degeneration**

For explant cultures: For each explant at each time point, two non-overlapping fields were imaged using a 10x objective. The percentage of axons with fragmented morphology as assayed by TuJ1 immunostaining was calculated for each image and averaged across both images for each explant. Degeneration index was calculated by dividing each value within an individual experiment by the average degeneration of wild-type cultures at the terminal time point.

For Campenot chambers: Chambers were imaged using a 10x objective and degeneration was calculated as the percentage of fragmented axons within an entire chamber.

For dissociated and re-aggregated cultures: Two non-overlapping fields were imaged for each culture and degeneration was calculated as the average percentage of fragmented axons across both images.

## ***In vivo* imaging**

Whole mount *in vivo* immunolabeling and imaging was performed according to previously established protocols within the lab (Renier et al., 2014). Briefly, embryos were fixed overnight at 4°C in 4% PFA and then for 1hr at room temperature the next day. Embryos were washed in PBS and treated with a methanol series (50%, 80%, 100%) for 1hr (2x each step). Following this, embryos were bleached in 5% H<sub>2</sub>O<sub>2</sub> in 20% DMSO/methanol (1 vol 30% H<sub>2</sub>O<sub>2</sub>/1 vol DMSO/4 vol methanol, ice cold) at 4°C overnight. Samples were then

rehydrated according to the published protocol and permeabilized in PBS/0.2% Triton X-100 for 1 hr twice before immunolabeling. Embryos were imaged on a light-sheet microscope (Ultramicroscope II, LaVision Biotec) equipped with a sCMOS camera (Andor Neo) and a 23/0.5 objective lens equipped with a 6 mm working distance dipping cap. Maximal projections were made in ImageJ (NIH, <http://imagej.nih.gov/ij/>).

