The role of IkappaB kinase epsilon and Tank-binding kinase 1 in regulating X-linked inhibitor of apoptosis during axonal degeneration

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ABSTRACT

The developing nervous system is highly malleable and requires constant feedback to achieve proper neural circuit assembly and functionality. Axonal degeneration is one of the mechanisms utilized by vertebrate nervous systems to maintain homeostasis, however it is also dysregulated in a number of devastating neurodegenerative diseases. While very little is known about the signaling cascade that controls it, axonal degeneration requires the activation of caspases, which are in turn regulated by XIAP. Recent evidence suggests a role for the non-canonical NF-κB kinases, IKKε and TBK1, in XIAP regulation in various settings. In this study, we show that IKKε and TBK1 directly interact with XIAP and phosphorylate it at Ser430. We also demonstrate that NGF withdrawal from DRG axons results in the phosphorylation of XIAP at this residue, which precedes its elimination through the proteasome. Additionally, we show that chemical inhibition of IKKε/TBK1 significantly delays axonal degeneration, further implicating them in this process. NGF-dependent axonal degeneration therefore requires the inhibition of XIAP by IKKε and TBK1 for caspase-driven neurite elimination to proceed. We propose a model in which NGF withdrawal leads to the activation of IKKε/TBK1, which then phosphorylate XIAP at Ser430 and target it for ubiquitination and degradation by the proteasome, thereby freeing caspases to proceed with axonal degeneration.

RÉSUMÉ

Durant son développement, le système nerveux est extrêmement malléable et nécessite un apport constant d'information, afin d'assurer l'assemblage et fonctionnement adéquats des circuits neuronaux. La dégénérescence axonale est l'un des mécanismes utilisés par les systèmes nerveux des vertébrés pour maintenir l'homéostasie, cependant le dérèglement de ce processus entraine de nombreuses maladies neurodégénératives dévastatrices. Bien que l'on en sache peu au sujet des signaux qui la contrôlent, la dégénérescence axonale nécessite l'activation des caspases, qui sont à leur tour régulées par XIAP. Récemment, on attribua un rôle aux kinases de la famille non-canoniques de NF-kB, IKKε et TBK1, dans la régulation de XIAP dans plusieurs contextes. Dans cette étude, nous démontrons qu'IKKε et TBK1 interagissent directement avec XIAP et phosphorylent le résidu Ser430. Nous démontrons aussi que le retrait de NGF des DRG permet la phosphorylation de ce résidu sur XIAP, ce qui précède son élimination par le protéasome. En outre, nous montrons que l'inhibition chimique d'IKKε/TBK1 retarde considérablement la dégénérescence axonale, les impliquant davantage dans ce processus. La dégénérescence axonale qui suit le retrait de NGF nécessite donc une inhibition de la protéine XIAP par IKKε et TBK1, pour permettre l'élimination des neurites par les caspases. Nous proposons un modèle dans lequel le retrait de NGF mène à l'activation d'IKKε/TBK1, qui ensuite phosphorylent XIAP sur Ser430 et entrainent son ubiquitination et dégradation par le protéasome, libérant ainsi les caspases qui peuvent désormais procéder à la dégénérescence axonale.

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1. INTRODUCTION

Axonal degeneration is an important physiological event responsible for proper neural development and function. It is defined as the active process of axon self-destruction similar to the self-elimination of cells during apoptosis (Saxena and Caroni 2007). Although not involving cell death, axon degeneration requires the activation and regulation of an intrinsic destruction machinery that will effectively eliminate specific neurite segments. This involves microtubule disassembly, axon swelling, fragmentation, and removal of debris by local phagocytes (Saxena and Caroni 2007). Mammalian neurons initially produce an overabundance of processes that must subsequently be pruned to ensure the functional organization of the neuronal circuit to which they contribute. The vertebrate nervous system thus utilizes several mechanisms to meet the operative needs of its projection fields, as well as to fine-tune its wiring (Cowan et al. 1984; Luo and O'Leary 2005). Apoptosis, synapse retraction and axonal degeneration are the nervous system's elimination mechanisms that collectively contribute to neural circuit assembly and developmental brain plasticity (Low and Cheng 2006).

Nascent axons are extremely dynamic and typically probe their surroundings through ongoing extensions and retractions of their processes, ultimately settling in a target area (O'Rourke et al. 1994). Early outgrowth and target invasion are highly plastic though, as local competition for target-derived neurotrophic factors results in the retraction or elimination of neurons and processes that receive inadequate support (Luo and O'Leary 2005; Saxena and Caroni 2007). Surviving axons are protected from degeneration by responding to target-derived nerve growth factor (NGF), which binds to its high-affinity surface receptor tropomyosin related kinase A (TrkA) and initiates a pro-survival signaling cascade (Yuan and Yankner 2000; Raff et al. 2002). Early findings suggested that outcompeted neurons withered passively in the absence of neurotrophic support due to a lack of survival signaling initiated by NGF; however, it is now

evident that axonal loss is an active process (Martin et al. 1988), dependent on a tightly controlled, sub-lethal and reversible destruction pathway involving cysteine aspartate protease (caspase) activation (Yuan and Yankner 2000; Wang et al. 2012; Neukomm and Freeman 2014).

It was also long assumed that neurite degeneration was a mere consequence of neuronal apoptosis, occurring only in instances where the cell body was destined to die. However, accumulating evidence now highlights the ability of neurites to degenerate independently of the neuronal cell body (Wang et al. 2012), thereby emphasizing the compartmentalization of subcellular apoptotic events (Raff et al. 2002; McLaughlin et al. 2003). For instance, axon elimination is critical for generating the adult patterns of all vertebrate projection systems, including retinotopic maps in the superior colliculus (McLaughlin et al. 2003) and subcortical projections of the neocortex (O'Leary and Koester 1993). In response to guidance molecules, neurons extend long primary axons that navigate through a pathway along which their possible targets are positioned. By a process of delayed interstitial axon branching, collateral branches often found at great distances from the primary growth cone extend toward peripheral targets and arborize (Luo and O'Leary 2005). Eventually, the distal component of the primary axon and ectopically-positioned arbors are eliminated through local degeneration, leaving proper connections and cell bodies intact (Figure 1).

The selective destruction of neuronal processes, without the loss of the parent neuron, also occurs in response to injury and disease in the adult peripheral and central nervous systems. Although this pruning mechanism is critical for repairing and stabilizing affected circuits following injury (Luo and O'Leary 2005), its dysregulation characterizes a wide range of neurological pathologies that exhibit axonal and dendritic atrophy prior to neuronal death. For example, many neurological diseases such as amyotrophic lateral sclerosis (ALS) are subject to

dying back degeneration, a process by which axons degenerate in a distal-to-proximal progressive fashion (Cavanagh 1964; Fischer and Glass 2007). Likewise, many neurodegenerative diseases that ultimately culminate in neuronal loss, such as Alzheimer's (Tsai et al. 2004), Huntington's (Li et al. 2001) and Parkinson's (Burke and O'Malley 2013) disease, exhibit significant loss of distal axons well before the neuronal cell body dies. While trophic factor withdrawal plays a prominent role in axonal degeneration during development, there is little evidence to implicate it in the pathogenic mechanisms of neurodegenerative diseases. Indeed, pathological degeneration is most likely triggered by the co-option of late effector phases of apoptotic signaling pathways, such as free-radical generation and caspase activation (Yuan and Yankner 2000).

As of yet, the cell biological mechanisms that allow certain processes to be eliminated while others remain intact are still unknown. Unraveling the sequence of events that promote axonal degeneration during normal development may therefore further our understanding of the early stages of many neurodegenerative disorders, as well as shed light on ways to therapeutically delay or prevent this process when it becomes aberrantly activated.

1.1 Axon degeneration *in vitro*

Developmental axon degeneration is commonly modeled *in vitro* in embryonic dorsal root ganglion (DRG) sensory neurons. Afferent DRG axons are unique in their ability to relay sensory information from peripheral tissues, like the skin, to the spinal cord and brain. Similarly to other neurons of neural crest origin, DRG neurons require NGF for survival *in vivo*, although their dependency is transient and restricted to early embryonic development (Johnson Jr et al. 1986). Likewise, cultured embryonic DRG neurons are initially dependent on NGF for survival *in vitro*, and degenerate within 24 to 36 hours of NGF withdrawal from the growth media (Levi-Montalcini

and Booker 1960). The *in vitro* system utilized by Rita Levi-Montalcini was improved over the years to facilitate the specific study of local axon signaling in response to NGF withdrawal by enabling fluidic isolation of soma and axons, such that either could be withdrawn of NGF independently of the other (Campenot 1977). A similar culture method was introduced by Jeffery Twiss (Zheng et al. 2001), and consists of plating dissociated embryonic day 13.5 (E13.5) DRG explants on laminin-collagen-coated porous filters that allow the passage of growing axons but not neuronal cell bodies (Figure 2). For the first 2.5 days *in vitro* the culture medium is supplemented with NGF to support axonal growth through the filter. At the start of NGF deprivation, the culture medium is replaced with NGF-free media supplemented with anti-NGF antibody to inhibit any remaining NGF. Pure axonal processes (free of neuronal cell bodies and glia) that are present on the bottom surface of the filter can then be collected at various time points for immunocytochemical and biochemical analyses. The Twiss culture method is ideal for dissecting the molecular pathways that underlie axonal degeneration, as it allows for various pharmacological manipulations and genetic deletions of candidate proteins. As such, it engenders a direct examination of the signaling cascades activated in degenerating axons and is the method used throughout this study.

1.2 Caspases

Caspases are a family of cysteine proteases that function as downstream executioners of cell death pathways. They are highly conserved in multicellular organisms and can be activated in response to a multitude of intrinsic and extrinsic cell signals. Caspases, so called because they cleave substrates after specific aspartate residues, were first identified in *Caenorhabditis elegans* where Ced-3 (cell-death abnormality-3) was shown to orchestrate the programmed cell death of

somatic cells during development (Ellis and Horvitz 1986). Their role in apoptosis is also critical for proper tissue sculpting, homeostasis and regeneration. Inhibition of cell death signals can cause developmental defects such as abnormal heart formation, neural tube defects and limb malformations (Vaux and Korsmeyer 1999). However, the role of caspases extends beyond apoptosis. In fact, the first mammalian caspase to be found and identified as a Ced-3 homolog, interleukin-1β processing enzyme (ICE) (renamed caspase-1), was involved in both apoptotic and non-apoptotic processes (Cerretti et al. 1992; Thornberry et al. 1992). Additionally, increasing evidence suggests that the ability of caspases to actively regulate development stems from their non-apoptotic roles in differentiation, proliferation, cell shape, and cell migration (Miura 2012). Their dual roles in apoptosis and cell homeostasis are thought to be the reason for the constant evolutionary expansion of the caspase family (Degterev et al. 2003; Kuranaga 2011).

Thus far, 14 mammalian caspases have been identified and share a number of distinct features. All caspases are initially synthesized as inactive zymogens, or procaspases, that contain an N-terminal pro-domain, a central "large subunit", and a C-terminal "small subunit" (Figure 3). A series of proteolytic cleavage events at aspartate residues are necessary to activate caspases (Clarke and Tyler 2009). The first divides the procaspase into a large subunit (p20) and a small subunit (p10), while the second removes the N-terminal pro-domain. The catalytically active caspase dimer is then formed by the association of two identical caspase molecules that join their surface loops to create the active site.

Caspases are typically classified into two categories based on their structure and function. Executioner caspases (caspase-3, -6, -7) have a short N-terminal domain and are responsible for most of the proteolytic events that occur following a cell death signal, as well as for the proteolytic activation of cell dismantling enzymes. Meanwhile, initiator caspases (caspase-2, -8, -9, -10) have

a long N-terminal domain that provides a molecular platform for interaction with adaptor proteins and for their catalytic activation. They also have a few specific substrates, including the pro-forms of executioner caspases. Thus, initiator caspases activate executioner caspases, which in turn orchestrate apoptotic cell death.

Mammalian caspases can be activated either through extrinsic or intrinsic signaling pathways. In the extrinsic pathway, stimulation of death receptors (e.g. Fas) by the binding of extracellular ligands (e.g. FasL) leads to the intracellular interaction between initiator caspase-8, the death domain of the receptor, and adaptor proteins (e.g. Fas associated death domain, FADD). This oligomeric complex mediates the cleavage and activation of caspase-8, thereby granting it access to the effector caspases (Schafer and Kornbluth 2006). In the intrinsic pathway, apoptotic stimuli trigger the mitochondrial release of Cytochrome c, which interacts with Apaf-1 and pro-caspase 9 to form the apoptosome complex. Oligomerization of this complex and clustering of pro-caspase-9 induces the activation of caspase-9, which then activates caspase-3 that executes apoptosis (Elmore 2007).

1.2.1 The role of caspases in axonal degeneration

The role of caspases in neurite degeneration was initially discounted due to failure to detect caspase activation or observe protective effects of caspase inhibition during axonal degeneration (Finn et al. 2000; Saxena and Caroni 2007). However, increasing evidence now implicates these proteases in local sub-lethal neurite elimination. Their activity was first demonstrated in the developing nervous system of *Drosophila melanogaster*, which undergoes extensive neuronal remodeling during larval metamorphosis. Class IV sensory neurons in flies initially produce elaborate dendritic arbors that must be pruned and re-organized during metamorphosis to suit the

more complex adult nervous system (Truman 1990). The fly caspase DRONC, a functional homolog of mammalian caspase-9, was identified as the protease that selectively eliminates arbors and drives the pruning process during metamorphosis (Kuo et al. 2006). Subsequent studies demonstrated that axons of sensory and sympathetic neurons either lacking pro-members of the apoptotic signaling pathway or treated with caspase inhibitors were protected from degeneration after NGF withdrawal (Nikolaev et al. 2009; Vohra et al. 2010). Furthermore, initiator caspase-9 and executioner caspase-3 were recently established to play obligate roles in developmental axon degeneration, with executioner caspase-6 playing a more subsidiary role (Simon et al. 2012). In addition, a caspase-9-to-caspase-3 cleavage cascade is necessary for normal axon degeneration to proceed, while caspase-3 null DRGs are completely protected from NGF-dependent axonal degeneration (Unsain et al. 2013).

Taken together, caspase-dependent neurite destruction pathways appear to be phylogenetically-conserved and essential for local degenerative events in the nervous system. However, their over-activation can be extremely detrimental and therefore requires regulatory mechanisms that limit caspase activity and prevent the uncontrolled and aberrant signaling of apoptotic pathways.

1.3 IAPs: Endogenous caspase inhibitors

Once caspases are cleaved and activated, they are responsible for most of the proteolytic events that occur during apoptosis (Fischer et al. 2003). There are two main ways to terminate caspase activity: target them for destruction by the ubiquitin-proteasome machinery or directly inhibit their enzymatic activity (Eckelman et al. 2006). Viruses have long learned that interfering with caspases, the central components of apoptotic pathways, can prevent the destruction of a host

cell long enough for viral replication to take place. Many have thus evolved potent caspase inhibitors, able to bind and irreversibly lock caspases in an inactive conformation (Stennicke et al. 2002). Evolution has also selected for an elegant mechanism to modulate caspase activity in eukaryotes, specifically via the inhibitor of apoptosis (IAP) family of proteins. Members of the IAP family can confer protection from apoptosis in response to various stimuli (Deveraux and Reed 1999), in addition to playing important roles in unrelated processes such as signal transduction and cell cycle regulation (Verhagen et al. 2001). So far, eight IAP proteins have been identified in humans (Figure 4). The prototype IAP was identified two decades ago in baculovirus and contained two distinct motifs: the baculovirus IAP repeat (BIR) and the really interesting new gene (RING) domain (Crook et al. 1993). The BIR motif is a zinc-binding domain required for binding and inhibition of caspases, while the RING domain shows E3 ubiquitin ligase activity that allows IAPs to catalyze the ubiquitination and degradation of target proteins, including themselves. As such, IAPs have the ability to inhibit caspases both by targeting them to the ubiquitin-proteasome degradation machinery and by directly inhibiting their enzymatic activity (Salvesen and Duckett 2002; Vaux and Silke 2005). The evolutionarily conserved mechanism appears to be caspase elimination though, as the *Drosophila* IAP (DIAP1) can inhibit caspase activity by regulating the ubiquitination and degradation of the caspase DRONC (Meier et al. 2000). Interestingly, the mammalian homolog of DIAP1, termed X-linked IAP (XIAP), is the only human IAP to have evolved the ability to directly inhibit the enzymatic activity of caspases at both the initiator and executioner phases (Figure 5) (Deveraux et al. 1997). On activation, cleavage of caspase-3, caspase-7 and caspase-9 exposes a neo-epitope on the small subunit, termed IAP-binding motif (IBM), which serves as the BIR-binding domain. The second BIR domain (BIR2) of XIAP can bind and inhibit caspase-3 and caspase-7 via two active sites found around its IBM-

binding groove, while the third BIR domain (BIR3) of XIAP can bind and inhibit caspase-9 via an inhibitory element found immediately downstream of its IBM-binding groove (Deveraux et al. 1999). In so doing, XIAP inhibits caspase-3 and caspase-7 through steric hindrance of the active site (Scott et al. 2005), whereas it inhibits caspase-9 activity through inhibition of dimer formation (Shiozaki et al. 2003). While other closely related IAPs, such as cIAP1 and cIAP2, contain IBM-binding grooves that are highly conserved with those of XIAP, they lack the functional inhibitory elements that are required to lock caspases in an inactive conformation (Shiozaki et al. 2003).

