Regulation of Regenerative and Degenerative Responses to Axonal Injury

by

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In memory of my grandfather Mr. Wenzhi Hao

To my parents and my brother

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PREFACE

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LIST OF ABBREVIATIONS

AC: adenylyl cyclase

AKAP: A-Kinase Anchoring Proteins

AP-1: activator protein-1

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

CSPG: chondroitin sulfate proteoglycan

CREB: cAMP response element binding protein

Dcp-1: *Drosophila* caspase-1

DLK: dual leucine zipper kinase

DRG: dorsal root ganglion

ER: endoplasmic reticulum

HIF-1α: hypoxia-inducible factor 1α

Hiw: Highwire

JAK/STAT: Janus kinase/ signal transducer and activator of transcription

JNK: Jun N-terminal Kinase

MAPKKK: mitogen activated protein kinase kinase kinase

MTORC1: mechanistic target of rapamycin complex 1

NGF: nerve growth factor

NMJ: neuromuscular junctions

Nmnat: nicotinamide mononucleotide adenylyltransferase

Phr1: Pam/Highwire/Rpm-1

PNS: peripheral nervous system

PKA: protein kinase A

PTEN: phosphatase and tensin

Puc: Puckered

RGC: retinal ganglion cell

Rtca: RNA 3'-terminal phosphate cyclase

SOCS3: suppressor of cytokine signaling 3

UPR: unfolded protein response

UPS: ubiquitin proteasome system

WldS: Wallerian degeneration slow

Wnd: Wallenda

WT: wild type

ABSTRACT

Axons allow neurons to communicate over long distances, however their long length makes them vulnerable to injury, since damage at any location leads to loss of a neuron's function within a circuit. Repair from axonal damage requires that damaged axon gains an ability to initiate new growth which is termed axonal regeneration. This involves the activation of signaling pathways in the injured neurons which promote a 'regenerative' state, but many neuronal types in the mammalian central nervous system show a failure to initiate this state. Functional repair also requires that the distal axonal stumps, which have lost connection with cell bodies hence are non-functional, undergo degeneration and clearance via a process termed Wallerian degeneration. This degeneration takes place via a cell autonomous self-destructive pathway, akin to apoptosis, but with distinct, and still poorly characterized molecular components.

My thesis work has focused on understanding the cellular mechanisms by which neurons detect and respond to axonal damage. A conserved axonal kinase, named DLK in mammals or Wallenda (Wnd) in *Drosophila*, appears to function as a 'sensor' of axonal damage in neurons. However, the mechanism that activates Wnd/DLK is unknown. I have discovered that the cAMP-regulated protein kinase A (PKA) is a conserved and direct upstream activator of Wnd/DLK: PKA is required for the induction of Wnd/DLK signaling in injured axons, and directly stimulates its activation via phosphorylation of its activation loop. Elevation of intracellular cAMP level is a broadly known but poorly understood method to stimulate the growth potential of axons. In this study, I found that DLK is essential for the regenerative effects

of cAMP. These findings link two important mediators, DLK/Wnd and cAMP/PKA, into a unified and evolutionarily conserved molecular pathway for regulating axonal regeneration upon axonal injury.

My work has also identified a new regulator of Wallerian degeneration, from the fortuitous discovery of a mutation that strongly inhibited axonal degeneration in the strain background of *dcp-1* mutant. Genetic mapping, whole genome sequencing and rescue analysis pinpoint this phenotype to a mutation in the putative transmembrane protein, Raw. Raw functions as a negative regulator of the transcription factor AP-1, and this activity mediates its role in axonal degeneration. While Raw does not have an obvious mammalian homologue, the basic mechanism of axonal degeneration is highly conserved between *Drosophila* and mammalian neurons, so understanding the mechanism for Raw in degeneration may lead to new insight for understanding and treating nerve damage in humans.

Chapter 1 :

Introduction

1.1 Abstract

Axons connect neurons over long distances in the brain and body, and because of this are vulnerable to damage and injury. In some cases, damaged axons can initiate new growth and reconnect with their targets. However, in cases such as in the mammalian central nervous system (CNS), damaged axons fail to regrow and the disconnected neurons undergo cell death. Both regeneration and cell death involve a large-scale transcriptional response of the neuron to a signal that is, in most cases, far away from the cell body. How does the cell body 'know' that a distant site in the axon is injured? In this thesis work, I seek to understand the fundamental question of how neurons sense and cope with the axonal damage. Understanding the mechanisms of neuronal injury response is of great interest as it will shed light on the development of strategies to repair neuronal damage after injury. In this chapter of my dissertation, I will give a general introduction to how neurons response to axonal injuries and initiate regenerative growth. The first part of this chapter will focus on mechanisms that promote axonal regeneration after injury, with particular emphasis on the known signaling pathways that are involved. The second part of this chapter will focus on one crucial and evolutionarily conserved signaling cascade which senses and coordinates responses to axonal damage. A central regulator of this pathway is an axonal mitogen activated protein kinase kinase kinase (MAPKKK), which is named Wallenda

(Wnd) in *Drosophila* or Dual Leucine Zipper Kinase (DLK) in mammals. I will review regulatory mechanisms of Wnd/DLK, which are the focus of my thesis work. The novel mechanism of DLK regulation and its functional consequences that I discovered will be described in the following chapters of the dissertation. The third part of this chapter will focus on the mechanisms of axonal degeneration.

1.2 Axonal injury signaling and regeneration

Neurons are post mitotic cells with axons that transmit information over long distances. Therefore, failure of repair after axonal injury results in permanent functional deficits. There is a dichotomy in the ability of the nervous system to undergo repair between peripheral nervous system (PNS) and the central nervous system (CNS). In the PNS, the injured axon is able to initiate new axonal growth and the newly grown axon can reach and ultimately reconnect with its original target. This form of repair is referred to as 'axon regeneration'. However, axon regeneration generally fails to occur in many cases, including in the adult mammalian CNS. Hence, understanding the cellular mechanisms of injury response can provide potential strategies for stimulating axon regeneration and even functional recovery after CNS injury.

1.2.1 Extrinsic and intrinsic factors for axon regeneration

The dichotomy between the regenerative capacity of the CNS and the PNS inspires much interest in understanding the differences between them. Injury of axons in the PNS induces large-scale transcriptional-dependent responses in gene expression and axonal transport that promote effective growth by enhancing the intrinsic regenerative state. A key observation that injured CNS axons were able to regenerate in the PNS environment (Richardson and Issa, 1984) suggested that the growth environment might also play a role in axon regeneration. Indeed, it is

now widely accepted that both intrinsic and extrinsic factors contribute to the axon regeneration in the CNS.