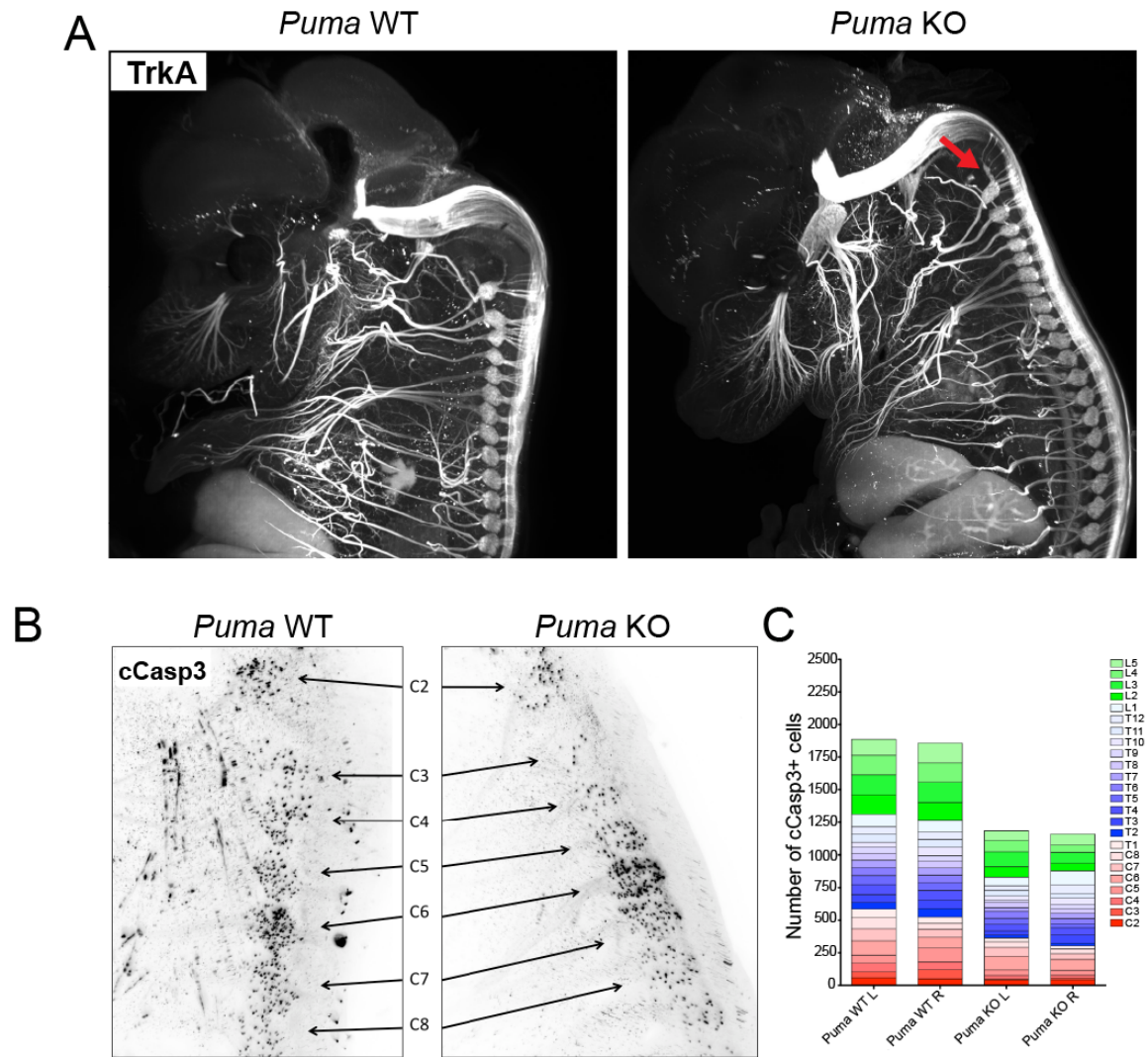
### **Statistical analysis**

All statistical analysis was performed in Prism version 5.0 (<http://www.graphpad.com/scientific-software/prism/>).

## **Appendix 1: Effect of Puma deletion on neuronal apoptosis *in vivo***

This experiments listed in Chapters 3 and 4 clearly establish a central role for Puma in TD induced axon degeneration *in vitro*. We utilized iDISCO, a newly developed protocol that allows for immunolabeling and volume imaging of whole embryos (Renier et al., 2014), to image TrkA neurons within embryonic day 14.5 (E14.5) *Puma* WT and KO embryos. Axon projections to the periphery appeared grossly similar in WT and KO embryos, in accordance with that seen in *Bax* and *Caspase-3* mutants (Figure A1.1A, Nicolas Renier and Zhuhao Wu, unpublished data). This is possibly due to the limits of resolution associated with the imaging technique (ie. small nociceptive axon numbers within large bundles might not be captured fully). Notably however, genetic deletion of *Puma* led to partial preservation of an entire ganglion and its axonal projections (Figure A1.1A red arrow). This rostrally located ganglion, known as “Froriep’s ganglion”, is normally eliminated during development (Geffen and Goldstein, 1996). The fact that it remains in KO embryos is a striking demonstration of necessity of *Puma* for neuronal apoptosis in the developing embryo. This is further emphasized by the ~40% reduction of Caspase-3 positive cells in DRGs at this stage of development in *Puma* mutants (Figure A2.1B,C). Future experiments could examine the effect of Puma across the entire range of development and quantify axonal Caspase-3 *in vivo*.

Imaging individual degenerating DRG sensory axons during development is extremely challenging, but two approaches could be used in future experiments to overcome this limitation. First, *in vivo* pruning defects could be examined by crossing *Puma* knockouts to *Plexin-A3/A4* double knockouts, which show defasciculation and misprojection of peripherally extending axons. These misprojected axons are largely eliminated during development in a Bax-dependent fashion (Schoenmann et al., 2010). If deletion of *Puma* produces a similar phenotype, it would provide compelling evidence for an *in vivo* role of *Puma* in axon pruning. Secondly, developmental axon degeneration could be assessed *in vivo* using electron microscopy to determine the number of myelinated and unmyelinated axons in the sciatic nerve of knockout and wild-type mice across the spectrum of development (E15, postnatal day 0 (P0), P7, P14 and at six weeks). Using this method, the Tessier-Lavigne lab showed that P14 *Caspase-3* knockout mice showed an increase in unmyelinated fibers in the sciatic nerve compared to wild-type littermate controls (David Simon, unpublished). Examining *Puma* knockouts in this paradigm represents an intriguing avenue of future experimentation.