Given that all IAPs can inhibit the activity of caspase-3 and -7, and that certain IAPs can inhibit the activity of caspase-9 as well (Deveraux et al. 1997), it is not surprising that IAPs have the ability to block both the intrinsic and extrinsic apoptotic pathways. The mechanisms by which IAPs other than XIAP regulate apoptosis are still largely unclear, but likely involve targeting pro-apoptotic molecules for degradation by the ubiquitin-proteasome machinery and activating pro-survival signaling such as the nuclear factor of κB (NF-κB) pathway (Salvesen and Duckett 2002; Gyrd-Hansen and Meier 2010). As such, overexpression of XIAP, cIAP1, cIAP2, NAIP and Survivin has been shown to suppress apoptosis in a variety of settings that typically induce cell death, including the addition of tumor necrosis factor α (TNFα), FasL, DNA damaging agents, and growth factor withdrawal (Duckett et al. 1996).

1.3.1 The role of XIAP in axonal degeneration

The ability of XIAP to directly bind and inhibit active caspases places it at the center of an important regulatory pathway involved in degenerative processes. In Drosophila, DIAP1 plays a crucial role inhibiting the caspase DRONC during nervous system development (Kuo et al. 2006). DRONC is constitutively active in fly neurons, which requires XIAP to restrain its activity during

periods of neuronal growth. In the context of NGF-dependent axonal degeneration in mammals, it was recently shown that XIAP plays an important role blocking caspase activity within axons (Unsain et al. 2013). NGF deprivation leads to a sharp drop in XIAP protein levels that correlates with an accumulation of cleaved and active caspase-3. This loss of XIAP is not blocked in caspase-3 null ($CASP3^{-/-}$) axons but can be largely prevented with proteasomal inhibitors, indicating that the ubiquitin-protease system is the primary pathway used to eliminate XIAP following NGF withdrawal. If XIAP is overexpressed in DRG neurons prior to NGF withdrawal, caspase-3 activity is strongly suppressed while axonal integrity is significantly enhanced. Furthermore, $XIAP^{-/-}$ DRGs degenerate more quickly and accumulate more active caspase-3 than their wild-type counterparts.

As such, XIAP plays a phylogenetically-conserved role in caspase inhibition and the removal of XIAP via the proteasome is a prerequisite for caspase-driven axonal degeneration. This suggests that a signaling cascade initiated by NGF withdrawal results in the activation of yet unknown mechanisms that target XIAP for degradation, thereby liberating caspases to proceed with axonal degeneration (Figure 6).

1.3.2 Endogenous IAP inhibitors

Owing to the important role of IAPs in caspase inhibition, cells require a mechanism that can relieve this IAP brake that prevents cell death processes from proceeding. A number of proteins have thus been identified with the proposed primary function of promoting caspase activation by blocking IAPs during apoptosis. In *Drosophila*, the Reaper (rpr), head-involution defective (hid), grim and Sickle (skl) genes encode a group of pro-apoptotic factors that can bind to DIAP1 and antagonize its ability to inhibit DRONC, thereby promoting apoptosis (Wing et al. 2002). In

mammals, IAPs can be inhibited by a family of IBM-containing proteins that include second mitochondria-derived activator of caspase (SMAC) and second homolog of 'high temperature requirement-like' (HTRA2). These pro-apoptotic molecules are released from the mitochondria in response to apoptotic stimuli and bind XIAP to displace it from caspases (Figure 7) (Srinivasula et al. 2001). Both Smac and HTRA2 are synthesized in the cytosol and contain an N-terminal mitochondrial targeting sequence that is removed following mitochondrial transport (Du et al. 2000; Suzuki et al. 2001). Cleavage of this sequence results in the maturation of Smac and HTRA2, as well as exposure of a conserved motif that corresponds to the IBM consensus sequence. As such, interaction between XIAP and Smac or HTRA2 completely inhibits the binding of caspase-9 to BIR3, and caspases-3 and -7 to BIR2 through steric hindrance of the active site (Scott et al. 2005). XIAP associated factor 1 (XAF1) is another IAP inhibitor known to bind and sequester XIAP in the nucleus (Liston et al. 2001). Overexpression of XAF1 in cells blocks XIAP-mediated inhibition of apoptosis, however it remains unclear whether this is a direct result of the physical separation of XIAP from cytosolic caspases or whether there are additional consequences of XIAP sequestration (Liston et al. 2001)

While endogenous IAP inhibitors appear to be evolutionary conserved, the signaling cascades that activate them induce high levels of caspase activation that result in apoptosis. They are therefore not suited for the activation of sub-lethal caspase activity required during axonal degeneration.

1.4 The role of IκB kinases in NF-κB activation

The innate immune system is activated in response to a variety of pathogen-associated molecular patterns (PAMPs) such as bacterial lipopeptides, viral infection, ultraviolet light and

double-stranded RNA (Peters et al. 2000; Brasier 2006). Signaling pathways triggered by these pathogens occur either through the Toll-like receptor (TLR) (Doyle and O'Neill 2006) or the cytosolic receptor termed retinoid-acid-inducible gene I (RIG-I) (Akira et al. 2006). Stimulation of these receptors results in the coordinated activation of transcription factors such as nuclear factor-κB (NF-κB) and IFN regulatory factors (IRFs).

The human NF-κB family of transcription factors is comprised of five structurally related subunits (Figure 8) that function in various combinations to induce the expression of genes involved in immunity, inflammation, cell survival and proliferation (Brasier 2006). NF-κB activation is cell-type and stimulus-specific, allowing for a wide range of physiological responses to assaults on the body. The NF-κB family members work as dimers in the cell and control the expression of inducible cytokines, cell adhesion molecules and anti-apoptotic proteins important in cellular stress responses. NF-κB can be activated by two distinct pathways, the "canonical" (Karin 1999) and the "non-canonical" (Senftleben et al. 2001) pathways. The canonical pathway controls the nuclear translocation of an NF-κB dimer composed of the trans-activating RelA subunit and the DNA-binding NF-κB1 subunit, while the non-canonical pathway controls processing and translocation of the NF-κB2-RelB complex. The two pathways are induced by different ligands and control the expression of distinct gene networks, however in both instances the nuclear translocation of NF-κB requires the dissociation of an inhibitor of NF-κB (IκB) protein from the complex. In resting cells, the NF-κB dimer is sequestered in the cytoplasm by one of several IκB family members (IκBα, β, or ε) (Figure 8). Following an inflammatory or immune stimulus of the canonical pathway, signal transduction will trigger the activation of a complex of IκB kinases (IKKs) that will phosphorylate IκB at two specific serines (Ghosh et al. 1998), leading to its ubiquitination and degradation by the proteasome (Karin 1999). As a result, free NF-κB can

translocate into the nucleus and activate gene expression at specific promoters. In the non-canonical pathway, IKKs phosphorylate the p100 subunit, which results in the proteolytic cleavage and the generation of the mature p52 NF-κB subunit. The active RelB-p52 (NF-κB2) complex can then translocate into the nucleus and proceed with targeted gene expression.

The IκB kinase (IKK) family of proteins can be divided into two groups based on their known functions. First to be identified and characterized, the canonical IKK is a multi-protein complex of approximately 700 kDa composed of 10-14 discrete proteins (Mercurio et al. 1999). The core of the canonical IKK complex consists of two catalytic serine-threonine kinases, IKKα and IKKβ (Mercurio et al. 1997), as well as the non-catalytic scaffolding protein known as IKKγ or the NF-κB essential modulator (NEMO) (Yamaoka et al. 1998) (Figure 8). The less understood non-canonical IKKs (also known as IKK-related kinases), IKKε and TANK-binding kinase 1 (TBK1), are distinct from their canonical counterparts and interact with them only weakly (Peters et al. 2000). The kinase domain of IKKε is 27% identical to those of IKKα and IKKβ (Shimada et al. 1999; Peters et al. 2000), while TBK1 exhibits 49% identity and 65% similarity to IKKε (Shen and Hahn 2011). Both IKKε and TBK1 contain an N-terminal kinase domain, a ubiquitin-like domain, a C-terminal leucine-zipper and a helix-loop-helix motif (Shen and Hahn 2011). The targets of these serine-threonine kinases in the NF-κB activation pathway are still largely unclear, although they do not appear to be IκBα (Bonnard et al. 2000). Nevertheless, the expression of either IKKε or TBK1 can activate NF-κB reporter genes (Shimada et al. 1999; Tojima et al. 2000), while they have also been proposed to phosphorylate the NF-κB p65 subunit directly, leading to NF- κB activation (Fitzgerald et al. 2003). Furthermore, a role for IKKε and TBK1 was identified in interferon regulatory factor 3 and 7 (IRF3 and IRF7) activation, which are required for the expression of type 1 interferons (IFNs), such as IFN-β, during viral infection (Fitzgerald et al.

2003; Kawai and Akira 2010). More recently, the IKK-related kinases have also been implicated in cell transformation and tumor progression (Shen and Hahn 2011).

The IKK family of proteins are therefore important regulators of the innate immune response due to their role in NF-κB and IRF activation. While the signaling mechanism and downstream targets of the canonical IKK complex (IKKαβγ) are well characterized, little is known about the roles of the non-canonical homologs IKKε and TBK1.

1.4.1 Non-canonical IKKs as novel XIAP regulators

Emerging work on the non-canonical IKKs, including their distinct interaction partners and phosphorylation targets, raises the possibility that their roles extent beyond NF-κB activation. As such, recent studies have found that a member of the fly IKK family, termed DmIKKε, is a novel antagonist of DIAP1 (Kuranaga et al. 2006; Oshima et al. 2006). DmIKKε was shown to phosphorylate DIAP1, leading to its ubiquitination and degradation by the proteasome. Its mammalian ortholog TBK1 (also known as NF-κB-activating kinase (NAK)) also depleted DIAP1 from cultured cells and could phosphorylate the mammalian ortholog XIAP (Kuranaga et al. 2006). *In vivo*, DmIKKε was important for proper sensory organ precursor (SOP) development in the wing imaginal disc, which requires regulated DRONC caspase activity (Kuranaga et al. 2006). Moreover, DmIKKε activity is crucial for F actin assembly and proper development of the fly tracheal system (Oshima et al. 2006). Therefore, DmIKKε activity mediates caspase activation via inhibition of IAPs, however its role does not appear to be regulation of apoptosis. Unlike the Reaper/Hid/Grim pathway, DmIKKε-mediated degradation of DIAP1 does not induce cell death, but rather promotes the non-apoptotic functions of the caspase DRONC, such as regulation of cell morphology, migration, proliferation and differentiation (Kuranaga et al. 2006; Oshima et al.

2006).

Interestingly, the mammalian IKKε was also recently shown to promote the phosphorylation and degradation of XIAP in virus-infected cells, resulting in their sensitization to apoptosis (Nakhaei et al. 2012). Meanwhile, its closely-related homolog TBK1 could also target XIAP for phosphorylation but without triggering cell death. Both IKKε and TBK1 were shown to phosphorylate XIAP at serine 430 (Ser430) located within its RING domain, *in vitro* and *in vivo* (Nakhaei et al. 2012). Phosphorylation of XIAP by IKKε mediated its ubiquitination on lysines 322 (K322) and 328 (K328), followed by its degradation by the proteasome. Most notably, kinase-deficient mutants of IKKε and TBK1 were not able to mediate XIAP phosphorylation on Ser430, which protected cells from apoptosis.

IKKε and TBK1 thus appear to have roles beyond the NF-κB signaling pathway, including a phylogenetically conserved role in XIAP regulation in various settings. As of yet however, their roles in regulating IAP levels within neurites have not been elucidated, but current findings raise the possibility that they orchestrate the XIAP-mediated sub-lethal caspase activation observed in degenerating axons following NGF withdrawal.

1.5 Purpose of this study

Axonal degeneration is a tightly controlled event, crucial for the proper development and function of the nervous system. Due to initial overabundance of neuronal processes, degenerative mechanisms are needed to correctly shape neural circuits and maintain homeostasis following injury. Nevertheless, axon degeneration can become aberrant at any step of the signaling cascade that regulates it and is known to characterize a wide range of neurological and neurodegenerative diseases that exhibit axonal and dendritic atrophies. Therefore, understanding the signaling

cascade that drives this process may lead to the discovery of ways to therapeutically delay or prevent it when it becomes abnormally activated. While significant progress has been made to identify the key regulators of this sub-lethal apoptotic pathway (Kuo et al. 2006; Unsain et al. 2013), much still remains unknown.

Caspases are known as central regulators of apoptotic cell death pathways, however increasing evidence demonstrates their ability to carry out non-apoptotic functions in both mammals and *Drosophila* (Miura 2012). The list was recently extended to include the role of caspases in developmental axon degeneration, where their activation was shown to be indispensable for dendritic pruning (Kuo et al. 2006) and axon elimination following NGF withdrawal (Simon et al. 2012; Unsain et al. 2013).

As an endogenous caspase inhibitor, XIAP plays an important role regulating caspases in a variety of settings. In neurites, it has been shown to mediate inhibition of the caspase DRONC during periods of neural growth (Kuo et al. 2006), while also being critical for inhibition of cleaved caspase-3 in response to NGF signaling (Unsain et al. 2013). During morphogenesis or following NGF withdrawal, XIAP-mediated caspase inhibition is relieved, allowing caspases to cleave their substrates and proceed with axonal degeneration. The role of XIAP in caspase regulation appears to be phylogenetically conserved, yet little is known about the mechanisms that relieve this XIAP brake and allows for sub-lethal caspase activation without prompting neuronal apoptosis.

Recent studies on the non-canonical IKK family members, IKKε and TBK1, began unraveling their roles beyond NF-κB activation. As such, IKKε and TBK1 were shown to mediate caspase activation by inhibiting XIAP *in vitro* and *in vivo* (Kuranaga et al. 2006; Oshima et al. 2006; Nakhaei et al. 2012). Phosphorylation of XIAP by IKKε and TBK1 mediates its auto-ubiquitination and degradation by the proteasome, thereby liberating caspases to proceed with their

non-apoptotic cellular functions. The roles of IKKε and TBK1 in neurites are still largely unknown and new functions continue to emerge regularly. Given their evolutionarily conserved ability to phosphorylate XIAP, their function might include regulation of sub-lethal caspase activity following NGF withdrawal.

The purpose of this study is to investigate the signaling mechanisms that regulate XIAP during axonal degeneration in mammalian neurons. Globally, the loss of XIAP from axons must result from specific signaling events that are initiated following NGF withdrawal. Specifically, the working hypothesis is that NGF withdrawal results in the activation of IKKε and TBK1, which induce XIAP phosphorylation on Ser430, thereby targeting it for ubiquitination and destruction by axonal proteasomes. Elimination of XIAP liberates caspases, which are free to proceed with axonal degeneration. Using a direct method of investigation, the following questions were addressed:

1. Is XIAP phosphorylation at Ser430 by IKKε and TBK1 specific?
2. Is XIAP phosphorylated at Ser430 in degenerating axons in respond to NGF withdrawal?
3. Does chemical inhibition of IKKε/TBK1 affect axonal degeneration?