One major environmental distinction between the PNS and the CNS is the composition of the myelin sheath. While axons in the PNS are wrapped by Schwann cells, axons in the CNS are myelinated by oligodendrocytes. Multiple ligands and receptors are involved in the inhibition of axon growth by affecting cytoskeleton rearrangements through a signaling pathway that involves Rho and Rho-associated kinase (ROCK) (Wills et al., 2012, Domeniconi et al., 2005). MAG, OMgp and Nogo are three prototypical CNS myelin-associated axon regeneration inhibitors that have been studied extensively during the past few decades. MAG is a transmembrane glycoprotein that functions as an age-dependent switch in certain neuronal types: it promotes axon growth during neuronal development and inhibits axon growth once the axon is mature (Mukhopadhyay et al., 1994). OMgp is a glycosylphosphatidylinositol (GPI)-anchored protein which is involved in the regulation of synaptic plasticity (Raiker et al., 2010). Nogo has three isoforms (Nogo-A, Nogo-B and Nogo-C), among which Nogo-A is expressed most abundantly in oligodendrocytes (Huber et al., 2002). Nogo-A is a transmembrane protein that is expressed in endoplasmic reticulum and cell surface. Multiple Nogo knockout mice lines all exhibit reduced inhibitory effects on neurite growth (Lee and Zheng, 2012). These three ligands share some of the receptors including NgRs and PirB (Geoffroy and Zheng, 2014). However, genetic studies targeting one or even multiple of these inhibitors and receptors demonstrate very limited effects on axon regeneration in CNS (Geoffroy and Zheng, 2014).

In addition to myelin-associated inhibitors, another major deterrent to axon regeneration is the glial scar, which forms at the injury site after the CNS injury. The scar is composed of astrocytes, oligodendrocytes precursors, microglia, meningeal cells and stem cells (Fawcett and

Asher, 1999). Reactive astrocytes in the glial scar generate many inhibitory molecules in scar extracellular matrix, such as tenascins, semaphorin 3A and chondroitin sulfate proteoglycans (CSPGs) (Sandvig et al., 2004). Among them, CSPGs are of great importance as they are dramatically increased in scar tissue in CNS after injury (Galtrey and Fawcett, 2007). They specifically bind to and activate functional receptors including leukocyte common antigen-related (LAR) phosphatase (Fisher et al., 2011) and NgRs (Dickendesher et al., 2012), suggesting they might share similar signaling pathways with myelin-associated inhibitors. Moreover, these large molecules concentrate and form non-permissive perineuronal nets that physically prevent axons from regenerating (Ohtake and Li, 2015). Therefore, CSPGs inhibit axon regeneration through several different mechanisms, making them extremely difficult to target therapeutically. In addition, some studies suggest that the lack of supportive growth factors (Gordon, 2009, Harvey et al., 2012) and persistent inflammation (Donnelly and Popovich, 2008, Benowitz and Popovich, 2011) also contribute to the extracellular inhibitory environment for axon regeneration.

To sustain axonal growth over long distances, a cell body response appears to be indispensable in both the PNS and the CNS. A cell body needs to coordinate the raw material synthesis and transportation to the injury site. Thus, the cellular intrinsic pathways that are activated by axonal injury are of great importance. I will discuss the known signaling pathways involved in injury response in details (section 1.1.3) after introduction of 'conditioning lesion' (section 1.1.2).

1.2.2 Conditioning lesion and regeneration

The conditioning lesion experiment of primary mammalian adult sensory neurons from dorsal root ganglion (DRG) has been a classic model to study the intrinsic mechanism of axon regeneration. DRG neurons are unique as they have two axonal branches, one extends to the

PNS and the other projects to the CNS. Richardson and Issa first discovered that the central branches of DRGs could regenerate in a peripheral nerve graft if the peripheral branch has been previously injured (called a conditioning lesion) (Richardson and Issa, 1984). Then Neumann and Woolf demonstrated that both central and peripheral branches could regenerate robustly, as long as the conditioning lesion is carried out in the peripheral branch (Neumann and Woolf, 1999). In addition, more recent study suggests that a PNS injury that is induced after the CNS injury is still sufficient to promote axon regeneration in the CNS (Ylera et al., 2009). Therefore, it is of great interest from a therapeutic perspective to understand the molecular mechanisms by which such regenerative potential can be released.

Injuries in PNS axons trigger transcriptional changes in many genes, including transcription factors (such as ATF-3, c-Jun and Sox11), Arginase 1 (a key enzyme in polyamine synthesis), translation regulators, actin and tubulins (reviewed by (Ma and Willis, 2015)). In contrast, there are only minor changes of gene transcription after injury in the CNS (Mason et al., 2002, Starkey et al., 2009, Schreyer and Skene, 1993, Geeven et al., 2011). It is unknown why the changes in gene expression are different. However, it is well known that genetic manipulations such as PTEN deletion (Park et al., 2008, Sun et al., 2011) or elevation of intracellular cyclic adenosine monophosphate (cAMP) (Neumann et al., 2002, Cai et al., 1999, Gao et al., 2003) strongly promote axon regeneration in the CNS. Therefore, understanding the intrinsic cellular pathways that are stimulated by the conditioning lesion in the PNS could shed light on the therapeutic strategies for CNS injuries.

1.2.3 Intrinsic axonal injury signaling pathways

Robust axon regeneration in the PNS is associated with the broad activation of gene transcription and other molecular changes in the cell body (Blackmore, 2012), suggesting that

injury induces complex reactions in neurons. What are the core upstream signaling pathways that coordinate transcriptional programs and stimulate axon regeneration? This section will review the central signaling pathways, including recent discoveries, in different model organisms.

1.2.3.1 JNK/c-Jun pathway

Transcription factor c-Jun is activated by axonal injury and required for axon regeneration (Raivich et al., 2004). c-Jun is phosphorylated and activated by a mitogen-activated protein kinase (MAPK) called c-Jun N-terminal kinase (JNK). Previous studies have suggested that JNK can be locally activated in axon by injuries and retrogradely transported to the cell body (Lindwall and Kanje, 2005, Cavalli et al., 2005). In support of this, JNK is found to be linked to the motor protein dynein by the scaffolding protein JIP3 (Sunday driver in *Drosophila*) (Cavalli et al., 2005).

1.2.3.2 JAK/STAT pathway

Many studies have been focusing on inflammation responses which are demonstrated to be beneficial to axon regeneration (Richardson and Lu, 1994). For example, gp130 cytokines (leukemia inhibitory factor, LIF and interleukin-6, IL-6) knockout mice show reduced axon regeneration after injury (Zhong et al., 1999, Cafferty et al., 2004). Recent studies showed that overexpression of a monocyte chemokine CCL2 in DRG neurons promotes neurite growth (Niemi et al., 2016, Kwon et al., 2015). Both gp130 cytokines and CCL2 function through the downstream Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway (Niemi et al., 2016, Shuai and Liu, 2003). The JAK-STAT pathway can be activated acutely by peripheral axon injuries and is essential for the conditioning injury effects *in vivo* (Qiu et al., 2005).

The suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of the JAK-STAT pathway (Baker et al., 2009). Smith et al. showed that deletion of SOCS3 strongly enhanced axon regeneration in retinal ganglion cells (RGCs) *in vivo* (Smith et al., 2009). However, no axon regeneration is observed in SOCS3 and gp130 double deletion RGCs, suggesting that gp130 cytokines are essential for axon regeneration in SOCS3 mutants (Smith et al., 2009).

1.2.3.3 PTEN/mTOR

To prevent neurons from overgrowth when they are mature, several growth inhibitory pathways are engaged, including the tumor suppressor phosphatase and tensin homolog (PTEN)/mTOR pathway. Does axon regeneration require a re-activation of the growth program? To answer this question, Park et al. did a candidate-based genetic screen in RGCs and found that deletion of the gene *PTEN* promoted robust axon regeneration (Park et al., 2008). PTEN is a phosphatase that converts PIP_3 to PIP_2. Deletion of PTEN leads to accumulation of PIP_3 which can activate AKT. mTOR is one of the downstream targets of AKT. Application of rapamycin, a mTOR inhibitor, abolishes the regeneration effects (Park et al., 2008), indicating that the enhanced axon regeneration in *PTEN* mutants is mTOR dependent. Indeed, axotomy triggers a stress response in which mTOR levels are significantly reduced in RGCs (Park et al., 2008). As a central regulator of protein translation, mTOR down-regulation could be the mechanism by which axon regeneration fails in the CNS. However, it is unknown that whether or not this is the only reason.

1.2.3.4 Newly identified axon regeneration regulators: Hif-1α and Activin

A recent study has identified that hypoxia-inducible factor 1α (HIF-1α) positively regulates axon regeneration and contributes to preconditioning lesion effects (Cho et al., 2015). HIF-1α is activated by hypoxia and controls multiple injury-induced gene expression (Cho et al., 2015). Therefore, hypoxia could be a new tool to treat axonal injuries.

Another research group found axons regenerate significantly better in a specific mice line CAST/Ei (Omura et al., 2015). Omura et al. performed a whole-genome expression screen and found gene *Activin* is highly correlated with axon regeneration ability (Omura et al., 2015). However, the underlying molecular mechanisms need further investigation.

1.2.3.5 svh-1/svh-2 in *C.elegans*

In *C.elegans*, the growth factor svh-1 and its corresponding receptor tyrosine kinase svh-2 were identified to be upstream regulators of the JNK/c-Jun pathway that regulates axon regeneration (Li et al., 2012). The svh-2 expression level is induced by axonal injuries (Li et al., 2012). Svh-1 does not have an obvious homolog in mammals, but based on structural analysis, it belongs to the hepatocyte growth factor (HGF), macrophage stimulating protein (MSP) and plasminogen super family. Svh-2 is homologous to the HGF receptor Ron in mammals. A recent study suggested that svh-2 expression is regulated by transcription factors ETS-4 and CEBP-1, both of which are activated by axonal injuries (Li et al., 2015). However, this pathway has not yet been studied in mammals.

1.2.3.6 RNA splicing/repair pathway

Song et al. used *Drosophila* as a model organism and identified the RNA 3'-terminal phosphate cyclase (Rtca) as an inhibitor of axon regeneration (Song et al., 2015). Rtca is an RNA processing enzyme whose function is not fully understood. Interestingly, another

independent study in *C.elegans* found that RtcB, an RNA ligase, inhibits axon regeneration (Kosmaczewski et al., 2015). RtcB is involved in the processing of the mRNA of the transcription factor Xbp-1, which can activate the unfolded protein response (UPR) (Kosmaczewski et al., 2014). Importantly, a previous study suggested that Xbp-1 is induced by optical nerve injury in RGCs and ectopic activation of Xbp-1 promotes axon regeneration (Hu et al., 2012). Therefore, neurons might adopt the RNA processing and repair pathway to cope with axonal injuries.

1.2.3.7 cAMP signaling pathway

It has been known for a long time that cAMP plays an important role in axon regeneration. The Filbin group was the first to show that elevated level of cAMP enhances axon regeneration ability in a variety of mammalian neurons, including DRG neurons growing on inhibitory MAG (Cai et al., 1999, Cai et al., 2001). Followed by Neumann et al., they showed that increased cAMP level promotes axon regeneration in the mouse CNS *in vivo* (Neumann et al., 2002). Neumann et al. demonstrated that injection of membrane permeable cAMP into DRG neurons can mimic the pre-conditioning effects of peripheral nerve injury (Neumann et al., 2002), suggesting that manipulation of intracellular cAMP level could be a potential therapeutic method in clinic. Moreover, a recent study, using zebrafish as a model, demonstrated an optogenetic method that can precisely increase cellular cAMP level and stimulate axon regeneration *in vivo* (Xiao et al., 2015b).

The Filbin group also dissected the underlying molecular mechanism by identifying the transcription factor cAMP response element binding protein (CREB) as a downstream factor (Gao et al., 2004). Their research has suggested that constitutively activated CREB in injured axons is sufficient to overcome the inhibitory myelin environment *in vivo* (Gao et al., 2004).

They also reported that the level Arginase I (ArgI), a key enzyme in the polyamine synthesis, is highly correlated with the activation of CREB (Gao et al., 2004). Interestingly, a recent study suggests that cAMP's effects on axon regeneration is transcriptional complex activator protein 1 (AP-1) dependent. Moreover, AP-1 directly binds to the promoter region of *ArgI* gene and enhances ArgI expression (Ma et al., 2014). Therefore, multiple molecular mechanisms could contribute to the effects of cAMP on axon regeneration.

My thesis work has identified a novel mechanism for cAMP to promote axon regeneration (discussed in detail in Chapter 3). This mechanism involves activation of a stress-responding MAPKKK kinase, which is called dual leucine zipper kinase (DLK). DLK is strongly required for axon regeneration in multiple model organisms (Xiong et al., 2010, Shin et al., 2012a, Hammarlund et al., 2009, Yan et al., 2009). Apart from that, DLK also plays multiple roles in other important cellular processes, which are reviewed in next section (section 2).