**Figure A1.1 An *in vivo* role for Puma in neuronal apoptosis during development.** (A) *Puma* is required for degeneration of Froriep's ganglion. Whole mount labeling of E14.5 *Puma* WT and KO embryos for TrkA using the iDISCO protocol. (B and C) *Puma* deletion reduces the number of Caspase-3 positive cells in DRGs during development. Whole mount labeling of E14.5 *Puma* WT and KO embryos for cCasp3 using the iDISCO protocol. Max projections of DRGs were acquired on a light sheet microscope (B) and quantified (C). n=1 for both genotypes.

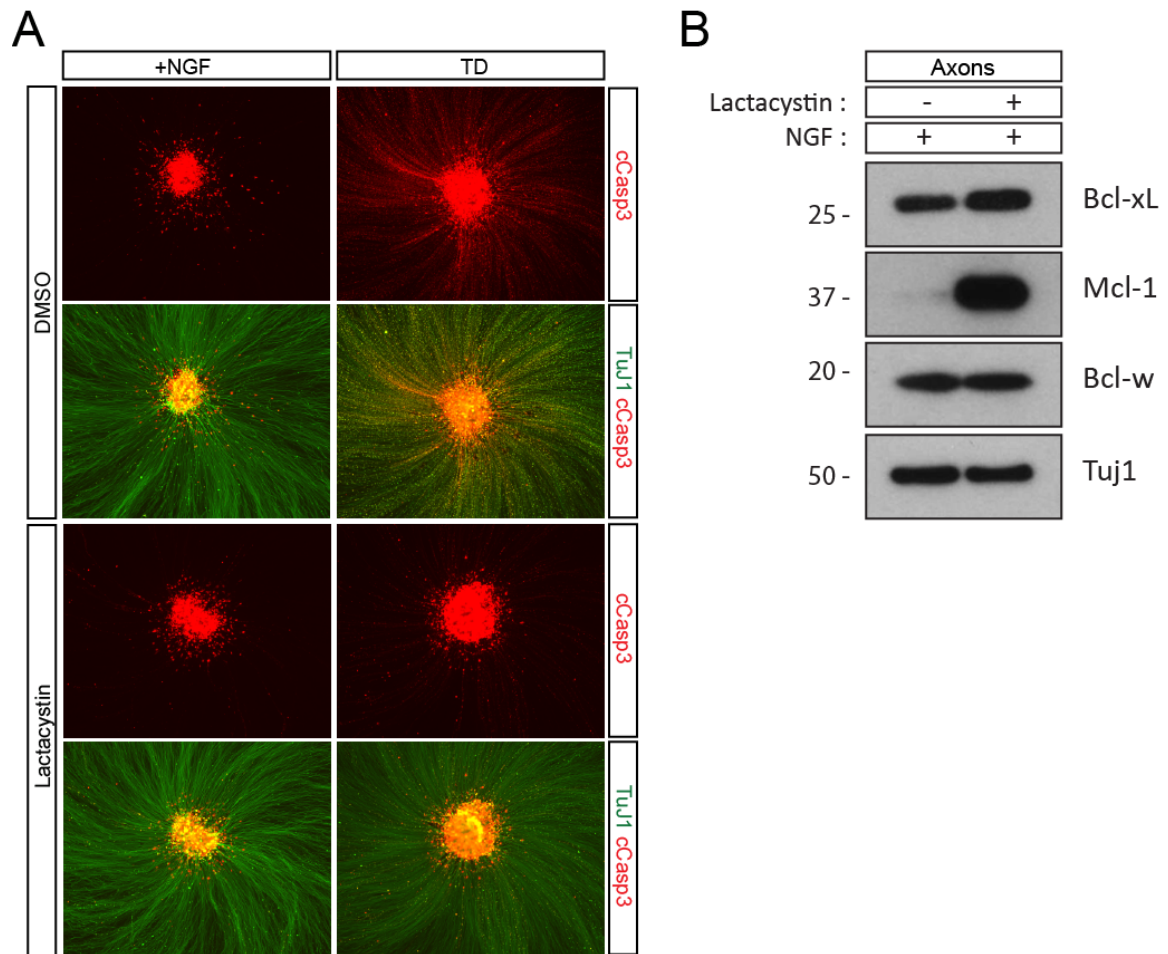
## **Appendix 2: Involvement of the ubiquitin proteasome system in axon degeneration**