2. MATERIALS AND METHODS

Mice. CD-1 mice were purchased from Charles River Laboratories. All animal procedures were approved by the Canadian Council of Animal Care, and efforts were made to minimize the number of manipulations and animals used.

Cell culture and transfection. HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% bovine calf serum (BCS), 1% L-glutamate and 1% penicillin/streptomycin and were maintained in 5% CO_2 at 37 °C. Cells were plated on 10 cm plates with a density of 1 x 10^6 cells/plate 24 hours prior to transfection. The cell media was changed 4 hours prior to addition of the Ca_2PO_4 precipitate to the cells. The precipitate for each plate was formed by mixing 530 µl ddH_2O with 10 µg of plasmid DNA, followed by the dropwise addition of 600 µl 2x HBS (pH 7.05; 283 mM HEPES, 1.5 mM Na_2PO_4) and 60 µl 2M $CaCl_2$. After 20 minutes of incubation at room temperature, the entire volume was added to the plate in a dropwise fashion and mixed gently. Finally, the media in the plates was replaced with fresh media approximately 18 hours after transfection.

Plasmid construction and mutagenesis. Human XIAP cDNA was amplified and cloned into Myc pcDNA3. The XIAP S430A point mutation was introduced using the Q5 site-directed mutagenesis kit (NEB) and confirmed by DNA sequencing. It was then cloned into Myc pcDNA3.1. Flag-IKKε was constructed by cloning human IKKε cDNA into Flag pDest12.2. IKKε K38A point mutation was introduced using the Q5 site-directed mutagenesis kit (NEB) and verified by DNA sequencing. It was then cloned into pDest12.2. Flag-TBK1 and Flag-TBK1 K38A plasmids were a gift from Dr. Nathalie Grandvaux (University of Montreal).

Immunoprecipitation of phosphorylated XIAP from HEK 293T cells. Approximately 48 hours after transfection, the cells were placed on ice and washed once with 1 ml cold PBS-EDTA. Then, 1 ml cold RIPA (Radio-Immunoprecipitation Assay) lysis buffer supplemented with protease inhibitors (0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 20 μg/ml phenol methyl sulfonyl fluoride (PMSF) and 100 μg/ml benzamidine) and phosphatase inhibitors (50 μM sodium fluoride, 1mM sodium orthovanadate, 5 mM sodium pyrophosphate and 50 μM β-glycerophosphate) was added to each plate and incubated with shaking at 4 °C for 20 minutes. Next, the lysates were scraped off the plates and transferred to eppendorf tubes, vortexed, and spun down at 13,200 rpm for 5 minutes. 1000 μl of lysate was transferred to new tube and designated IP (for immunoprecipitation), while 100 μl of lysate was set aside and designated as input. An equal amount of 2x Laemmli loading buffer (4% SDS, 100 mM Dithiothreitol, 120 mM Tris pH 6.8, Bromophenol Blue, 10% Glycerol) was added to the input tubes, which were then boiled for 5 minutes and kept at -20 °C. Next, 5 μl of anti-XIAP^{S430} (phospho-XIAP) antibody was added to the IP tubes which were incubated on a rotator at 4 °C overnight. The following morning, 50 μl of protein-A sepharose bead slurry (50%) was added and an additional incubation of 1 hour at 4 °C was performed. Then, the supernatant was removed and the beads were washed 4 times with 1 ml RIPA lysis buffer. After the last wash, the beads were re-suspended in 50 μl 2x Laemmli loading buffer, boiled for 5 minutes, spun down and stored at -20 °C.

DRG culture and treatments. Dissociated DRG explants were collected from E13.5 mouse embryos and grown on tissue culture filter inserts (1 μm pore size; BD-Falcon) that fit in 6-well plates. The filter was coated sequentially with poly-D-lysine (1 mg/ml; Sigma-Aldrich), laminin (10 μg/ml; Sigma-Aldrich), and collagen (0.1 mg/ml, PureCol; Advance BioMatrix). The basal

culture media consisted of Neurobasal (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1% L-glutamate (Wisent), 1% penicillin/streptomycin (Wisent) and 20 μM 5-Fluoro-2'-deoxyuridine (FDU) (Sigma-Aldrich). For the first 2.5 days of axonal growth, the media was also supplemented with 12.5 ng/ml NGF (Alomone). NGF deprivation was achieved by changing the media in the wells to an NGF-free basal medium supplemented with anti-NGF antibody Rab1 (1 μg/ml). Treatment with MRT67307 (Tocris Bioscience) consisted of pre-treating DRGs 30 minutes prior to NGF withdrawal and then treating with 0.5 μM MRT67307 at the start of NGF deprivation.

Immunocytochemistry and quantification of axonal degeneration. DRGs grown in plastic 6-well plates were fixed with 4% paraformaldehyde for 10 minutes at room temperature and then blocked in blocking solution containing TBS-T, 5% skim milk and 0.3% Triton X-100. The DRGs were then incubated overnight at 4 °C with anti-β-III-tubulin antibody, diluted 1:10,000 in blocking solution, followed by incubation with Alexa488-conjugated goat anti-mouse secondary antibody (The Jackson Laboratory) for 1 hour at room temperature. The wells were then filled with PBS and DRGs were imaged using a wide-field microscope (Axioscope 2; Zeiss). Quantification of axonal degeneration was performed by imaging whole explants. Using a script created in our lab, one quarter of each DRG explant was divided into 50-pixel bins starting from the explant cell body. The density in each bin was then calculated and plotted on a graph, thereby demonstrating the area occupied by axons in each treatment condition. Statistical analysis consisted of one-way ANOVA with post-hoc Tukey test.

Biochemical analysis of axonal preparations. Filters were washed with ice-cold PBS, and cell bodies and axons from the top of the filter were scraped off and discarded. The filter was then

removed from the insert and incubated in 100 µl cold RIPA buffer for 30 minutes at 4 °C with occasional vortexing. The filter was then removed following centrifugation at 13,200 rpm for 5 minutes. The volume was increased to 1 ml with RIPA buffer and was followed by another round of centrifugation. Then, 100 µl of lysates was set aside and designated as input. An equal amount of 2x Laemmli loading buffer was added and stored at -20 °C. The remaining volume was used for immunoprecipitation of phosphorylated XIAP as described above.

Western blot analysis. 293T samples (Figures 10-12) were separated on a 5%-16% SDS-acrylamide gradient maxi gel and transferred to a nitrocellulose membrane. The membranes were then washed in TBS-T and blocked in blotto solution containing TBS-T and 5% skim milk for 30 minutes. The blots were incubated in appropriate antibodies for 2 hours at room temperature, washed 3 times with TBS-T, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies diluted in blotto solution. Following 3 washes in TBS-T, reactive bands were detected using enhanced chemiluminescence (ECL). DRG and other 293T samples were separated on 10% SDS-acrylamide gel. Each experiment was replicated a minimum of 3 independent times and a representative western blot was chosen.

3. RESULTS

3.1 NGF withdrawal results in XIAP loss and axonal degeneration

Recent studies on the physiological nature of axonal and dendritic pruning indicate that caspases play a phylogenetically-conserved and essential role in local degenerative events of the nervous system. To examine the role of XIAP, the only mammalian IAP family member capable of directly inhibiting caspases, NGF-dependent DRG cultures were established using the Twiss method (Zheng et al. 2001) (Figure 2). As such, embryonic day 13.5 (E13.5) DRG sensory neuron explants were plated on porous filters that allow the passage of growing axons but not cell bodies. Survival of DRG sensory neurons and robust axonal growth were supported by the presence of NGF in the culture media for 2.5 days *in vitro*, following which NGF was removed from the media and NGF deprivation was performed for 6, 12 and 24 hours. In this setting, DRG sensory neurons undergo rapid degeneration as observed by immunocytochemical analysis (Figure 9A). In addition, collection of DRG axons from the bottom of the filter for biochemical analysis revealed that NGF withdrawal causes caspase-3 cleavage and a significant loss of XIAP protein during this time period, most notable after 12 hours of NGF deprivation (Figure 9B). These results are consistent with recent findings asserting that NGF withdrawal results in the elimination of XIAP by the ubiquitin-proteasome system and that a caspase-IAP regulatory loop actively drives degenerative events in the nervous system (Meier et al. 2000; Kuo et al. 2006; Simon et al. 2012; Unsain et al. 2013). Moreover, it indicates that the signaling mechanisms leading to the removal of XIAP are critical for caspase-driven axonal degeneration in mammals.

3.2 IKKε and TBK1 phosphorylate XIAP at Serine 430 in vitro

Accumulating evidence implicates the IκB kinases IKKε and TBK1 in the downregulation

of XIAP leading to caspase activation, however their role in sub-lethal axonal degeneration remains unclear. For instance, the *Drosophila* dmIKKε was shown to directly target DIAP1 for phosphorylation and degradation by the proteasome to promote non-apoptotic functions of DRONC such as regulation of cell morphology, actin dynamics and cell differentiation (Kuranaga et al. 2006; Oshima et al. 2006). Likewise, the mammalian IKKε and TBK1 were shown to promote the phosphorylation and degradation of XIAP in virus-infected cells, resulting in their sensitization to apoptosis (Nakhaei et al. 2012). To begin examining the role of IKKε and TBK1 in axonal degeneration, their interaction with XIAP was first assessed *in vitro* in human embryonic kidney (HEK) 293T cells. Co-expression of either wild type IKKε (Figure 10A) or wild type TBK1 (Figure 10B) decreased XIAP migration in SDS-PAGE, resulting in a molecular weight shift indicative of protein modification. Subsequent treatment with calf intestine phosphatase (CIP) increased XIAP migration, suggesting a phosphorylation event (Figure 10). Additionally, kinase-deficient mutants of IKKε ($IKK\varepsilon^{K38A}$) (Figure 11A) and TBK1 ($TBK1^{K38A}$) (Figure 11B) were unable to decrease XIAP migration in SDS-PAGE, further demonstrating their innate ability to phosphorylate XIAP. It was recently shown by microcapillary liquid chromatography (LC)-MS/MS that XIAP could be phosphorylated by IKKε at Serine 430 (Ser430) (Nakhaei et al. 2012), and that the residues surrounding the site of XIAP phosphorylation by IKKε conformed with the consensus site (x-x-x-Y-x-pS-L-x-Y-x) previously identified as the site of IKKε phosphorylation in the cylindromatosis deubiquitinating enzyme CYLD (Hutti et al. 2009). The Ser430 site in XIAP is located within its RING domain and phosphorylation of this site has been shown to activate the auto-ubiquitination function of the E3 ubiquitin ligase (Nakhaei et al. 2012). To verify the specificity of this phosphorylation site, the XIAP Ser430 residue was mutated to an alanine (XIAP S430A) and a phospho-specific antibody against the XIAP Ser430 phosphopeptide was generated.

Immunoprecipitation of phosphorylated XIAP using the Ser430 phospho-specific antibody detected phosphorylated XIAP only in cells co-expression either wild type IKKε (Figure 12B) or wild type TBK1 (Figure 12C). However, both IKKε and TBK1 still decreased the migration of XIAP S430A in SDS-PAGE (Figure 12A), raising the possibility that other residues within XIAP may also be susceptible to phosphorylation by IKKε and TBK1. Although this finding is in contradiction with current literature on the subject (Nakhaei et al. 2012), protein sequence analysis (Figure 13) revealed that XIAP contains several sites similar to the previously identified IKKε phosphorylation consensus sequence (x-x-x-Y-x-pS-L-x-Y-x). In addition, investigation of known post-translational modifications of XIAP revealed that Threonine 429 (Thr429), adjacent to Ser430, is also a potent XIAP phosphorylation site (PhosphoSitePlus®, www.phosphosite.org) (Hornbeck et al. 2012) and could be a target of IKKε and TBK1 phosphorylation.

Taken together, these results validated the phospho-specific XIAP antibody and demonstrated the ability of the two homologous IKK family members to phosphorylate XIAP at Ser430, a site known to activate XIAP auto-ubiquitination and degradation by the proteasome. Most importantly, it established XIAP as a physiological substrate of IKKε and TBK1, underlining the possibility that they play a role in XIAP regulating during axonal degeneration.

3.3 XIAP is phosphorylated at Ser430 in vivo following NGF withdrawal

To determine whether IKKε and TBK1 mediate XIAP phosphorylation following NGF withdrawal in DRG axons, the Twiss culturing method was used. An initial reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that both IKKε and TBK1 messenger RNA (mRNA) was present in DRG sensory neurons (data not shown). Nevertheless, because transcriptional regulation is not the only predictor of cellular protein levels (Vogel et al. 2010),

others being mRNA decay, translation and protein degradation, mRNA levels were not investigated further and were simply used to confirm the presence of the transcripts in DRGs. NGF-dependent DRG axons were deprived of NGF for 3 and 6 hours, and collected for biochemical analysis (Figure 14). As observed above, XIAP protein levels decline progressively, and XIAP loss is most noticeable after 12 hours of NGF deprivation (Figure 9). As such, XIAP loss cannot be observed at early time points of NGF withdrawal, and immunoprecipitation of phospho-XIAP revealed basal levels of the phosphorylated protein across all time points (Figure 14A). However, blocking proteasomal degradation with epoxomicin resulted in the accumulation of phosphorylated XIAP specifically in axons deprived of NGF, while total XIAP protein levels remained unchanged (Figure 14B). This suggests that robust XIAP phosphorylation occurs early during the axonal degeneration process and must precede XIAP loss via the ubiquitin-proteasome system. Furthermore, the presence of both IKKε and TBK1 mRNA following NGF withdrawal points to the possibility of either (or both) responding to an early signal initiated by NGF withdrawal and phosphorylating XIAP at the start of axonal degeneration.

3.4 Chemical IKKε/TBK1 inhibitor delays axonal degeneration in vivo

To determine whether phosphorylation of XIAP at Ser430 is required for axonal degeneration, cultured DRG sensory axons were treated with the IKKε/TBK1 inhibitor MRT67307, which inhibits IKKε and TBK1 with IC_{50} values of 160 and 19 nM, respectively (Clark et al. 2011). Initial validation of the compound *in vitro* in 293T cells revealed that 0.5 and 1 μM of the inhibitor effectively reduced XIAP phosphorylation by TBK1, but has little to no effect on XIAP phosphorylation by IKKε (Figure 15). This might be expected considering that IKKε and TBK1 were greatly overexpressed in that setting, and that MRT67307 is a much more

potent inhibitor of TBK1 than IKKε. Furthermore, immunocytochemical analysis revealed that application of as little as 0.5 μM of MRT67307 significantly reduced axonal degeneration after 24 hours of NGF deprivation and enhanced cell body integrity (Figure 16).

Taken together, it suggests that XIAP phosphorylation at Ser430 is critical for the initiation of events leading to XIAP loss, caspase activation and axonal degeneration. Additionally, the ability of MRT67307 to significantly delay axonal degeneration at such a low concentration indicates that IKKε/TBK1 play critical roles in the regulation of XIAP and activation of caspase-dependent neurite destruction pathways.

4. DISCUSSION

Axonal degeneration is an essential biological process that drives proper nervous system development and repair. It is critical for the generation of all vertebrate projection systems, as well as accurate neural circuit assembly and plasticity. Much like cellular apoptosis, axonal degeneration is a tightly controlled event that requires integration of multiple external and internal signals that orchestrate the ordered destruction of cellular components. Although it does not involve cell death, it includes cytoskeleton disassembly, membrane swelling, fragmentation, and removal of debris by local phagocytes (Saxena and Caroni 2007). Axonal degeneration therefore represents a fine balance between life and death, wherein dysregulation at any step of the signaling pathway that regulates it can shift this balance and engender a cascade of undesirable consequences. Aberrant activation of degenerative pathways is extremely detrimental to neurons and characterizes a multitude of neurological and neurodegenerative disorders such as ALS (Luo and O'Leary 2005), Parkinson's (Burke and O'Malley 2013) and Alzheimer's (Tsai et al. 2004) disease. While the pathological degeneration observed in disease is not initiated in the same way as developmental axon degeneration, it shares many of the same features and likely involves the co-option of late effector phases of apoptosis (Yuan and Yankner 2000). Unraveling the sequence of events that occurs during developmental axon degeneration is thus necessary for the development of innovative therapies that can delay or prevent this process when it becomes pathologically activated.