1.3 DLK/Wnd signaling pathway

DLK, which is named Wallenda (Wnd) in *Drosophila*, is a neuronal-enriched MAPKKK that controls multiple fundamental cellular processes, including cell death, axon degeneration and axon regeneration. How does a kinase regulate these seemingly contradictory processes? This section will review the known functions of DLK with specific emphasis on the regulation of DLK signaling, which is the interest of my thesis research.

1.3.1 The Hiw/Wnd signaling pathway plays an important role in synaptic development.

The function of neuronal circuits relies on the assembly of synapses, which include both pre-synaptic terminals and post-synaptic structures. The size of the pre-synaptic structure is crucial for determining the amount of neurotransmitter release and the development of post-

synaptic structures. Previous studies have identified an evolutionarily conserved E3 ubiquitin ligase, which is named Highwire (Hiw) in *Drosophila*, in regulating pre-synaptic structures. Mutations of *hiw* in flies lead to dramatic pre-synaptic overgrowth (Wan et al., 2000). Hiw homologs in *C.elegans* (Rpm-1), zebrafish (Esrom) and mice (Phr1) all have similar functions in regulating axon guidance and synaptogenesis (Bloom et al., 2007, Lewcock et al., 2007, Zhen et al., 2000, D'Souza et al., 2005).

In an effort to identify suppressors of the synaptic overgrowth phenotype in Hiw mutants, Collins et al. designed a genetic screen in *Drosophila* and found mutations in *wnd* can suppress the synaptic overgrowth, indicating that Wnd is a target of Hiw (Collins et al., 2006). Hiw plays an important role in keeping Wnd level low in axons to ensure proper synaptic development, as overexpression of Wnd in neurons phenocopies the synaptic defects in Hiw mutants (Collins et al., 2006). Studies in *C.elegans* have suggested that Hiw regulates Wnd level by promoting its ubiquitination via the RING domain of Hiw (Nakata et al., 2005). However, other studies indicate that the regulation mechanisms could be more complicated (Bloom et al., 2007, Lewcock et al., 2007, DiAntonio et al., 2001).

1.3.2 DLK mediates both cell death and axon regeneration after injury

The MAP kinase pathways in cells are organized as cascades: including MAP Kinase Kinase Kinases (MAPKKK), which phosphorylate MAP Kinase Kinases (MAPKK) which phosphorylate MAP Kinases. These pathways regulate a myriad of important cellular processes, including cell differentiation, cell proliferation, cell survival, mitosis, apoptosis and wound healing. DLK and its downstream factors, MKK4/7 (MAPKK) and JNK (MAPK), function as a stress response pathway. In response to nerve growth factor (NGF) withdrawal or axotomy, the DLK/JNK signaling cascade leads to rapid cell death and axon degeneration both *in vitro* and *in*

vivo (Ghosh et al., 2011, Itoh et al., 2011, Miller et al., 2009). Moreover, DLK has been identified as a key mediator of retinal ganglion cells (RGC) death in glaucoma model (Welsbie et al., 2013) and excitotoxicity-induced neuronal degeneration (Pozniak et al., 2013). DLK is also involved in DRG neuron and motoneuron apoptosis, neuronal migration and axon path-finding during development (Ghosh et al., 2011, Itoh et al., 2011, Hirai et al., 2006).

Interestingly, recent studies in diverse model systems suggest that DLK/Wnd plays a key role in axon regeneration. DLK homologous in *C.elegans* (DLK-1) and *Drosophila* (Wnd) have been shown to regulate axon regeneration after axotomy (Yan et al., 2009, Xiong et al., 2010, Hammarlund et al., 2009). Importantly, Shin et al. has demonstrated that DLK is required for the conditioning lesion effects in mouse sciatic nerves (Shin et al., 2012a). In addition, DLK is also crucial for axon regeneration in CNS neurons where regeneration can be ectopically induced by mutations on PTEN (Watkins et al., 2013). Therefore, DLK's role in axon regeneration is evolutionarily conserved among all the model organisms.

1.3.3 DLK functions as a sensor of axonal damage

Given the functions mentioned above, DLK has been thought to act as a 'sensor' of axonal damages and thus should become activated upon axonal injuries. Indeed, there are several lines of evidence to support that. First, Wnd is both anterogradely and retrogradely transported in axons (Xiong et al., 2010). Moreover, Wnd is required acutely in injured axons to retrogradely transport signals to cell bodies that facilitate axon regeneration (Xiong et al., 2010, Shin et al., 2012a). However, how DLK senses axonal damages is still not clear.

1.3.4 Regulation of DLK signaling

DLK is predominantly expressed in neurons in cytosol, plasma membranes and nuclei. DLK is actively transported in axons (Xiong et al., 2010, Holland et al., 2016) and its level

increases acutely in neurons upon axonal injury (Xiong et al., 2010, Hao et al., 2016) or nerve growth factor (NGF) withdrawal from medium in cultured neurons (Huntwork-Rodriguez et al., 2013). So it is known as a sensor for neuronal damage and NGF withdrawal induced stress. And the acute up-regulation of DLK levels involves posttranscriptional mechanisms (Huntwork-Rodriguez et al., 2013, Xiong et al., 2010). Despite great interest, the mechanisms by which DLK is activated and stabilized are still not fully understood. My thesis work has focused on seeking evolutionarily conserved upstream components of the DLK signaling pathway. Before diving into the details of my studies, I review here the known regulatory mechanisms of DLK.

1.3.4.1 DLK dimerization and auto-phosphorylation

DLK is a serine/threonine kinase that belongs to mixed lineage kinase (MLK) subfamily of protein kinases (Holzman et al., 1994). It is a large protein (888 amino acids in mice) that characterized by a catalytic domain (amino acids 156-404) and two Leucine zipper motifs. DLK forms dimers through the Leucine zippers and the two dimers can phosphorylate each other (called auto-phosphorylation). It has been shown that the auto-phosphorylation is required for the activation of DLK downstream targets (Nihalani et al., 2000). However it is not clear which sites are phosphorylated during the auto-phosphorylation.

1.3.4.2 regulation of DLK by calcium

One study in *C.elegans* has identified novel DLK-1 isoform DLK-1S, which binds to DLK-1L and inhibits DLK function (Yan and Jin, 2012). Increased intracellular calcium level switches the heteromeric protein complex to homomeric protein complex, and DLK-1 is therefore activated. Although the C-terminal calcium binding hexapeptide is conserved in one isoform of DLK (MAP3K13), so far there is no evidence suggesting mammalian DLK can be

regulated by calcium. Instead, some studies suggest that DLK can be activated through a calcium independent mechanism (Valakh et al., 2015).