The Ubiquitin proteasome system (UPS) is the main mechanism for directed proteolysis in eukaryotic cells. Proteins are targeted for degeneration by attachment of ubiquitin chains to lysine residues through a mechanism involving substrate specific E3 ligases (Finley, 2009). This system also contains substrate specific deubiquitinating enzymes (DUBs), also called ubiquitin proteases, that can remove ubiquitin chains from substrates. In addition to proteolytic functions, ubiquitination also plays key roles in cell signaling (Chen and Sun, 2009), including playing a role in TrkA internalization that is necessary for endocytosis and signaling (Wooten and Geetha, 2006).

Recent studies have shown that ubiquitination of Caspase-8 plays a major role in initiating the extrinsic apoptotic pathway in human cell lines (Jin et al., 2009) and factors that activate proteasome activity play a key role in the caspase activation necessary for spermatogenesis in *Drosophila* (Bader et al., 2011). The UPS also plays key regulatory roles in intrinsic apoptotic pathways, including regulation of anti-apoptotic Bcl-2 family members Bcl-xL, Bcl-2, and Mcl-1, as well as pro-apoptotic members including Bim and Bid (Neutzner et al., 2012; Vucic et al., 2011). Moreover, ubiquitination of Bax by the E3 ligase Parkin has an anti-apoptotic effect in neuronal cell lines (Johnson et al., 2012). Additionally, over expression of a specific DUB can reduce stereotyped pruning in *Drosophila* mushroom bodies and inhibition of the proteasome with the irreversible inhibitor

lactacystin protects mouse sympathetic axons from degeneration *in vitro* both in response to physical injury and in response to TD (Watts et al., 2003; Zhai et al., 2003).

To gain a better mechanistic insight into the role of the UPS in axon degeneration we assayed Caspase-3 activation after TD in the presence of the proteasome inhibitor lactacystin. Proteasome inhibition delays axon degeneration after TD, confirming earlier studies, and it appears to act upstream of Caspase-3 activation, as the immunoreactivity of cleaved Caspase-3 in axons was also noticeably reduced (Figure A2.1A). We reasoned that this effect could be a result of increasing concentration of Bcl-2 family members normally degraded by the UPS. While only producing a small increase in Bcl-xL and no noticeable increase in Bcl-w, levels of Mcl-1 were dramatically upregulated in axons of DRG cultures treated with the proteasome inhibitor lactacystin in the presence of NGF (Figure A2.1B). This was surprising in that Mcl-1 could only be observed at extremely low levels basally in DRGs untreated with lactacystin and Mcl-1 levels in cell bodies were not affected by NGF withdrawal (Figure A2.1B, 3.8A.B). This may indicate that the mechanism by which proteasome inhibition protects axons is by raising the threshold of activation of the mitochondrial apoptotic pathway through increased anti-apoptotic protein concentration. Further, it may indicate that Mcl-1 is normally restricted to the cell body in healthy neurons by the activity of the proteasome, providing a mechanism whereby cell bodies are more resistant to apoptotic stressors than axons. Both of these possibilities warrant further study.



**Figure A2.1 The role of the proteasome in Caspase activation in axons after TD.** (A) Proteasome inhibition delays activation of axonal Caspase-3 after TD. 2DIV DRG explant cultures were subject to 9hr TD or pretreated for 1hr with 10 $\mu$ M lactacystin and subjected to 9hr TD in the presence of lactacystin as indicated. Caspase-3 activation was assayed by immunofluorescence. (B) 7DIV DRG explants were treated with 10 $\mu$ M lactacystin or DMSO for 15hr and highly enriched axonal preparations were collected. Levels of anti-apoptotic Bcl-2 family proteins were assayed by immunoblot.

## **Appendix 3: Rights and permissions**

### **Figure 1.1**

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### **Figure 1.2A**

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### **Figure 1.2B, 1.4A**

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