One unknown aspect of the axonal degeneration signaling pathway, and the main focus of this study, is the mechanism by which the endogenous caspase inhibitor XIAP gets regulated in this process. Given the recent discovery of the roles of IKK-related kinases IKKε and TBK1 in the activation of non-apoptotic functions of caspases, it is likely that their roles are similarly conserved

in mammalian neurons. This study demonstrates the ability of IKKε and TBK1 to phosphorylate XIAP on Ser430, a site that is phosphorylated following NGF withdrawal from DRG axons. Furthermore, inhibition of IKKε/TBK1 significantly delays axonal degeneration, strongly suggesting their involvement in regulation of XIAP during caspase-dependent axonal degeneration.

It has previously been established that physiological neurite degeneration involves the sub-lethal activation of caspases, which are essential for degenerative events of nervous systems across phylogeny (Kuo et al. 2006; Williams et al. 2006; Unsain et al. 2013). As endogenous caspase inhibitors, IAPs appear to be have a conserved role in caspase regulation in a variety of settings, and their inhibition is required for caspase activation (Unsain et al. 2013). Their role was initially demonstrated in *Drosophila*, where DIAP1-mediated inhibition of the caspase DRONC must be relieved in order for DRONC to actively drive neurite loss (Kuo et al. 2006). In the context of mammalian axonal degeneration, this and previous studies have found that NGF deprivation results in the elimination of XIAP from degenerating axons, with a concurrent accumulation of cleaved and active caspase-3 (Figure 9B) (Unsain et al. 2013). Loss of XIAP in DRG axons is most prominent after 12 hours of NGF deprivation, and DRG axons degenerate completely within 24 to 36 hours of NGF withdrawal (Figure 9A). It has previously been reported that XIAP can be cleaved by caspase-3 (Deveraux et al. 1999; Hornle et al. 2011), raising the possibility that XIAP degradation is a consequence of caspase-3 accumulation. However, XIAP loss is not blocked in $CASP3^{-/-}$ axons deprived of NGF (Unsain et al. 2013). Additionally, XIAP mRNA levels remain unchanged after NGF withdrawal, but proteasomal inhibition prevents XIAP protein loss (Unsain et al. 2013), suggesting that XIAP is regulated at the posttranslational level. As such, XIAP loss essentially precedes caspase accumulation, and NGF deprivation results in the destruction of XIAP

through the ubiquitin-proteasome system. The subsequent accumulation of active caspases is thought to drive degenerative processes that were thus far blocked by XIAP. The caspase-IAP regulatory loop is therefore phylogenetically conserved and requires the removal of XIAP via the proteasome for caspase-driven axonal degeneration to proceed (Meier et al. 2000; Unsain et al. 2013).

The *Drosophila* DmIKKε has recently been identified as a novel antagonist of DIAP1, and thus a positive regulator of caspase activity (Montell 2006). Overexpression of DmIKKε results in increased caspase activity and apoptotic cell death in wing imaginal discs (Kuranaga et al. 2006). It has also been implicated in regulation of F-actin polymerization, where its overexpression causes loss of apical-basal polarity in tracheal epithelial cells, thereby disrupting organization of the tracheal system (Oshima et al. 2006). In both studies, DmIKKε-mediated caspase activation was shown to result from reduction of the DIAP1 protein through its phosphorylation and subsequent degradation. Interestingly, the physiological caspase functions activated by DmIKKε did not involve apoptosis, but rather promoted previously defined non-apoptotic functions of caspases in cell differentiation, actin dynamics and morphogenesis (Kuranaga et al. 2006; Oshima et al. 2006). Recently, the mammalian homologs IKKε and TBK1 were shown to phosphorylate XIAP in the context of a viral-induced immune response (Nakhaei et al. 2012), raising the possibility that their role in XIAP regulation is conserved across phylogeny. The involvement of IKKε and TBK1 in axonal degeneration was thus assessed *in vitro* and *in vivo*. Consistent with previous studies (Nakhaei et al. 2012), IKKε and TBK1 were shown to interact with XIAP in 293T cells, where their overexpression induced a molecular weight shift in XIAP that could be rescued by treatment with a phosphatase (Figure 10). Furthermore, this interaction was dependent on the kinase function of IKKε and TBK1, as overexpression of kinase-deficient mutants did not modify the migration

of XIAP in SDS-PAGE (Figure 11). Identification of the Ser430 residue as the site of XIAP phosphorylation by IKKε (Nakhaei et al. 2012) prompted further investigation of this phosphorylation site located within the RING domain of XIAP. Using a Ser430 phospho-specific antibody, this study demonstrated that IKKε and TBK1 unambiguously phosphorylate XIAP at this residue (Figure 12B and 12C). However, IKKε and TBK1 also affected the migration of the XIAP S430A mutant in SDS-PAGE (Figure 12A), hinting at the existence of other yet unidentified IKKε/TBK1 phosphorylation sites within XIAP. The IKKε phosphorylation motif (x-x-x-Y-x-pS-L-x-Y-x) was initially identified in the deubiquitinating enzyme CYLD, and contains an aromatic residue at the -2 position and a hydrophobic residue at the +1 position of the phosphorylation site (Hutti et al. 2009). CYLD usually functions as a negative regulator of NF-κB, deubiquitinating adaptor proteins involved in NF-κB signaling such as TNF receptor associated factor (TRAF) 2, TRAF6 and IKKγ (Brummelkamp et al. 2003; Kovalenko et al. 2003), as well as the NF-κB family member BCL-3 (Massoumi et al. 2006). Phosphorylation of CYLD by IKKε at Ser418 was shown to decrease its deubiquitinase activity, thereby increasing NF-κB signaling and promoting tumorigenesis (Hutti et al. 2009). In contrast, phosphorylation of XIAP by IKKε at a similar motif on Ser430 was shown to activate the auto-ubiquitination function of the E3 ligase RING domain, leading to Lys^{48}-linked ubiquitination and degradation of XIAP (Nakhaei et al. 2012). Interestingly, phosphorylation at the Ser430 residue by TBK1 did not result in cell death, suggesting that the two homologs function through distinct mechanisms. TBK1 is known to activate Akt following its recruitment to the exocyst complex (Ou et al. 2011), while activated Akt has been shown to phosphorylate XIAP at Ser87 (Dan et al. 2004). Phosphorylation at this residue inhibits the auto-ubiquitinating function of XIAP, resulting in protein stability and cell survival. As such, Akt appears to be the TBK1 substrate that mediates its pro-survival effects, which is

seemingly independent of the XIAP Ser430 site. Another possibility is that XIAP Ser430 phosphorylation by TBK1 is equally important for its pro-survival functions, perhaps even in addition to Ser87 phosphorylation by Akt. While very little is known about XIAP phosphorylation, the XIAP Thr429 residue has been reported as a endogenous phosphorylation site *in vitro* and *in vivo* (PhosphoSitePlus®, www.phosphosite.org) (Hornbeck et al. 2012), making it a potential target of the serine-threonine kinases IKKε and TBK1. However, the nature and physiological relevance of this XIAP phospho-peptide has yet to emerge. Additionally, protein sequence analysis revealed that XIAP contains a second site similar to the previously identified IKKε phosphorylation consensus sequence, at Ser406 (x-x-x-Y-x-pS-L-x-x-x) (Figure 13), with an aromatic residue at the -2 position and a hydrophobic residue at the +1 position of the phosphorylation site. Although there is currently no evidence to suggest that this site is a target of IKKε or TBK1 phosphorylation, it could prove to be a novel site of XIAP regulation and therefore requires further investigation.

Following validation of the phospho-specific XIAP antibody and confirmation of the interaction between XIAP and IKKε/TBK1 *in vitro*, XIAP phosphorylation at Ser430 was assessed *in vivo* in DRG axons deprived of NGF (Figure 14). As such, phosphorylated XIAP was shown to accumulate specifically in DRG axons deprived of NGF, while total XIAP protein levels remained unchanged (Figure 14B). Given that a decrease in XIAP protein levels was not observed at early time points of NGF deprivation (Figure 9 and Figure 14A), it suggests that XIAP phosphorylation occurs early following NGF withdrawal, and must precede XIAP loss. Furthermore, XIAP phosphorylation at Ser430 has previously been shown to result in its auto-ubiquitination and degradation via the proteasome (Nakhaei et al. 2012), which likely also happens in degenerating axons. This is demonstrated by blocking proteasomal degradation in degenerating axons, which

results in the accumulation of phosphorylated XIAP (Figure 14B) that normally undergoes quick turnover (Figure 14A). The known ability of IKKε and TBK1 to phosphorylate XIAP at Ser430 directly implicates them in this process, further suggesting that their activation must result from a signaling cascade initiated by NGF withdrawal.

To observe whether IKKε and TBK1 have an effect on axonal degeneration *in vivo*, the chemical inhibitor MRT67307 was applied to DRG axons deprived of NGF for 24 hours (Figure 16). Although initial application of 0.5 and 1 μM of the compound on 293T cells seemed to have a minimal effect on XIAP phosphorylation by IKKε (Figure 15), NGF-dependent axonal degeneration was significantly delayed with 0.5 μM of MRT67307 (Figure 16). Furthermore, cell body integrity was greatly enhanced in the presence of the compound, suggesting an inhibitory effect on cell death pathways. Given the specificity of the compound for TBK1 (IC_{50} = 19 nM) and IKKε (IC_{50} = 160), it indicates that inhibition of the IKK-related kinases blocks caspase-dependent axonal degeneration *in vivo*, likely via to the inhibitory actions of XIAP. It is not surprising that MRT67307 could not completely inhibit XIAP phosphorylation in 293T cells, particularly because XIAP, IKKε and TBK1 were greatly overexpressed in that setting. However, a minimal dose of the compound significantly reduced axonal degeneration in the presence of endogenous IKKε and TBK1, suggesting that they play important roles regulating XIAP in this process. It will therefore be necessary to observe whether different doses of MRT67307 have a proportional effect on XIAP phosphorylation in DRG axons *in vivo*, as well as on caspase-driven axonal degeneration.

These findings provide novel insight into the mechanistic role of caspases and their regulators in developmental axon degeneration, however they also raise a number of compelling questions. For example, how are IKKε and TBK1 activated in this process? Is there only one

activation pathway or does it dependent on the biological context? Do IKKε and TBK1 play similar or distinct roles in this setting? These and many other questions will need to be answered in order to improve current knowledge on the subject.

In the context of viral infection, binding of foreign nucleic acids (e.g. RNA or DNA from viruses or damaged cells) to RIG-I and the melanoma differentiation-associated gene 5 (MDA-5) leads to the activation of their caspase-recruitment domain (CARD)-like domains, which can interact with various adaptor proteins such as TRAF3, TRAF6 and mitochondrial antiviral signaling (MAVS) (Chau et al. 2008). These adaptors can in turn modulate the activation of various kinases, including IKKε and TBK1. IKKε has been characterized in association with the mitochondrial reticular network, particularly with MAVS (Lin et al. 2006), creating a signaling platform that mediates an apoptotic response (Nakhaei et al. 2012). In contrast, RIG-I activation of TBK1 mediates the recruitment of TBK1 to the exocyst, where it interacts with Akt and mediates cell survival through XIAP protein stability (Nakhaei et al. 2012). Through a distinct mechanism, signaling via TLRs results in the activation of TRAF3 and recruitment of IKKε and TBK1 through interaction with the adaptor protein TANK (Shen and Hahn 2011). Additionally, activated MAVS can also signal to TRAF3, thereby bridging the NF-κB and IFN responses. IKKε and TBK1 can further be activated through TNF signaling, via the association of TRAFs to the TNFR-associated death domain (TRADD) adaptor protein (Chau et al. 2008). As such, the ability of IKKε and TBK1 to be activated by a variety of signaling pathways likely contributes to their complexity and functional diversity.

In the context of axonal degeneration, it remains unclear how NGF withdrawal triggers activation of IKKε/TBK1. IKKε and TBK1 activity is regulated through phosphorylation of Ser172 within the classical kinase activation loop (Peters and Maniatis 2001). Earlier findings

proposed that platelet-derived growth factor (PDGF) could stimulate TBK1 activity through protein kinase C (Tojima et al. 2000), which lies downstream of TNFR signaling. Moreover, the ability of TRAFs (e.g. TRAF3 and TRAF6) to bind to most members of the TNFR superfamily, including the neurotrophin receptor p75NTR, could allow them to mediate recruitment and activation of IKKε/TBK1 following NGF withdrawal. Cell death pathways are known to be activated by binding of TRAFs to the death domain of p75NTR, thereby creating a platform for recruitment of adaptors and kinases in a stimulus- and cell-dependent manner. It has recently been reported that the multi-domain protein SARM (sterile α-motif-containing and armadillo-motif containing protein), a member of the TLR adaptor protein family, associates with neuronal mitochondria and is crucial for NGF-dependent and injury-induced axonal degeneration (Gerdts et al. 2013). Interestingly, SARM was also shown to inhibit TRIF (Toll/interleukin 1 receptor (TIR)-domain-containing adaptor protein inducing IFN-β), which is thought to recruit TBK1 to TRAF3 following signaling through TLR3 (O'Neill and Bowie 2007). In doing so, SARM appears to be a negative regulator of IRF and NF-κB signaling, which is perhaps necessary for re-directing IKKε and TBK1 to the axonal degeneration signaling pathway. While the mechanism by which IKKε and TBK1 are activated are thus far only speculative, it is probable that their activation results from stimulus-specific interactions with surface receptors and adaptor proteins that bring their targets in close proximity.

The distinct mechanisms of action of IKKε and TBK1 also implicate them differently in pathogenesis. IKKε is amplified in 30% of breast cancers and promotes tumorigenesis through the activation of NF-κB (Eddy et al. 2005; Shen and Hahn 2011). Meanwhile, the association of TBK1 with the exocyst is essential for the transformation and survival of KRAS-driven cancer cell lines (Bodemann and White 2008). Moreover, duplication of the *TBK1* gene leads to normal tension

glaucoma (NTG), which may result in retinal ganglion cell death by activation of autophagy or altered NF-κB signaling (Ritch et al. 2014). Interestingly, the only other known NTG protein, optineurin, is a known substrate of TBK1 phosphorylation. More recently, loss-of-function mutations in *TBK1* were found to be implicated in familial ALS and dementia (Freischmidt et al. 2015). The identified mutations affected the C-terminal CCD2 domain of TBK1, which mediates interactions with multiple adaptors proteins and regulate the activation of downstream signaling pathways (Goncalves et al. 2011). Mutations of the CCD2 domain affected binding to optineurin, but had no effect on phosphorylation and activation of IRF3. Dysregulation of optineurin is also known to cause ALS (Maruyama et al. 2010), thereby linking the two proteins in this process. As such, there is a strong role for TBK1 in maintaining neuronal homeostasis, probably through its functions in regulated axonal degeneration.

While this study offers a novel interpretation of XIAP regulation in neurites, further investigations are required. For instance, it will be important to define other phosphorylation sites on XIAP, particularly given that IKKε and TBK1 appear to phosphorylate more than one residue (Figure 12A). Furthermore, it will be relevant to biochemically assess XIAP phosphorylation *in vivo* in the presence of MRT67307, as well as quantify the degeneration of axons that occurs in parallel. Additionally, upstream activation of IKKε and TBK1 following NGF withdrawal could be monitored using Ser172 phospho-specific antibodies that are commercially available (e.g. Cell Signaling Technology #5483 and #8766) and have already been used in different contexts (e.g. (Xie et al. 2011)). Eventually, XIAP phosphorylation status and axonal degeneration should be observed in DRGs derived from TBK1 and IKKε null animals, which will reinforce their implication in NGF-dependent axonal degeneration. It may also help discern their mutual involvement in the process, which has so far been difficult to achieve. Ultimately, linking NGF

withdrawal from surface receptors to TBK1/IKKε activation and subsequent phosphorylation of XIAP could be achieved by genetic and pharmacological manipulations of upstream targets, such as p75NTR and adaptor protein TRAF3.