1.3.4.3 JNK stabilizes DLK via a feedback mechanism

JNK is a MAP kinase (MAPK) that functions downstream of DLK in mediating retrograde axonal injury signaling (Xiong et al., 2010, Holland et al., 2016). In the cultured embryonic DRG neurons, withdrawal of NGF from serum leads to DLK-dependent cell death (Watkins et al., 2013). A recent study discovered that upon the NGF withdrawal, DLK level increases dramatically within 3 hours in DRGs (Huntwork-Rodriguez et al., 2013). Moreover, the stabilization of DLK requires JNK, since block of JNK with inhibitors abolished this effect. Further studies suggested that JNK may directly phosphorylate DLK at T43 and S533 outside of the kinase domain, and this phosphorylation changes DLK's sensitivity to degradation via the ubiquitin proteasome system (UPS) (Huntwork-Rodriguez et al., 2013). However, JNK activation alone does not lead to the activation of DLK.

1.3.4.4 Palmitoylation of DLK

A recent study showed that DLK is palmitoylated at a highly conserved site (C127) near the kinase domain (Holland et al., 2016). The point mutation DLKC127A fails to associate with vesicles and fails to be transported in axons. Since DLK is required for the retrograde transport of injury signaling in axons, the palmitoylation of DLK is crucial for its function. However, it is not clear that whether the activation of DLK requires palmitoylation..

1.3.4.5 DLK functions as both a sensor and a mediator of microtubule dynamics

Disruption of microtubule by either treating cultured neurons with cytoskeleton destabilizing drug in vitro or mutating microtubule stabilizing protein spectraplakin in *Drosophila* in vivo activates DLK (Valakh et al., 2015, Valakh et al., 2013). Interestingly, DLK is also required for the increased the microtubule dynamics after axonal injury (Ghosh-Roy et al., 2012). This section is discussed in detail in Chapter 2.

1.3.4.6 mTOR and Akt

Mechanistic target of rapamycin complex 1 (MTORC1) and PI3K/Akt are known for their roles in regulating cell growth (Wong et al., 2015). Interestingly, studies suggest that both of them can directly phosphorylate DLK and regulate its activity under specific conditions. MTORC1 modulates synaptic growth via regulating the DLK pathway — MTORC1 phosphorylates DLK at its kinase domain (Wong et al., 2015). Akt regulates embryonic stem cell proliferation via phosphorylation DLK at its C-terminal (Wu et al., 2015).

1.4 Axon degeneration

After acute axonal injury, the distal axonal stump that lost connection undergoes a self-destruction process called Wallerian degeneration. After a lag phase (the length of the period varies in different types of neurons and also in different species), the distal axonal stumps undergo a rapid degeneration process characterized by disappearance of cytoskeleton and fragmentation of axonal membrane. Then the axonal debris are cleared away by glia and other immune cells. This process morphologically resembles the axon degeneration process in many neurodegenerative diseases and neuropathies.

The discovery of the gain-of-function mutation Wallerian degeneration slow (WldS), which slows down Wallerian degeneration for weeks in mice suggested that axon degeneration

may be a regulated active process, akin to apoptosis. Remarkably, WldS is also able to suppress axon degeneration in neurodegenerative diseases and neuropathies models, suggesting that these processes share similar molecular mechanisms with Wallerian degeneration. Therefore, Wallerian degeneration can be used as a model to study the mechanism of axon degeneration. Given WldS is a gain-of-function mutation, there must be other endogenous mechanisms that regulate axon degeneration. In the past decade, studies using various axonal injury models in multiple organisms have identified several critical regulators of axon degeneration. Here I review the most significant discoveries that influenced our understanding of this fundamental process.

1.4.1 Nmnat

Since the discovery of WldS, extensive studies have been focused on the molecular mechanisms by which it protects axon degeneration. Now it is widely acknowledged that the nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) is the functional component of WldS (Conforti et al., 2009). Nmnat is the enzyme for Nicotinamide adenine dinucleotide (NAD) biosynthesis, catalyzing nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN) with ATP to form NAD or NaAD (Schweiger et al., 2001). There are three isoforms of Nmnat proteins in mammals (Nmnat1, Nmnat2 and Nmnat3). While Nmnat1 mainly resides in the nuclei and Nmnat3 localizes in mitochondria, Nmnat2 is in the cytosol and actively transported in axons (Berger et al., 2005). Nmnat2 is believed to be the 'survival factor' for axons, since knockout of Nmnat2 led to spontaneous axon degeneration (Gilley and Coleman, 2010, Gilley et al., 2013). The protective effect of WldS is due to the mislocalization of Nmnat1 into axons. However, the mechanism by which Nmnat regulates axon degeneration is not clear, yet of great interest. While some studies suggested its enzymatic activity for NAD+ synthesis is

important (Conforti et al., 2014), other studies proposed that it functions molecular chaperon (Ali et al., 2011, Ali et al., 2016).

1.4.2 Hiw

Hiw, an E3 ubiquitin ligase that plays a key role in modulating synapse development (Collins et al., 2006, DiAntonio et al., 2001), was identified as a promoter of axon degeneration via down-regulation of Nmnat protein through ubiquitination in *Drosophila* (Xiong et al., 2012). This mechanism is demonstrated to be conserved in mammals (Babetto et al., 2013). The role of Hiw in promoting axon degeneration has also been identified through an elegant unbiased genetic screen using *Drosophila* adult wing injury model (Neukomm et al., 2014), suggesting Hiw is one of the key regulators for axon degeneration.

1.4.3 Sarm1

An elegant genetic screen in *Drosophila* has identified dSarm (*Drosophila* sterile alpha and Armodillo motif containing protein) as a core regulator of Wallerian degeneration (Osterloh et al., 2012). dSarm is highly conserved between flies and mammals as the mammalian homolog Sarm1 is also required for axon degeneration (Osterloh et al., 2012, Gerdts et al., 2013). Interestingly, a recent study suggests that Sarm1 promotes axon degeneration by locally breaking down NAD+, the metabolite of Nmnat (Gerdts et al., 2015). Moreover, there are other compelling data suggest Nmnat2 protects axon degeneration via inhibition of Sarm1 (Gilley et al., 2015, Gilley et al., 2013) (Sasaki et al., 2016). These studies tie Sarm1 and Nmnat together into what appears to be a common pathway for axonal destruction.

1.5 Overview

In this thesis, I will describe my findings about the mechanisms of axon regeneration as well as axon degeneration.

Chapter 2 will be a review about the intrinsic mechanisms of axon regeneration, with specific emphasis on the recent significant findings in *Drosophila*. This chapter also discusses the merits of using *Drosophila* as a model organism to study molecular mechanisms of axonal injuries.

Chapter 3 will mainly focus on identification of a novel regulator of DLK in modulating axon regeneration. DLK is known to play a critical role in axon regeneration, however it is not clear how it is activated and stabilized after injury. In this chapter, I will demonstrate that cAMP and protein kinase A (PKA) are direct upstream activators of DLK upon axonal injury. Moreover, PKA also stabilizes DLK via a JNK independent mechanism.