The ultimate goal of unraveling the sequence of events leading to axonal degeneration is to find therapeutic targets that can help prevent or manage the devastating consequences of neurodegenerative diseases. This, and previous work, has identified the IκB kinases IKKε and TBK1 as positive regulators of caspase activity, and were shown to be dysregulated in a number of settings. Furthermore, given the ability of the MRT67307 inhibitor to delay axonal degeneration, it is tempting to propose IKKε and/or TBK1 as targets for therapeutic treatment of diseases displaying axonal atrophy. However, such assertions should be made with caution, as much still remains to be learned about these kinases. Nevertheless, increasing evidence suggests that blocking the functions of these non-canonical IKKs can have positive effects on the body. For instance, inhibition of IKKε/TBK1 leads to increased liver and adipose tissue inflammation, which promotes weight loss and improved glucose tolerance in obese mice (Reilly et al. 2013). MRT67307 has also been shown to block autophagy in cancer cells, which they require to combat tumorigenesis-induced stress (Petherick et al. 2015). However, IKKε and TBK1 have also been shown to be targeted by viruses to prevent inflammation and promote productive infection (Ma et al. 2012). Given their important roles in immunity and inflammation, further investigations will therefore be required in order to find ways to inhibit their activity in pathological settings with minimal side effects.

Taken together, it appears that IKKε and TBK1 can mediate caspase-dependent axonal degeneration through a mechanism resembling incomplete apoptosis. In this manner, cells harness the activity of caspases and their regulators to accomplish housekeeping and developmental tasks

utilizing a subset of the features of cell death pathways. In the context of axonal degeneration, this study proposes a mechanisms by which IKKε and TBK1 mediate sub-lethal caspase activation by regulating XIAP (Figure 17). As such, NGF withdrawal results in the initiation of a signaling cascade that activates IKKε and TBK1, which in turn phosphorylate XIAP and target it for ubiquitination and degradation by axonal proteasomes. The elimination of XIAP liberates caspases, which are free to proceed with the destruction of specific neurite segments. While further mechanistic investigations are required, it seems likely that IKKε/TBK1-mediated caspase activation pathways are conserved across phylogeny, and play a critical role in XIAP regulation during developmental axon degeneration.

Finally, the work accomplished in this study brings us a little bit closer to understanding the signaling pathways that are activated during axonal degeneration. While previous work focused on the role of IKKε and TBK1 in immunity and cellular caspase activation, here we show that their function as XIAP regulators is conserved in mammalian neurons, and required for activation of caspase-dependent degenerative processes. The novel use of the IKKε/TBK1 inhibitor in this setting further implicates these kinases in axon degeneration, and opens the door to future work that can unravel other aspects of this critical developmental pathway. Ultimately, it may also aid in the development of novel therapies that will improve the management and progression of many devastating diseases of the nervous system.

5. FIGURES AND LEGENDS

Figure 1

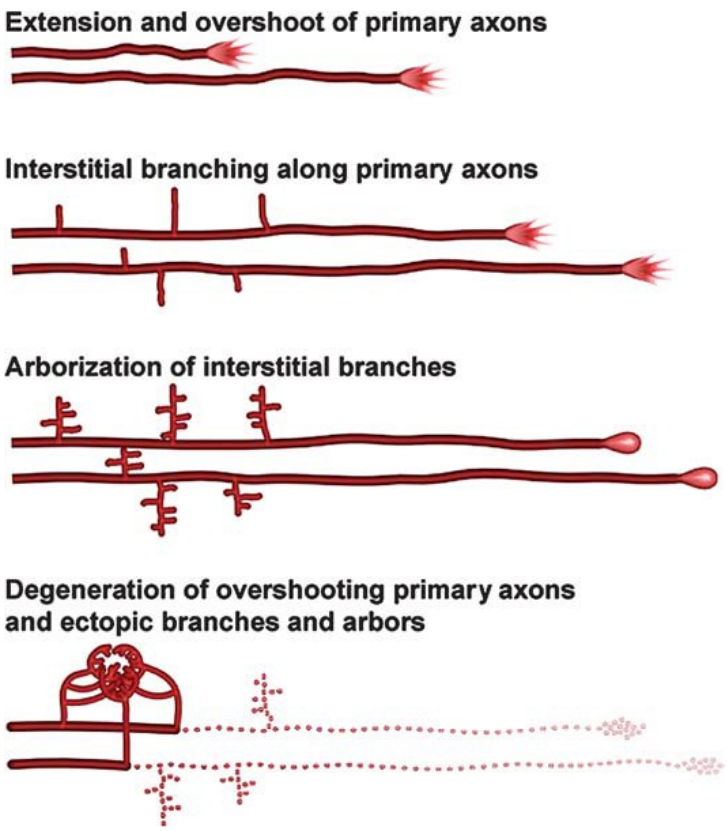

Figure 1: Overview of developmental axon degeneration. Primary axons extent long processes in response to guidance molecules in the environment. Interstitial branches that extend along the primary axons arborize and form local connections. Distal components of the primary axons are then eliminated to retain only proper connections (Adapted from (Luo and O'Leary 2005)).

Figure 2

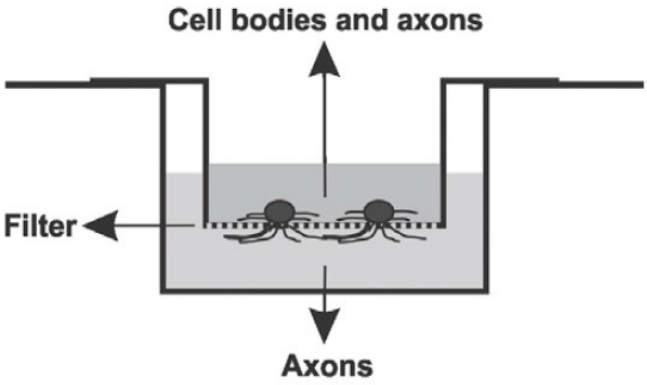

Figure 2: Twiss culturing method. Dissociated DRG explants are plated on porous filters as illustrated. Following NGF deprivation, axons from the bottom of the filter can be collected for immunocytochemical and biochemical analysis (Adapted from (Unsain et al. 2013)).

Figure 3

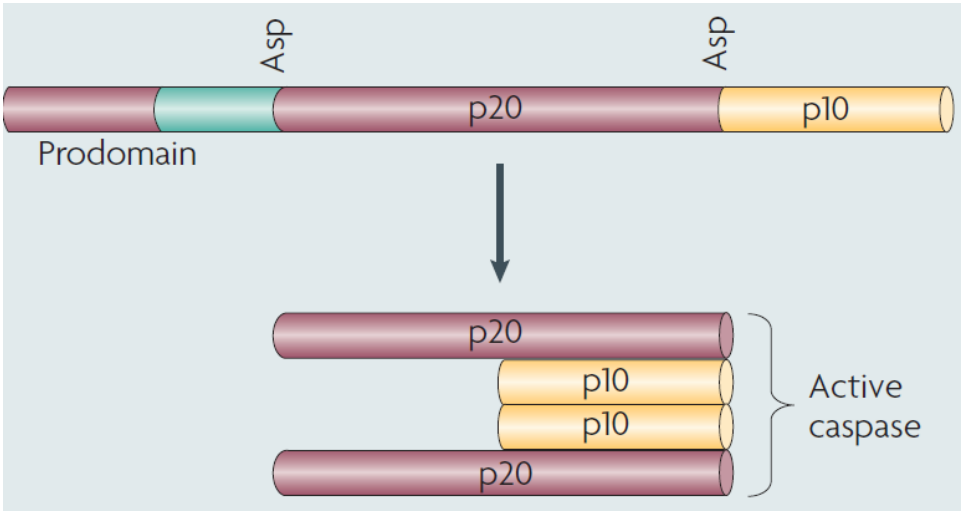

Figure 3: Caspase structure and activation. Caspases are synthesized as pro-enzymes that contain an N-terminal pro-domain, a central "large subunit" and a C-terminal "small subunit". Activation of procaspases requires two proteolytic cleavage events at aspartate residues, typically by other caspases. Dimerization of cleaved caspase subunits is essential for caspase activity (Adapted from (Clarke and Tyler 2009)).

Figure 4

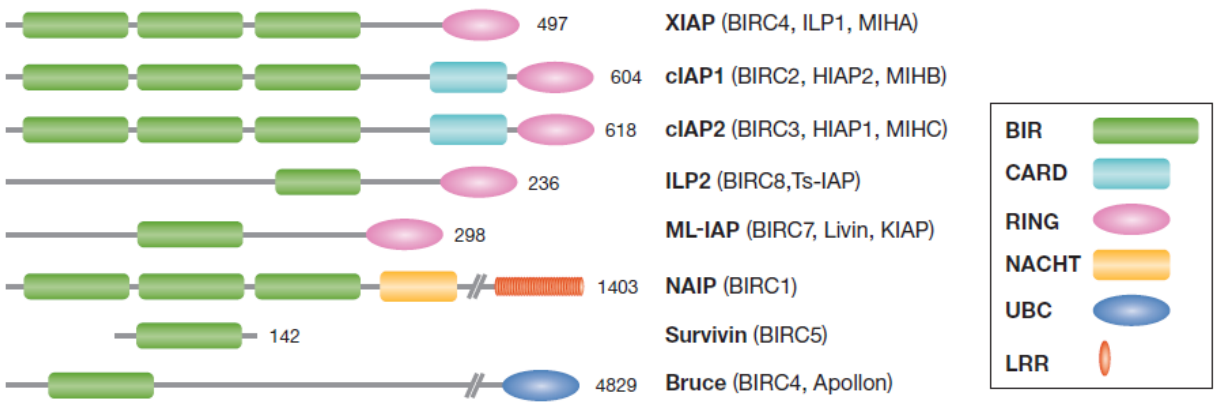

Figure 4: Schematic representation and structure of human IAP family. All IAP family members have at least one BIR domain, which mediates interactions with caspases. The RING domain is an E3 ligase that can direct targets to the ubiquitin-proteasome degradation system, while caspase-recruitment domains (CARDs) can mediate homotypic protein–protein interactions, although the binding partners of cIAP1 and cIAP2 have not been elucidated (Adapted from (Eckelman et al. 2006)).

Figure 5

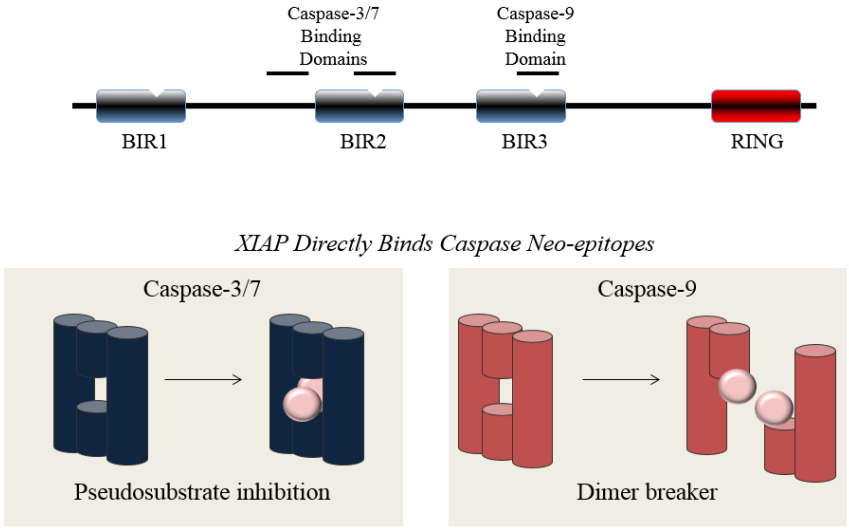

Figure 5: Functional domains of XIAP protein. BIR1 can interact with various IBM-containing proteins to regulate cell signaling. BIR2 and BIR3 contain IBM-binding grooves that interact with caspases and lock them in an inactive conformation via adjacent inhibitory elements, while the RING domain can target proteins for proteasomal degradation (Courtesy of Dr. Phil Barker).

Figure 6

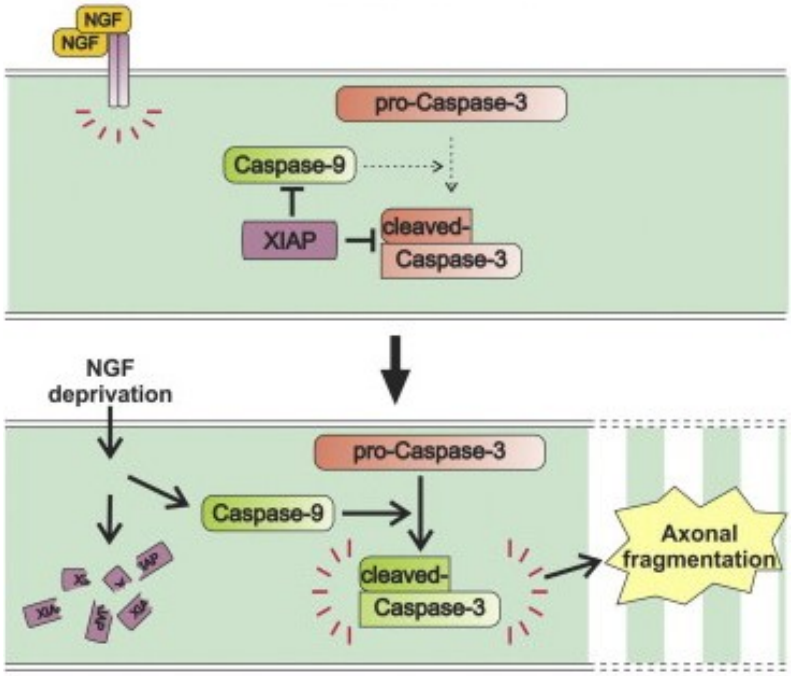

Figure 6: Model of caspase regulation by XIAP in degenerating axons. XIAP normally binds to and inhibits active caspases. Upon NGF withdrawal, XIAP is eliminated via the proteasome and axon degeneration can process through the action of caspases (Adapted from (Unsain et al. 2013)).

Figure 7

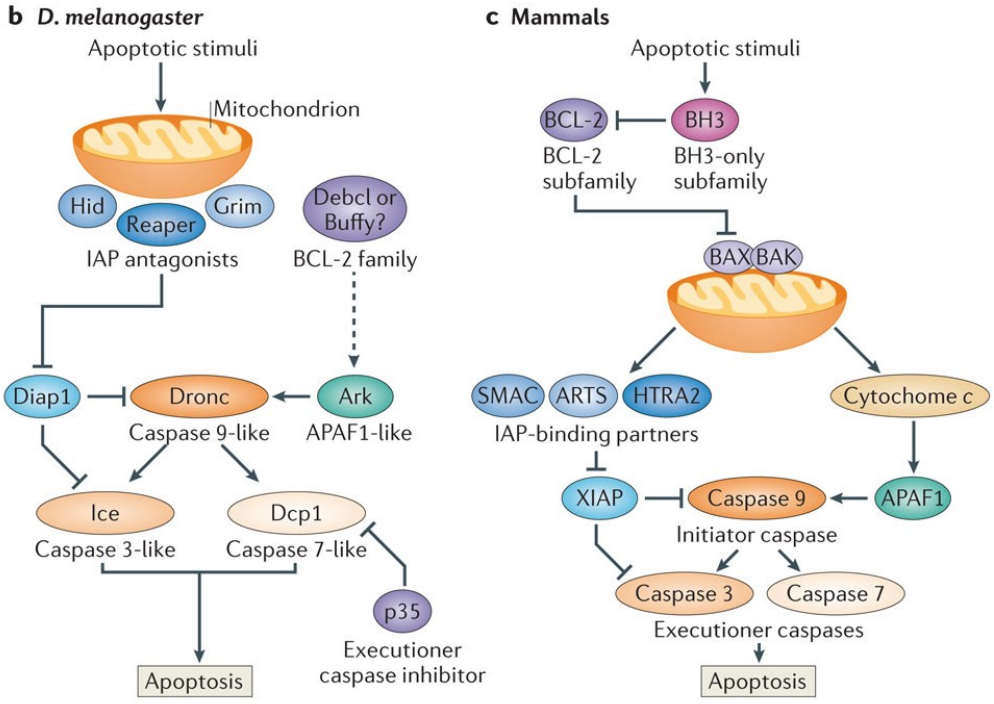

Figure 7: Endogenous IAP inhibitors in *Drosophila* and mammals. Homologous proteins have evolved across phylogeny to inhibit the activity of IAPs following an apoptotic stimulus. Binding to IAPs displaces them from caspases, which are free to proceed with cell death (Adapted from (Fuchs and Steller 2015)).