Chapter 4 will focus on identification of a novel axon degeneration promoter in *Drosophila*. This protein is named Raw in *Drosophila* and known to play important roles during development. I will show that Raw is required for axon degeneration in different types of neurons via function upstream of a highly conserved transcription factor Fos.

Chapter 5 will summarize these findings and discuss relevant studies in the field, as well as future research directions.

Chapter 2 :

Intrinsic mechanisms for axon regeneration: insights from injured axons in *Drosophila*

2.1 Abstract

Axonal damage and loss are common and negative consequences of neuronal injuries, and also occur in some neurodegenerative diseases. For neurons to have a chance to repair their connections, they need to survive the damage, initiate new axonal growth, and ultimately establish new synaptic connections. This review discusses how recent work in *Drosophila* models have informed our understanding of the cellular pathways used by neurons to respond to axonal injuries. Similarly to mammalian neurons, *Drosophila* neurons appear to be more limited in their capacity regrow (regenerate) damaged axons in the central nervous system, but can undergo axonal regeneration to varying extents in the peripheral nervous system. Conserved cellular pathways are activated by axonal injury via mechanisms that are specific to axons but not dendrites, and new unanticipated inhibitors of axon regeneration can be identified via genetic screening. These findings, made predominantly via genetic and live imaging methods in *Drosophila*, emphasize the utility of this model organism for the identification and study of basic cellular mechanisms used for neuronal repair.

2.2 Introduction

As the fundamental conduit for communication with other neurons, a neuron's axon is one of its most vulnerable features. An injury at any position in an axon's length leads to a silencing of its function. How do nervous systems cope with axonal damage? Neurons are expected to last for an animal's lifetime, so re-development and replacement of the damaged neuron is not an option in the adult nervous system. Instead, neuronal repair, when it occurs, requires that the neuron maintain survival through the damage and then initiate new axonal growth (termed 'axon regeneration') to re-form its lost connection. A neuron's ability to do this varies widely depending on what type of neuron it is, where the damage occurs, the presence of both extrinsic and intrinsic inhibitors of regeneration, and the cell's ability to organize its cytoskeleton to initiate new axonal growth. Towards the most idealistic goal of stimulating repair after nervous system damage, there is much interest in understanding what these factors are. Over the past decade, studies in invertebrate model organisms such as *Drosophila* and *C. elegans* have made increasingly important contributions to this goal, with discoveries made through genetic screens and live imaging techniques in these model organisms that take advantage of their simplified nervous systems and powerful genetic tools. In cases where it has been examined thus far, mechanisms identified in the invertebrate models have later been confirmed to be important in mammalian neurons.

In this review, we highlight recent and provocative discoveries made in *Drosophila* around the topic of responses to axonal and dendritic injuries. A number of different injury assays have been developed in the adult and larval nervous system and are reviewed in more detail in (Brace and DiAntonio, 2016, Fang and Bonini, 2012, Rooney and Freeman, 2014). Studies of axon degeneration in *Drosophila* have made groundbreaking discoveries and are also reviewed in more detail in (Rooney and Freeman, 2014). Here we focus on axon regeneration,

and also some of the interesting comparisons that can be made between injuries to axons verses injuries to dendrites.

2.3 *Drosophila* as a model to study intrinsic mechanisms for repair

A central feature of the mammalian nervous system is that axon regeneration occurs readily in the peripheral nervous system (PNS), yet fails to occur in the central nervous system (CNS). There has been great interest in understanding this dichotomy, since regeneration failure in the adult mammalian CNS is a major debilitating aspect of many neuronal injuries. One known reason for the dichotomy is the presence of proteins in CNS myelin that inhibit axonal growth, termed 'extrinsic' inhibitors (reviewed by (Silver et al., 2015)). Another is an 'intrinsic' incapability of neurons to initiate new axonal growth after damage in the CNS. Several landmark studies have shown that the intrinsic incapacity to regenerate can, at least for some neurons, be overcome through manipulations to cAMP or mTOR intracellular signaling pathways (Cai et al., 1999, Park et al., 2008, Qiu et al., 2002, Sun et al., 2011). How this capacity is either locked or unlocked, including how these pathways are regulated and utilized for this gating, is a topic of great interest in the field.

The *Drosophila* nervous system lacks myelin and many of the known extrinsic inhibitors of regeneration that are expressed by oligodendrocytes (eg. Nogo, OgMp, and MAG). However, despite this absence, the PNS/CNS dichotomy for may potentially also exist in *Drosophila*: new axonal growth can be observed in several different injury methods to motor and sensory neurons in the larval and adult PNS (reviewed in (Brace and DiAntonio, 2016)). However, in two injury assays described thus far in the larval and adult CNS, limited axonal sprouting was observed after injury (Song et al., 2012, Ayaz et al., 2008), and this lack of regeneration can be at least

partially bypassed by manipulations to the cAMP or the mTOR pathways (Song et al., 2012, Ayaz et al., 2008), echoing observations in mammalian neurons. Injuries in the CNS are inherently more likely to disrupt multiple neurons and synapses, and it is difficult to make direct comparisons across different injury methods and locations, so further studies are needed. However the similarities noted thus far suggest that neurons across the animal kingdom use similar intrinsic mechanisms to promote or inhibit their capacity to regenerate. *Drosophila* is therefore a reasonable model system to study these intrinsic mechanisms, and it benefits from the vast number of existing genetic tools to manipulate cellular signaling pathways on a single cell level in the *Drosophila* nervous system.

2.4 Intrinsic mechanisms of axon regeneration: discoveries from *Drosophila*

2.4.1 Cellular rearrangements in microtubules and organelles

An important technique for studying cellular responses to axonal damage is live imaging, which allows one to track changes in the structure of damaged neurons over time, and changes in the localization and abundance of their organelles and cytoskeletal components. For such studies, the dendritic arborization (da) neurons that line the larval body wall have been an excellent model, since these cells can be imaged in their entirety (cell bodies, dendrites, and axons) through the relatively transparent larval cuticle. Based on dendritic branching complexity, these sensory neurons are divided into four classes (Class I: most simple; Class IV: most complex) (Grueber et al., 2003). With specific GAL4 lines expressing in each type of these neurons, they can be genetically labeled and manipulated with single cell resolution. In these neurons, striking responses to axonal injury have been characterized to the microtubule cytoskeleton and to the endoplasmic reticulum (ER), some of which may be coupled to the neuron's ability to initiate

axon regeneration (Figure 2.2). Here we briefly review some of these changes that take place in neurons as they respond to axonal injury.

A useful tool to study the organization of microtubules in these neurons is to follow growth of individual microtubules via live imaging, using the microtubule plus-end binding protein EB1-GFP. In axons the microtubules are uniformly oriented with their growing ends ('plus ends') pointed away from the cell body, while microtubules in mature dendrites are oriented with an opposite polarity (Stone et al., 2008, Hill et al., 2012) (Figure 2.2). Strikingly, axonal injury (but not dendritic injury) induces a dramatic and global increase in the number of growing microtubules throughout the cell body, dendrites, and proximal axon (Stone et al., 2010) (Figure 2.2).

What is the function of this massive induction in new microtubule growth? It appears rapidly, within minutes, in neurons that are able to regenerate their axon (Stone et al., 2010), so it may reflect or occur alongside important cellular changes that establish this capacity. This capacity in the da sensory neurons is truly remarkable: if the axon is completely removed (leaving no axonal stump attached to the cell body) then a dendrite becomes restructured and transformed an axon (Stone et al., 2010), demonstrating a strong homeostatic drive to have an axon. This transformation entails a re-organization of microtubules within the dendrite to form a new process with the plus-end out polarity appropriate and specific to axons (Figure 2.2). Manipulations that inhibit microtubule growth, including knock-down of the microtubule polymerase msps, prevent the dendrite-to-axon transformation, however knock-down of gamma-tubulin, which inhibits the formation of new microtubules, does not inhibit the transformation (Stone et al., 2010, Chen et al., 2012). So the new microtubules may not be primary effectors of axonal regeneration, but may instead reflect broad and global effects of an axonal injury

response. In fact, current data favors the idea that the induced microtubule growth is a neuroprotective response: once the dynamics is induced, neurites that are damaged during subsequent injuries become resistant to the process of Wallerian degeneration (Xiong and Collins, 2012, Chen et al., 2012). Intriguingly, overexpression of expanded polyglutamine proteins (a form of proteotoxic stress) seems to engage the same protective response pathway, which involves microtubule nucleation via gamma-tubulin and is controlled by activation of Jun N-terminal kinase (JNK) signaling (Chen et al., 2012). After time (72 hours after axonal injury), the microtubule dynamics ultimately quiet down, and manipulations that prolong the response can actually inhibit axonal regeneration (Stone et al., 2010, Chen et al., 2012). This builds a model that major aspects of the axonal injury response, including the global changes in microtubule dynamics, may serve as an initial protective response but must ultimately subside before the neuron can initiate substantial new growth from its axon.

In addition to the global changes in microtubule structure induced by axonal injury, injury within any neurite (either axon or dendrite) also induces local changes in microtubule polarity (Song et al., 2012, Lu et al., 2015). This likely occurs via calcium-stimulated depolymerization of microtubules near the injury site, and treatments that block this polarity change in cultured neurons (eg., low concentrations of vinblastine applied immediately following axonal injury) completely inhibit axon regeneration (Lu et al., 2015). These local changes allow for microtubules of opposing polarities to orient at the new 'tips' of the injured neurite. This creates a scenario for microtubule sliding, driven by the kinesin-1 motor protein, which can link to oppositely polarized microtubules and power them further apart (Lu et al., 2015). The microtubule sliding ability of kinesin-1 plays an important role in promoting axonal outgrowth

during development (del Castillo et al., 2015, Winding et al., 2016), and genetic knock-down of kinesin-1 leads to failed axon regeneration in *in vitro* assays (Lu et al., 2015).

Another important factor in axon regeneration is the microtubule severing protein Spastin: deletion of a single copy of Spastin can dominantly impair regeneration, and over-expression of Spastin also impairs regeneration, suggesting that the process is very sensitive to gene dose of Spastin (Stone et al., 2012). Manipulations of Spastin have no obvious effects upon the global increase in microtubule dynamics induced by axotomy (Stone et al., 2012). Instead, a recent study suggests an important role for Spastin in its ability to associate with the ER as well as microtubules. Spastin and Atlastin, another protein with similar ER-microtubule association roles, mediate an enhanced localization of ER and microtubules into the growing tips of regenerating axons ((Rao et al., 2016) and Figure 2.2). Since the calcium-releasing function of ER in the growth cone has been shown to play an important role in growth cone guidance and axon regeneration (Sun et al., 2014, Takei et al., 1998), the localization of ER to a regenerating axon tip could promote regeneration by providing intracellular calcium locally.

2.4.2 Axonal damage signaling via the DLK kinase

Work in multiple model organisms from *C. elegans* and *Drosophila*, followed by later studies in mice has identified the dual leucine zipper kinase (DLK, also known as Wallenda (Wnd) in *Drosophila*) as an essential mediator of a neuron's ability to initiate new axonal growth (reviewed by (Tedeschi and Bradke, 2013)). This kinase is not required for axonal outgrowth during development, but its function is essential for neurons to initiate new growth after injury. Upon axonal injury, Wnd/DLK mediates the retrograde transport of the 'injury signals' in axons. The JNK MAP Kinase and transcription factor Fos are required for Wnd/DLK mediated axonal regeneration, suggesting a transcriptional response is involved (Xiong et al., 2010). Moreover,

studies in *C. elegans* found that the DLK pathway also regulates microtubule dynamics after injury via tubulin posttranscriptional modifications (Ghosh-Roy et al., 2012). This is consistent with the findings in *Drosophila* that JNK mediates the acute response of microtubule dynamics after injury (Stone et al., 2010). Therefore, Wnd/DLK appears to function as an upstream regulator of multiple cellular responses to axonal damage. Interestingly, Wnd/DLK is actively transported (associated with vesicles) in axons and becomes acutely activated upon axonal injury (Xiong et al., 2010, Holland et al., 2016). Thus, it may be able to 'sense' axonal damage locally in axons.

What determines DLK's activation after axonal damage? It is interesting that multiple studies in *Drosophila, C. elegans* and mammalian neurons have suggested that Wnd/DLK signaling is sensitive to manipulations that alter cytoskeletal structure in neurons, including mutations in spectraplakin, TCP1α or TCP1β, and treatment with nocodazole or cytochalasin D (Valakh et al., 2013, Valakh et al., 2015, Bounoutas et al., 2011, Kurup et al., 2015, Voelzmann et al., 2016, Massaro et al., 2009), hence axonal damage *per se* is not the only way to activate DLK. These observations suggest a central role for Wnd/DLK as a both a sensor and regulator of cytoskeletal structure in neurons. However, it is not yet clear how this kinase actually senses cytoskeletal changes on a mechanistic level. This is an interesting area of future work, particularly since several recent studies in mammalian neurons have implicated DLK in neuronal degeneration and death, including in models of glaucoma and excitoxicity (Watkins et al., 2013, Welsbie et al., 2013, Ghosh et al., 2011, Pozniak et al., 2013). Therefore, while DLK activation is important for axonal regeneration, it may be of greater interest to inhibit (rather than promote) in order to minimize pathology in neurodegenerative diseases and injury.