Figure 8

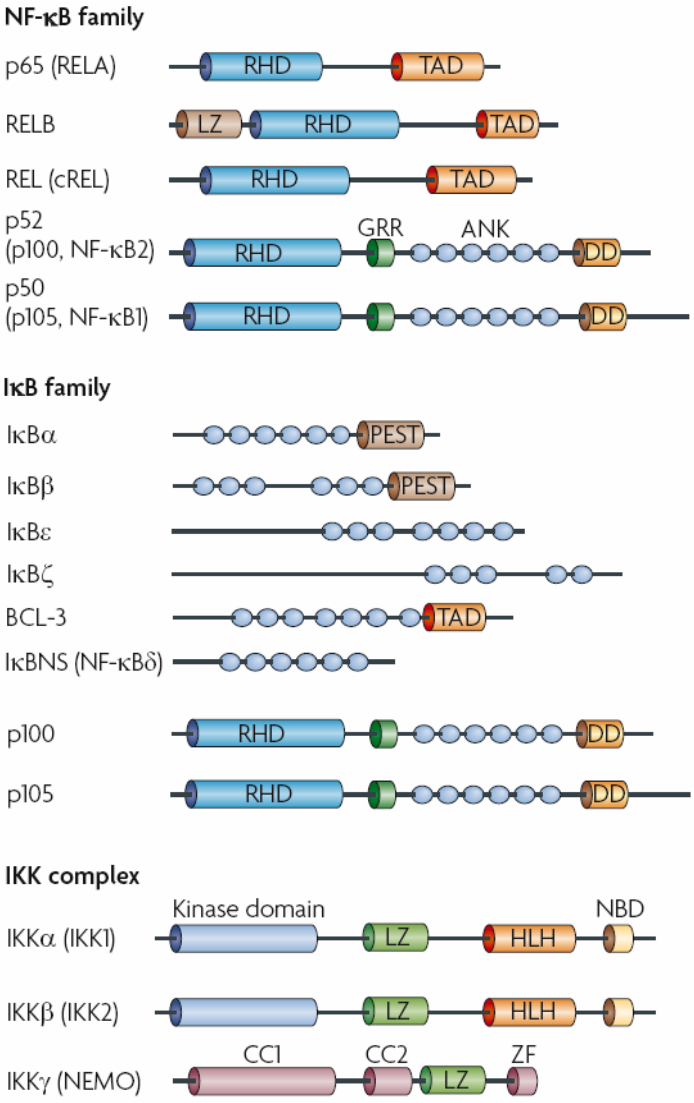

Figure 8: Structure of human NF-κB, IκB and canonical IKK protein families. Mature NF-κB subunits do not contain ANK domains, which are unique to IκB proteins. In the canonical pathway, IKKs mediate the phosphorylation of IκB, thereby targeting it for ubiquitination and degradation by the proteasome. In the non-canonical pathway, IKKs phosphorylate p100, thereby mediating its proteolytic cleavage and the activation of the NF-κB p52 subunit (Adapted from (Ghosh and Hayden 2008)).

Figure 9

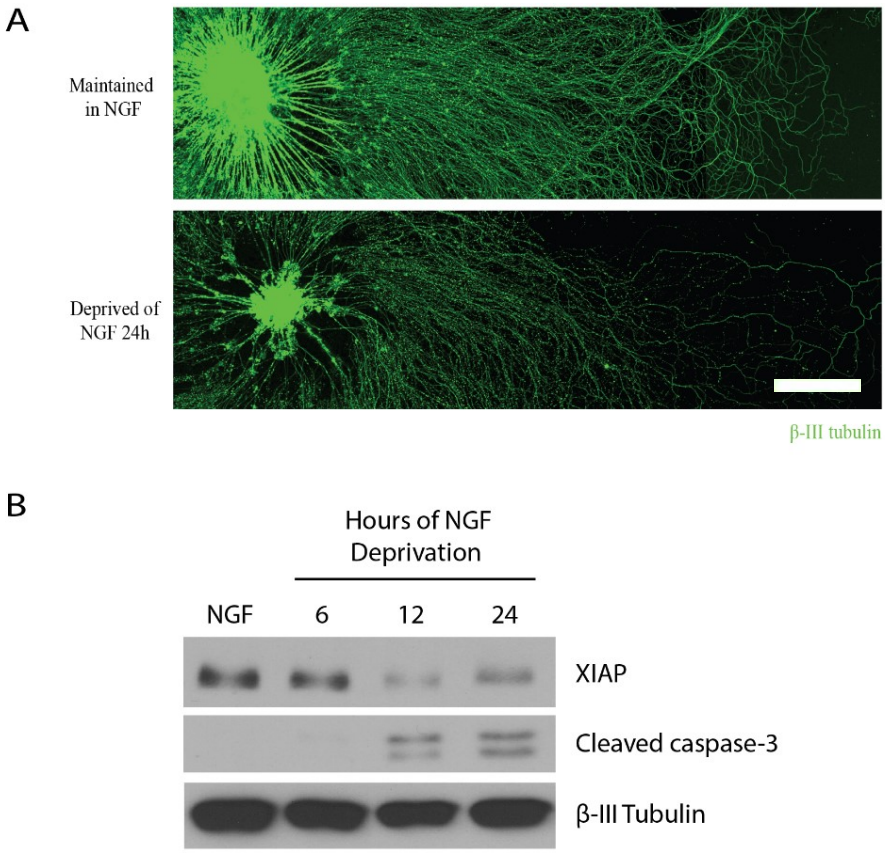

Figure 9: NGF withdrawal results in XIAP loss and axonal degeneration. (A) DRG explants derived from E13.5 embryos extend long processes in response to NGF, while NGF withdrawal results in the degeneration of axons. Immunocytochemical analysis was performed by staining explants with anti-β-III-tubulin antibody to visualize axons (courtesy of Aaron Johnstone). Scale bar, 200 μm. (B) DRGs axons were deprived of NGF for the indicated times and analyzed for levels of total XIAP and cleaved caspase-3 by immunoblot.

Figure 10

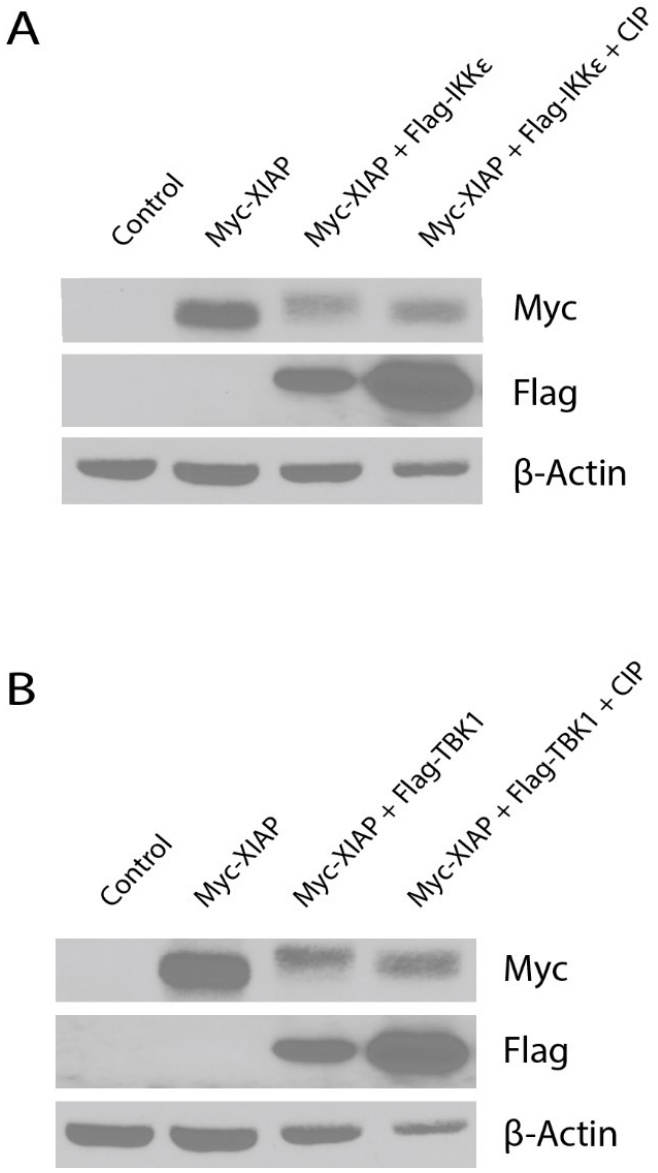

Figure 10: IKKε and TBK1 phosphorylate XIAP *in vitro*. (A) Overexpression of Flag-IKKε in 293T cells decreases the migration of XIAP in SDS-PAGE. Subsequent treatment with calf intestine phosphatase restores XIAP migration in SDS-PAGE. (B) Overexpression of Flag-TBK1 in 293T cells decreases the migration of XIAP in SDS-PAGE. Subsequent treatment with calf intestine phosphatase restores XIAP migration in SDS-PAGE.

Figure 11

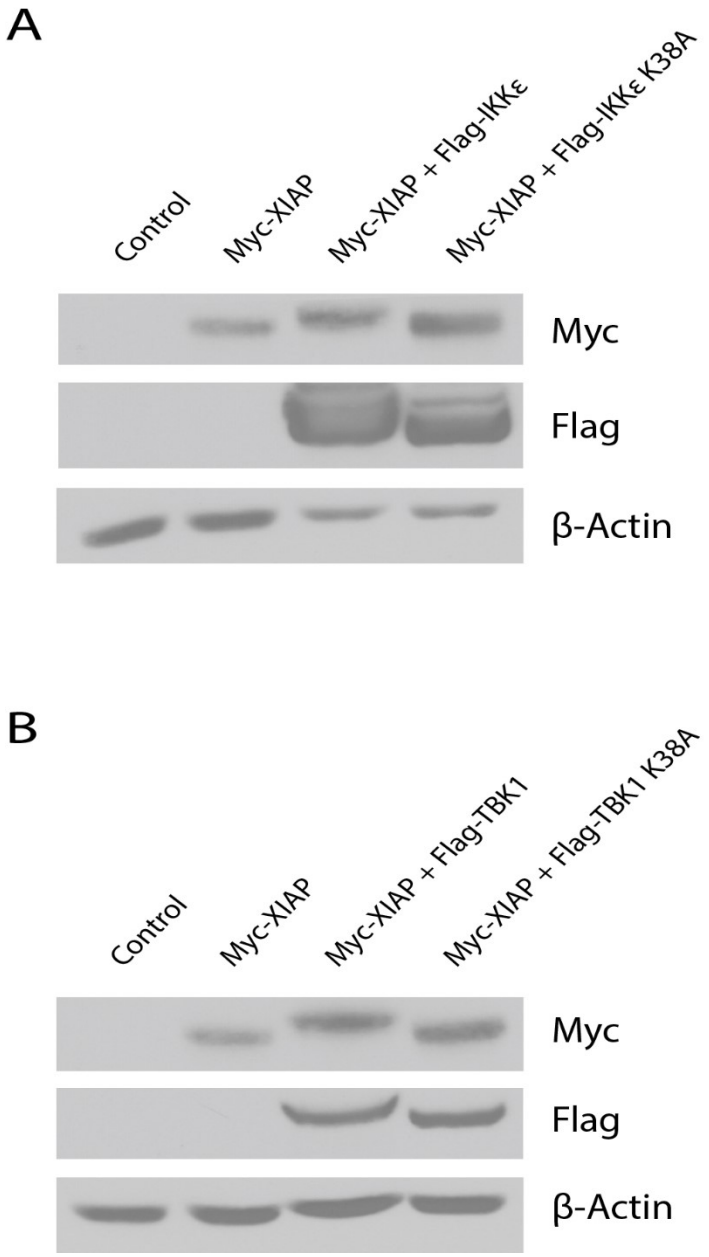

Figure 11: Kinase-deficient mutants of IKKε and TBK1 do not phosphorylate XIAP. (A) Overexpression of Flag-IKKε in 293T cells, but not Flag-IKKε^{K38A}, decreases the migration of XIAP in SDS-PAGE. (B) Overexpression of Flag-TBK1 in 293T cells, but not Flag-TBK1^{K38A}, decreases the migration of XIAP in SDS-PAGE.

Figure 12

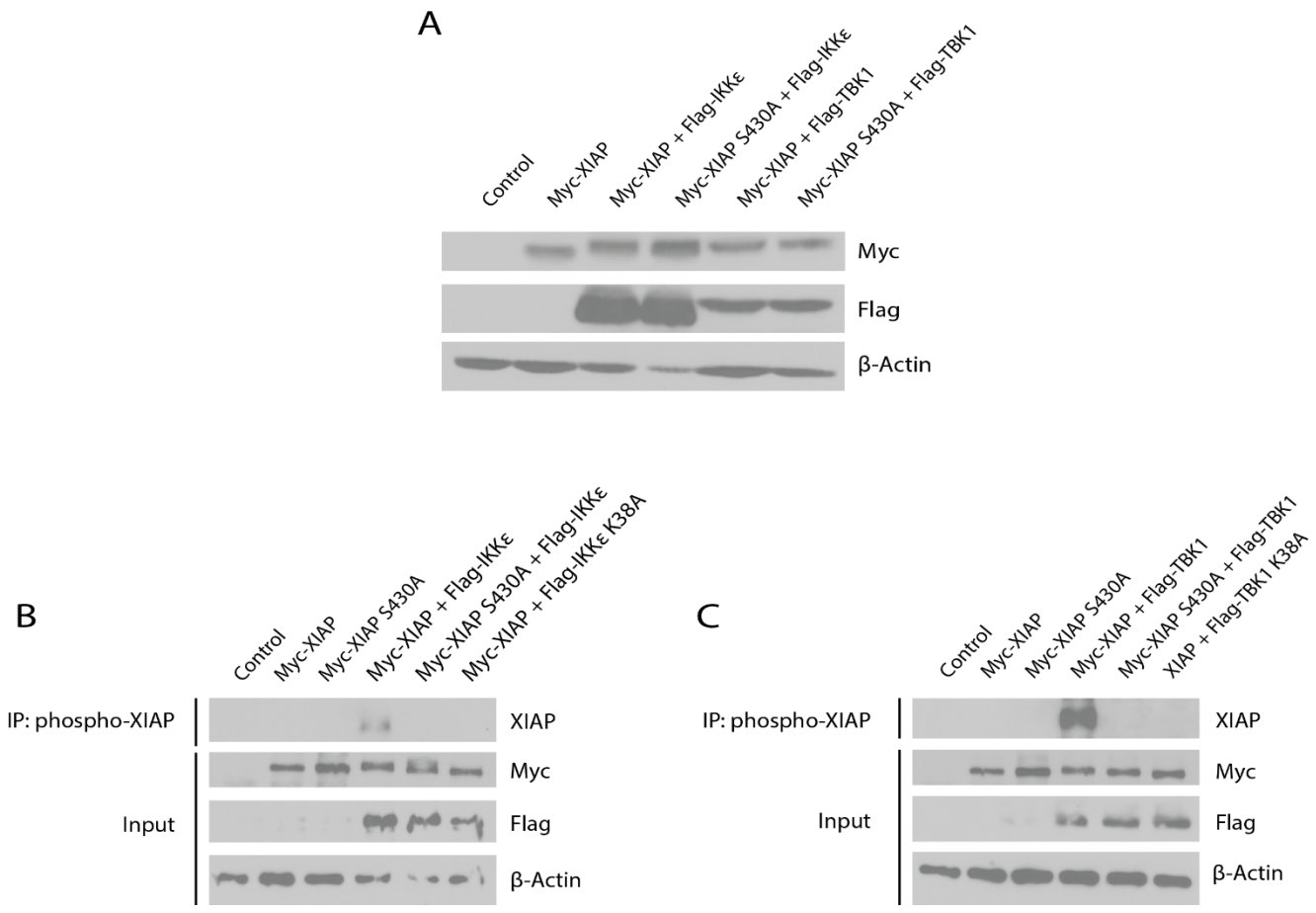

Figure 12: IKKε and TBK1 phosphorylate XIAP at Ser430. (A) Overexpression of Flag-IKKε and Flag-TBK1 in 293T cells decreases the migration of wild-type and S430A mutant XIAP in SDS-PAGE. Immunoprecipitation of phospho-XIAP detected XIAP phosphorylated at Ser430 only in cells co-expressing wild-type XIAP and wild-type IKKε (B) or TBK1 (C).

Figure 13

Figure 13: Potential sites of XIAP phosphorylation by IKKε and TBK1. The Ser430 site of XIAP phosphorylation by IKKε and TBK1 conforms to the consensus sequence (x-x-x-Y-x-pS-L-x-Y-x) previously identified as the site of IKKε phosphorylation in the enzyme CYLD. Sequence analysis of XIAP reveals another site that conforms to the x-x-x-Y-x-pS-L-x-Y-x consensus sequence at Ser406. Additionally, Thr429 has been identified as a potent XIAP phosphorylation site. Arrows points to phospho-serines within the phosphorylation sequence.

Figure 14

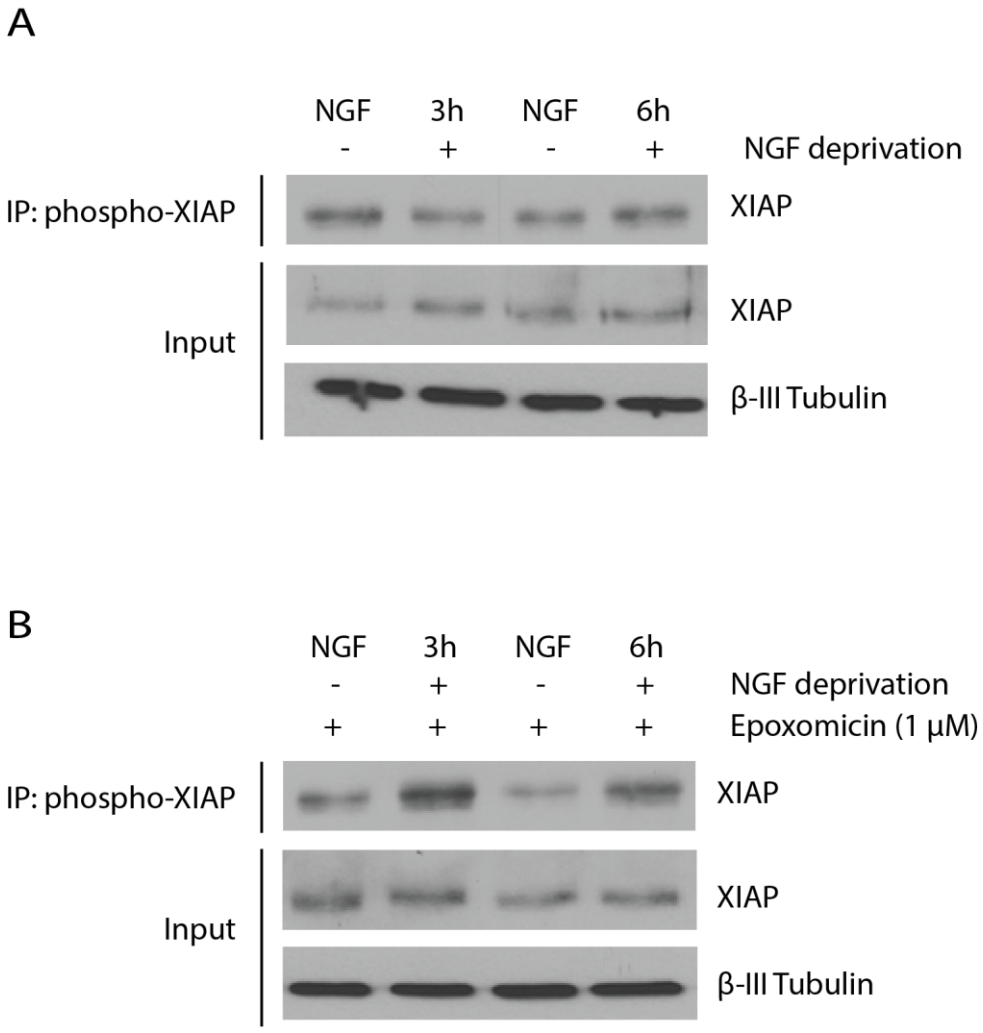

Figure 14: XIAP is phosphorylated at Ser430 following NGF withdrawal. (A) DRG axons were deprived of NGF for the indicated times and analyzed for levels of phosphorylated and total XIAP. At early times point, XIAP loss is not observed and only basal levels of phosphorylated XIAP are detected. (B) Inhibition of proteasomal degradation with 1 μM epoxomicin resulted in the accumulation of phosphorylated XIAP specifically in axons deprived of NGF, while total XIAP levels remained unchanged. Phosphorylated XIAP was detected by immunoprecipitation using the anti-phospho-XIAP antibody and immunoblotting with anti-XIAP antibody.

Figure 15

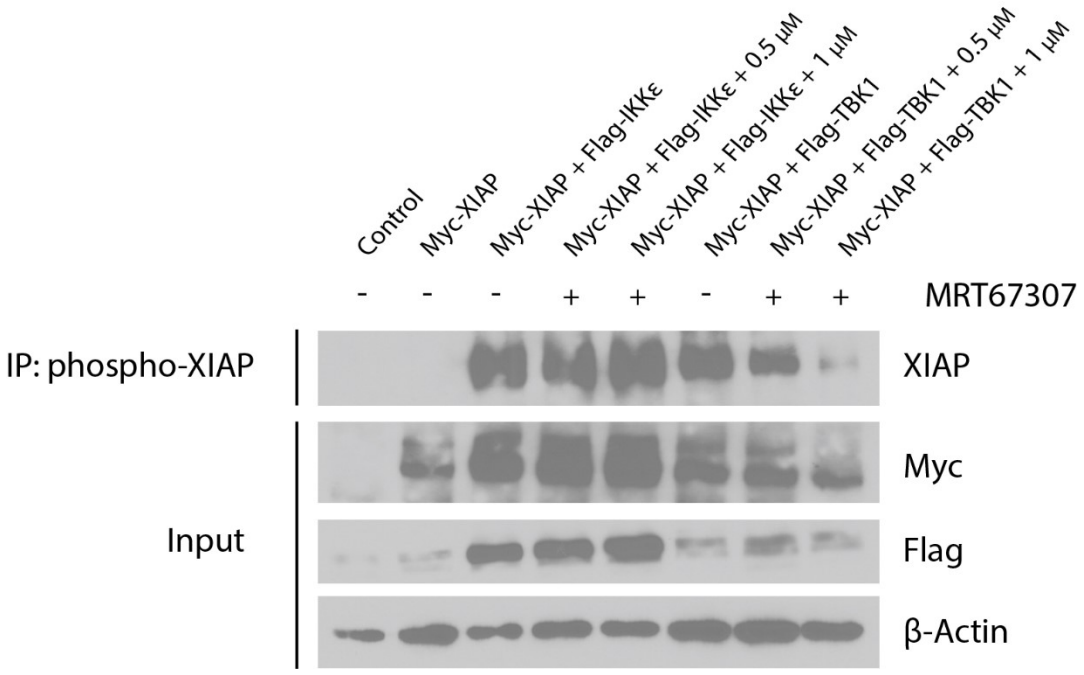

Figure 15: MRT67307 inhibits XIAP phosphorylation in 293T cells. Application of 0.5 and 1 μM of the chemical inhibitor of IKKε/TBK1 inhibits XIAP phosphorylation by TBK1 significantly, but has little to no effect on XIAP phosphorylation by IKKε.

Figure 16

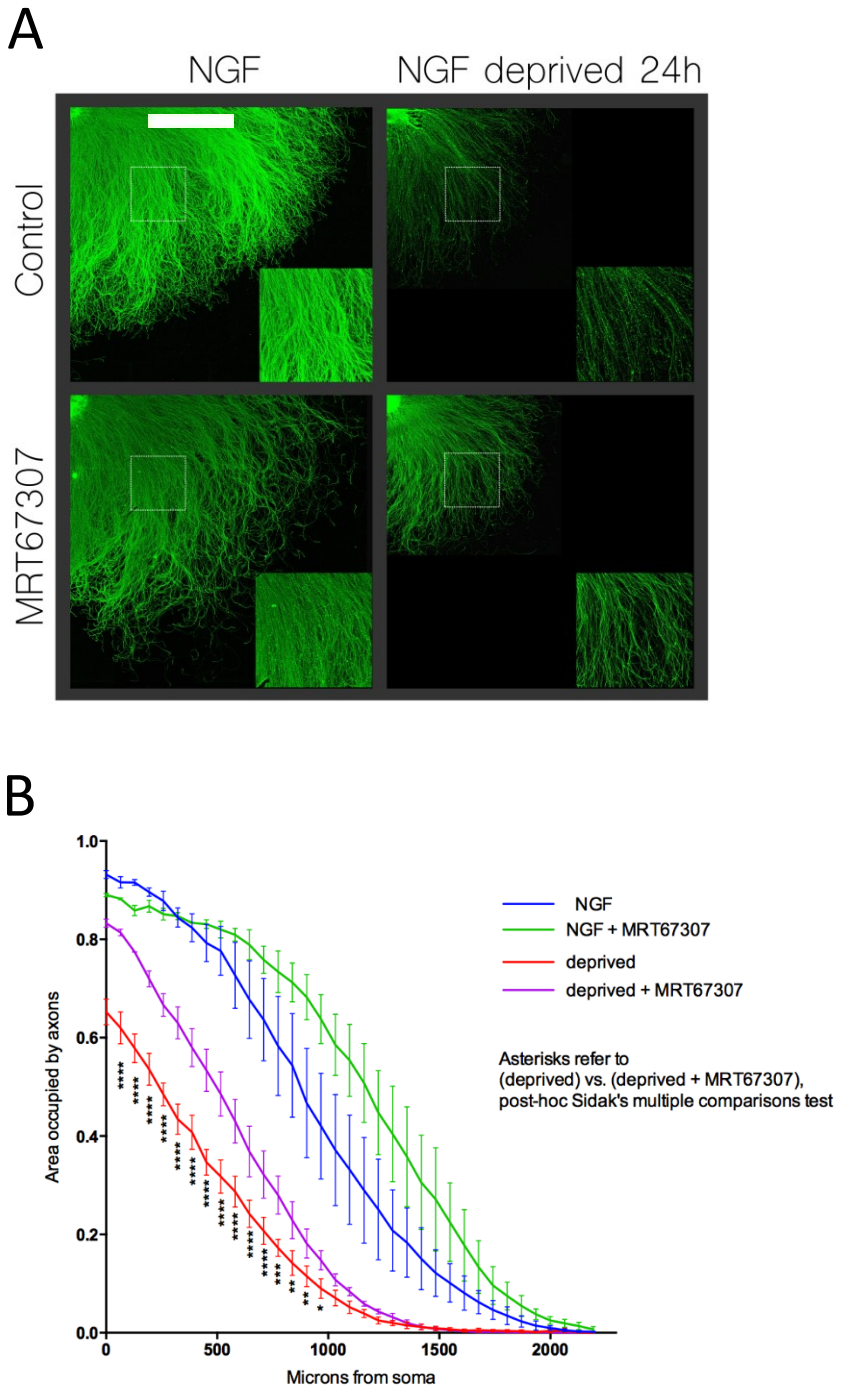

Figure 16: MRT67307 delays axonal degeneration *in vivo*. (A) Application of 0.5 µM of the chemical inhibitor of IKKε/TBK1 delays axonal degeneration and enhances cell body integrity. (B) Quantification of axonal loss (**** = $p < 0.0001$). DRG explants were stained with anti-β-III-tubulin antibody to visualize axons. Scale bar, 500 µm.

Figure 17

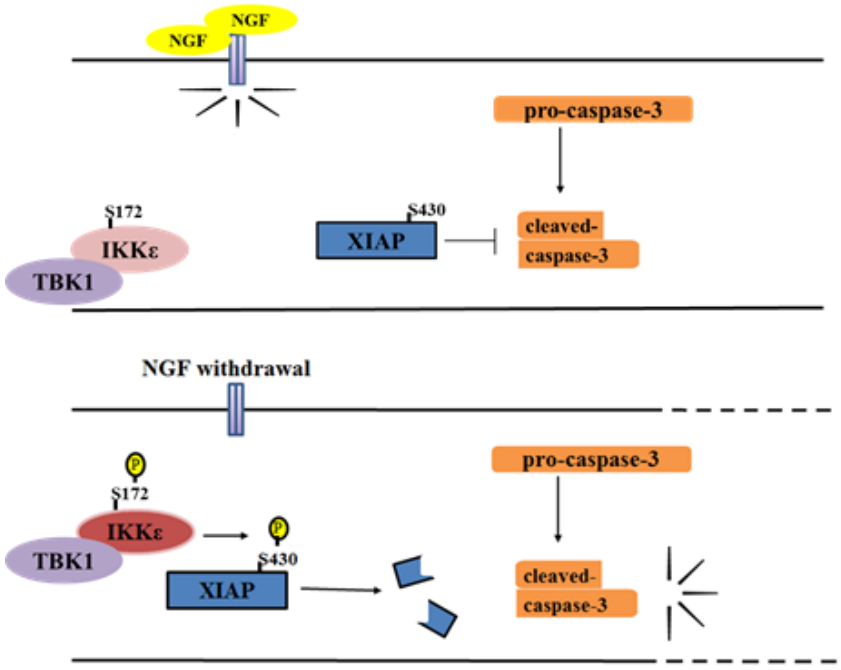

Figure 17: Proposed model of XIAP regulation by IKKε/TBK1 following NGF withdrawal. In the presence of NGF, IKKε and TBK1 are inactive, which allows XIAP to inhibit caspase activity. NGF withdrawal leads to the activation of IKKε and/or TBK1, which phosphorylate XIAP and target it for degradation. This frees cleaved caspase-3 to proceed with axonal degeneration

6. REFERENCES

Akira S, Uematsu S and Takeuchi O (2006). Pathogen Recognition and Innate Immunity. *Cell* 124(4): 783-801.

Bodemann BO and White MA (2008). Ral GTPases and cancer: linchpin support of the tumorigenic platform. *Nat Rev Cancer* 8(2): 133-140.

Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, Itie A, Wakeham A, Shahinian A, Henzel WJ, Elia AJ, Shillinglaw W, Mak TW, Cao Z and Yeh WC (2000). Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. *EMBO J* 19(18): 4976-4985.

Brasier AR (2006). The NF-kappaB regulatory network. *Cardiovasc Toxicol* 6(2): 111-130.

Brummelkamp TR, Nijman SM, Dirac AM and Bernards R (2003). Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. *Nature* 424(6950): 797-801.

Burke RE and O'Malley K (2013). Axon degeneration in Parkinson's disease. *Experimental Neurology* 246(0): 72-83.

Campenot RB (1977). Local control of neurite development by nerve growth factor. *Proc Natl Acad Sci U S A* 74(10): 4516-4519.

Cavanagh JB (1964). The significance of the "dying back" process in experimental and human neurological disease. *Int Rev Exp Pathol* 3: 219-267.

Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA and et al. (1992). Molecular cloning of the interleukin-1 beta converting enzyme. *Science* 256(5053): 97-100.

Chau T-L, Gioia R, Gatot J-S, Patrascu F, Carpentier I, Chapelle J-P, O'Neill L, Beyaert R, Piette J and Chariot A (2008). Are the IKKs and IKK-related kinases TBK1 and IKK-ε similarly activated? *Trends Biochem Sci* 33(4): 171-180.

Clark K, Peggie M, Plater L, Sorcek Ronald J, Young Erick RR, Madwed Jeffrey B, Hough J, McIver Edward G and Cohen P (2011). Novel cross-talk within the IKK family controls innate immunity.

Clarke P and Tyler KL (2009). Apoptosis in animal models of virus-induced disease. *Nat Rev Micro* 7(2): 144-155.

Cowan W, Fawcett J, O'Leary D and Stanfield B (1984). Regressive events in neurogenesis. *Science* 225(4668): 1258-1265.

Crook NE, Clem RJ and Miller LK (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J Virol* 67(4): 2168-2174.

Dan HC, Sun M, Kaneko S, Feldman RI, Nicosia SV, Wang HG, Tsang BK and Cheng JQ (2004). Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *J Biol Chem* 279(7): 5405-5412.

Degterev A, Boyce M and Yuan J (2003). A decade of caspases. *Oncogene* 22(53): 8543-8567.

Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS and Reed JC (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases.

Deveraux QL and Reed JC (1999). IAP family proteins—suppressors of apoptosis. *Genes Dev* 13(3): 239-252.

Deveraux QL, Takahashi R, Salvesen GS and Reed JC (1997). X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 388(6639): 300-304.

Doyle SL and O'Neill LAJ (2006). Toll-like receptors: From the discovery of NFκB to new insights into transcriptional regulations in innate immunity. *Biochemical Pharmacology* 72(9): 1102-1113.

Du C, Fang M, Li Y, Li L and Wang X (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102(1): 33-42.

Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM and Thompson CB (1996). A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *The EMBO Journal* 15(11): 2685-2694.

Eckelman BP, Salvesen GS and Scott FL (2006). Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO reports* 7(10): 988-994.

Eddy SF, Guo S, Demicco EG, Romieu-Mourez R, Landesman-Bollag E, Seldin DC and Sonenshein GE (2005). Inducible IkappaB kinase/IkappaB kinase epsilon expression is induced by CK2 and promotes aberrant nuclear factor-kappaB activation in breast cancer cells. *Cancer Res* 65(24): 11375-11383.

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

Elmore S (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology* 35(4): 495-516.

Finn JT, Weil M, Archer F, Siman R, Srinivasan A and Raff MC (2000). Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. *The Journal of Neuroscience* 20(4): 1333-1341.

Fischer LR and Glass JD (2007). Axonal degeneration in motor neuron disease. *Neurodegener Dis* 4(6): 431-442.

Fischer U, Janicke RU and Schulze-Osthoff K (2003). Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 10(1): 76-100.

Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao S-M and Maniatis T (2003). IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4(5): 491-496.

Freischmidt A, Wieland T, Richter B, Ruf W, Schaeffer V, Muller K, Marroquin N, Nordin F, Hubers A, Weydt P, Pinto S, Press R, Millecamps S, Molko N, Bernard E, Desnuelle C, Soriani MH, Dorst J, Graf E, Nordstrom U, Feiler MS, Putz S, Boeckers TM, Meyer T, Winkler AS, Winkelman J, de Carvalho M, Thal DR, Otto M, Brannstrom T, Volk AE, Kursula P, Danzer KM, Lichtner P, Dikic I, Meitinger T, Ludolph AC, Strom TM, Andersen PM and Weishaupt JH (2015). Haploinsufficiency of TBK1 causes familial ALS and fronto-temporal dementia. *Nat Neurosci* 18(5): 631-636.

Fuchs Y and Steller H (2015). Live to die another way: modes of programmed cell death and the signals emanating from dying cells. *Nat Rev Mol Cell Biol* 16(6): 329-344.

Gerdts J, Summers DW, Sasaki Y, DiAntonio A and Milbrandt J (2013). Sarm1-Mediated Axon Degeneration Requires Both SAM and TIR Interactions. *The Journal of Neuroscience* 33(33): 13569-13580.

Ghosh S and Hayden MS (2008). New regulators of NF-[kappa]B in inflammation. *Nat Rev Immunol* 8(11): 837-848.

Ghosh S, May MJ and Kopp EB (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 16: 225-260.

Goncalves A, Burckstummer T, Dixit E, Scheicher R, Gorna MW, Karayel E, Sugar C, Stukalov A, Berg T, Kralovics R, Planyavsky M, Bennett KL, Colinge J and Superti-Furga G (2011). Functional dissection of the TBK1 molecular network. *PLoS One* 6(9): e23971.

Gyrd-Hansen M and Meier P (2010). IAPs: from caspase inhibitors to modulators of NF-κB, inflammation and cancer. *Nat Rev Cancer* 10(8): 561-574.

Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V and Sullivan M (2012). PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res* 40(Database issue): D261-270.

Hornle M, Peters N, Thayaparasingham B, Vorsmann H, Kashkar H and Kulms D (2011). Caspase-3 cleaves XIAP in a positive feedback loop to sensitize melanoma cells to TRAIL-induced apoptosis. *Oncogene* 30(5): 575-587.

Hutti JE, Shen RR, Abbott DW, Zhou AY, Sprott KM, Asara JM, Hahn WC and Cantley LC (2009). Phosphorylation of the Tumor Suppressor CYLD by the Breast Cancer Oncogene IKKε Promotes Cell Transformation. *Molecular Cell* 34(4): 461-472.

Johnson Jr EM, Rich KM and Yip HK (1986). The role of NGF in sensory neurons in vivo. *Trends in Neurosciences* 9(0): 33-37.

Karin M (1999). The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *J Biol Chem* 274(39): 27339-27342.

Kawai T and Akira S (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11(5): 373-384.

Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D and Courtois G (2003). The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature* 424(6950): 801-805.

Kuo CT, Zhu S, Younger S, Jan LY and Jan YN (2006). Identification of E2/E3 ubiquitinating enzymes and caspase activity regulating Drosophila sensory neuron dendrite pruning. *Neuron* 51(3): 283-290.

Kuranaga E (2011). Caspase signaling in animal development. *Dev Growth Differ* 53(2): 137-148.

Kuranaga E, Kanuka H, Tonoki A, Takemoto K, Tomioka T, Kobayashi M, Hayashi S and Miura M (2006). Drosophila IKK-related kinase regulates nonapoptotic function of caspases via degradation of IAPs. *Cell* 126(3): 583-596.

Levi-Montalcini R and Booker B (1960). Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. *Proceedings of the National Academy of Sciences* 46(3): 384-391.

Li H, Li SH, Yu ZX, Shelbourne P and Li XJ (2001). Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *Journal of Neuroscience* 21(21): 8473-8481.

Lin R, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, Wilkinson P, Julkunen I, Vitour D, Meurs E and Hiscott J (2006). Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. *J Virol* 80(12): 6072-6083.

Liston P, Fong WG, Kelly NL, Toji S, Miyazaki T, Conte D, Tamai K, Craig CG, McBurney MW and Korneluk RG (2001). Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. *Nat Cell Biol* 3(2): 128-133.

Low LK and Cheng H-J (2006). Axon pruning: an essential step underlying the developmental plasticity of neuronal connections. *Philosophical Transactions of the Royal Society B* 361(1473): 1531-1544.
Luo L and O'Leary DDM (2005). Axon retraction and degeneration in development and disease. *Annual Review of Neuroscience* 28(1): 127-156.
Ma Y, Jin H, Valyi-Nagy T, Cao Y, Yan Z and He B (2012). Inhibition of TANK Binding Kinase 1 by Herpes Simplex Virus 1 Facilitates Productive Infection. *J Virol* 86(4): 2188-2196.
Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG and Johnson EM, Jr. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J Cell Biol* 106(3): 829-844.
Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, Kinoshita Y, Kamada M, Nodera H, Suzuki H, Komure O, Matsuura S, Kobatake K, Morimoto N, Abe K, Suzuki N, Aoki M, Kawata A, Hirai T, Kato T, Ogasawara K, Hirano A, Takumi T, Kusaka H, Hagiwara K, Kaji R and Kawakami H (2010). Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* 465(7295): 223-226.
Massoumi R, Chmielarska K, Hennecke K, Pfeifer A and Fassler R (2006). Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. *Cell* 125(4): 665-677.
McLaughlin T, Torborg CL, Feller MB and O'Leary DD (2003). Retinotopic map refinement requires spontaneous retinal waves during a brief critical period of development. *Neuron* 40(6): 1147-1160.
Meier P, Silke J, Leevers SJ and Evan GI (2000). The Drosophila caspase DRONC is regulated by DIAP1. *The EMBO Journal* 19(4): 598-611.
Mercurio F, Murray BW, Shevchenko A, Bennett BL, Young DB, Li JW, Pascual G, Motiwala A, Zhu H, Mann M and Manning AM (1999). IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. *Mol Cell Biol* 19(2): 1526-1538.
Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A and Rao A (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* 278(5339): 860-866.
Miura M (2012). Apoptotic and nonapoptotic caspase functions in animal development. *Cold Spring Harb Perspect Biol* 4(10).
Montell DJ (2006). A Kinase Gets Caspases into Shape. *Cell* 126(3): 450-452.
Nakhaei P, Sun Q, Solis M, Mesplede T, Bonneil E, Paz S, Lin R and Hiscott J (2012). IkappaB kinase epsilon-dependent phosphorylation and degradation of X-linked inhibitor of apoptosis sensitizes cells to virus-induced apoptosis. *J Virol* 86(2): 726-737.
Neukomm LJ and Freeman MR (2014). Diverse cellular and molecular modes of axon degeneration. *Trends in Cell Biology* 24(9): 515-523.
Nikolaev A, McLaughlin T, O/'Leary DDM and Tessier-Lavigne M (2009). APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature* 457(7232): 981-989.
O'Leary DDM and Koester SE (1993). Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex. *Neuron* 10(6): 991-1006.
O'Neill LAJ and Bowie AG (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7(5): 353-364.

O'Rourke NA, Cline HT and Fraser SE (1994). Rapid remodeling of retinal arbors in the tectum with and without blockade of synaptic transmission. *Neuron* 12(4): 921-934.
Oshima K, Takeda M, Kuranaga E, Ueda R, Aigaki T, Miura M and Hayashi S (2006). IKKε regulates F actin assembly and interacts with Drosophila IAP1 in cellular morphogenesis. *Current Biology* 16(15): 1531-1537.
Ou YH, Torres M, Ram R, Formstecher E, Roland C, Cheng T, Brekken R, Wurz R, Tasker A, Polverino T, Tan SL and White MA (2011). TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. *Mol Cell* 41(4): 458-470.
Peters RT, Liao S-M and Maniatis T (2000). IKKε is part of a novel PMA-inducible IκB kinase complex. *Molecular Cell* 5(3): 513-522.
Peters RT and Maniatis T (2001). A new family of IKK-related kinases may function as I kappa B kinase kinases. *Biochim Biophys Acta* 1471(2): M57-62.
Petherick KJ, Conway OJL, Mpamhanga C, Osborne SA, Kamal A, Saxty B and Ganley IG (2015). Pharmacological Inhibition of ULK1 Kinase Blocks Mammalian Target of Rapamycin (mTOR)-dependent Autophagy. *Journal of Biological Chemistry* 290(18): 11376-11383.
Raff MC, Whitmore AV and Finn JT (2002). Axonal self-destruction and neurodegeneration. *Science* 296(5569): 868-871.
Reilly SM, Chiang S-H, Decker SJ, Chang L, Uhm M, Larsen MJ, Rubin JR, Mowers J, White NM, Hochberg I, Downes M, Yu RT, Liddle C, Evans RM, Oh D, Li P, Olefsky JM and Saltiel AR (2013). An inhibitor of the protein kinases TBK1 and IKK-[epsiv] improves obesity-related metabolic dysfunctions in mice. *Nat Med* 19(3): 313-321.
Ritch R, Darbro B, Menon G, Khanna CL, Solivan-Timpe F, Roos BR, Sarfarzi M, Kawase K, Yamamoto T, Robin AL, Lotery AJ and Fingert JH (2014). TBK1 gene duplication and normal-tension glaucoma. *JAMA Ophthalmol* 132(5): 544-548.
Salvesen GS and Duckett CS (2002). IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* 3(6): 401-410.
Saxena S and Caroni P (2007). Mechanisms of axon degeneration: from development to disease. *Prog Neurobiol* 83(3): 174-191.
Schafer ZT and Kornbluth S (2006). The apoptosome: physiological, developmental, and pathological modes of regulation. *Dev Cell* 10(5): 549-561.
Scott FL, Denault J-B, Riedl SJ, Shin H, Renatus M and Salvesen GS (2005). XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *The EMBO Journal* 24(3): 645-655.
Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC and Karin M (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* 293(5534): 1495-1499.
Shen RR and Hahn WC (2011). Emerging roles for the non-canonical IKKs in cancer. *Oncogene* 30(6): 631-641.
Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J, Tatsumi Y, Kanamaru A and Akira S (1999). IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases. *Int Immunol* 11(8): 1357-1362.
Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, Alnemri ES, Fairman R and Shi Y (2003). Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell* 11(2): 519-527.

Simon DJ, Weimer RM, McLaughlin T, Kallop D, Stanger K, Yang J, O'Leary DDM, Hannoush RN and Tessier-Lavigne M (2012). A Caspase Cascade Regulating Developmental Axon Degeneration. *The Journal of Neuroscience* 32(49): 17540-17553.

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee R-A, Robbins PD, Fernandes-Alnemri T, Shi Y and Alnemri ES (2001). A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* 410(6824): 112-116.

Stennicke HR, Ryan CA and Salvesen GS (2002). Reprieval from execution: the molecular basis of caspase inhibition. *Trends Biochem Sci* 27(2): 94-101.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K and Takahashi R (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 8(3): 613-621.

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J and et al. (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356(6372): 768-774.

Tojima Y, Fujimoto A, Delhase M, Chen Y, Hatakeyama S, Nakayama K, Kaneko Y, Nimura Y, Motoyama N, Ikeda K, Karin M and Nakanishi M (2000). NAK is an IkappaB kinase-activating kinase. *Nature* 404(6779): 778-782.

Truman JW (1990). Metamorphosis of the central nervous system of Drosophila. *Journal of Neurobiology* 21(7): 1072-1084.

Tsai J, Grutzendler J, Duff K and Gan W-B (2004). Fibrillar amyloid deposition leads to local synaptic abnormalities and breakage of neuronal branches. *Nature Neuroscience* 7(11): 1181-1183.

Unsain N, Higgins J, Parker K, Johnstone A and Barker P (2013). XIAP regulates caspase activity in degenerating axons. *Cell Reports* 4(4): 751-763.

Vaux DL and Korsmeyer SJ (1999). Cell death in development. *Cell* 96(2): 245-254.

Vaux DL and Silke J (2005). IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* 6(4): 287-297.

Verhagen AM, Coulson EJ and Vaux DL (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2(7): 3009.3001-3009.3010.

Vogel C, Abreu Rde S, Ko D, Le SY, Shapiro BA, Burns SC, Sandhu D, Boutz DR, Marcotte EM and Penalva LO (2010). Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol Syst Biol* 6: 400.

Vohra BP, Sasaki Y, Miller BR, Chang J, DiAntonio A and Milbrandt J (2010). Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenylyltransferase 1-sensitive pathway. *J Neurosci* 30(41): 13729-13738.

Wang JT, Medress ZA and Barres BA (2012). Axon degeneration: Molecular mechanisms of a self-destruction pathway. *The Journal of Cell Biology* 196(1): 7-18.

Williams DW, Kondo S, Krzyzanowska A, Hiromi Y and Truman JW (2006). Local caspase activity directs engulfment of dendrites during pruning. *Nat Neurosci* 9(10): 1234-1236.

Wing JP, Karres JS, Ogdahl JL, Zhou L, Schwartz LM and Nambu JR (2002). Drosophila sickle Is a Novel grim-reaper Cell Death Activator. *Current Biology* 12(2): 131-135.

Xie X, Zhang D, Zhao B, Lu M-K, You M, Condorelli G, Wang C-Y and Guan K-L (2011). IκB kinase ε and TANK-binding kinase 1 activate AKT by direct phosphorylation. *Proceedings of the National Academy of Sciences* 108(16): 6474-6479.

Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ and Israel A (1998). Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* 93(7): 1231-1240.

Yuan J and Yankner BA (2000). Apoptosis in the nervous system. *Nature* 407(6805): 802-809.

Zheng JQ, Kelly TK, Chang B, Ryazantsev S, Rajasekaran AK, Martin KC and Twiss JL (2001). A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. *J Neurosci* 21(23): 9291-9303.