Other clues to DLK's activation mechanism may come from the biochemical identification of the upstream activators that can stimulate the activation loop of the Wnd/DLK kinase. Recent work has discovered such an activity for the cAMP effector kinase protein kinase A (PKA), which can induce Wnd's activation and function, and is required for its activation in injured axons (Hao et al., 2016). Genetic interaction studies have also led to the suggestion that Wnd may also be regulated by TORC1 (Wong et al., 2015). It is striking that, as discussed previously, both cAMP and mTOR signaling are known for abilities to unlock axonal regeneration potential. Importantly, DLK is also similarly regulated by cAMP and PKA in mammalian as well as *Drosophila* neurons (Hao et al., 2016). This suggests an interesting possibility that DLK activation could a key feature for the 'unlocking' mechanism, and a potentially universal mechanism for stimulating axonal regeneration.

2.4.3 Regulation of RNA-processing pathways

Use of *Drosophila* injury models for forward genetic screens allows for the discovery of new unanticipated regulators of axon regeneration. A recent example is the discovery of an RNA processing enzyme Rtca (RNA 3'-terminal phosphate cyclase), as a potent inhibitor of axon regeneration: mutations in Rtca allow for sensory neuron axons to grow in the CNS, and overexpression of Rtca causes an inhibition to axon regeneration in the PNS (Song et al., 2015). Following on this discovery, Song *et al* then demonstrated that the mammalian ortholog of Rtca functions similarly as an inhibitor of axonal regeneration in adult DRG neurons in culture and in the adult optic nerve *in vivo*. Known substrates of Rtca include mediators in the tRNA splicing pathway, and the ER-stress response factor X-box protein 1 (Xbp-1), whose non-canonical intron shares features and regulation with the tRNA splicing pathway (Yoshida et al., 2001). Song *et al* suggested that Xbp-1 may be the target of Rtca during axonal regeneration since the enhanced

regeneration observed in Rtca mutants was largely abolished in *Xbp-1, Rtca* double mutant flies. Such a role for Xbp-1 in axon regeneration would be consistent with other recent studies that have noted roles for ER stress pathways in regeneration (Onate et al., 2016, Ying et al., 2015). However, the genetic data alone cannot exclude other potential (and yet unknown) targets of Rtca regulation. Interestingly, a recent study in *C. elegans* identified the tRNA ligase Rtcb as an inhibitor of axon regeneration, but ruled out both Xbp-1 and tRNAs as functional targets for Rtcb in this function (Kosmaczewski et al., 2015). These findings suggest that much remains to be learned about the 'RNA dimension' of responses to axonal injury: we may have glimpsed only the tip of an iceberg that remains to be charted.

2.5 How are responses to dendritic injury different from axonal injury?

Many neuronal injuries entail loss of dendrites as well as axons. The highly characterized anatomy of *Drosophila* da neurons has turned into an exciting model system for comparing and contrasting the differences between dendritic and axonal injury. After laser-induced removal, da neurons show a robust ability to regrow their dendritic arbors (Thompson-Peer et al., 2016, Stone et al., 2014, Song et al., 2012), even when carried out in adult flies (Stone et al., 2014). While for some classes the regenerated dendrites cannot cover the entire lost territory and the architecture is not completely restored, the regenerated dendrites show a remarkable ability to re-grow the stereotyped pattern of primary branches according to their class type (Stone et al., 2014, Thompson-Peer et al., 2016). Strikingly, nearly all of the aspects of axon regeneration described above are not required for dendrite regeneration: dendrite regeneration does not require DLK or Spastin (Stone et al., 2012, Stone et al., 2014), and occurs independently of global microtubule rearrangements and ER relocalization (Stone et al., 2010, Rao et al., 2016). Dendrite regeneration and axon regeneration are therefore fundamentally different processes. This does

not mean that some basic cellular components may mediate both. The AKT-PTEN signaling, classically associated with cellular growth, is required both axon and dendrite regeneration (Song et al., 2012). Since mixed polarity microtubules form at the tips of injured dendrites (Song et al., 2012), kinesin-driven microtubule sliding is another candidate mediator of renewed dendrite growth, particularly since microtubules also have mixed (opposing) polarity in dendrites during their original developmental outgrowth (Hill et al., 2012). However these ideas remain to be further tested and require further basic understanding of the mechanisms that promote dendrite growth, and how these are distinct from axonal growth.

Comparison of dendritic injury with axonal injury has also revealed some differences in degeneration, defined here as the process by which the injured piece that is separated from the cell body becomes dismantled. This process requires a 'self-destruction' pathway which functions cell autonomously in the injured neurite, and studies of axon degeneration in *Drosophila* have identified many key players in this process (reviewed by (Neukomm and Freeman, 2014)). We know that at least some of the genetic manipulations that inhibit axon degeneration also inhibit dendrite degeneration (Tao and Rolls, 2011). However, studies of dendrite degeneration in *Drosophila* suggest the existence of aspects that are distinct from axons, such as specific requirement for the microtubule-severing protein fidgetin in dendrite but not axon degeneration (Tao et al., 2016). Live imaging data suggest that Fidgetin promotes an increase in microtubule number, presumably via its severing activity, followed by microtubule disassembly in distal dendrites. This response contrasts with axonal injury as described above, which promotes a global increase throughout the cell body (including all dendrites and the proximal stump), but not in distal axon stump (Stone et al., 2010). These differences further

emphasize that axons and dendrites respond distinctly to injuries and distinct mechanisms are involved.

2.6 Can functional reconnection occur after axon injury in *Drosophila*?

Initiation of new axonal growth is only the first step towards repairing lost connections. True repair requires that regenerating axons find their targets and form functional synapses. In general this process of 'synapse regeneration' is poorly studied in the field. To date, it has not been noted in any of the *Drosophila* models of injury, however the *Drosophila* system may present future opportunities to study this process. Earlier studies in other invertebrate, including cockroaches, leech, Aplysia, snails, crayfishes and crickets, suggest functional regeneration can indeed happen. By following the process in a system that is tractable to cell-specific genetic manipulations, live imaging and behavioral screening, one could determine whether synapse regeneration follows the same pathways as synapse development, whether axon regeneration pathways need to be turned off to initiate synapse formation, and what role remaining synapses within the injured circuit play in the process. We predict that exciting work lies ahead in this powerful model organism.

2.7 Figures

Figure 2.1

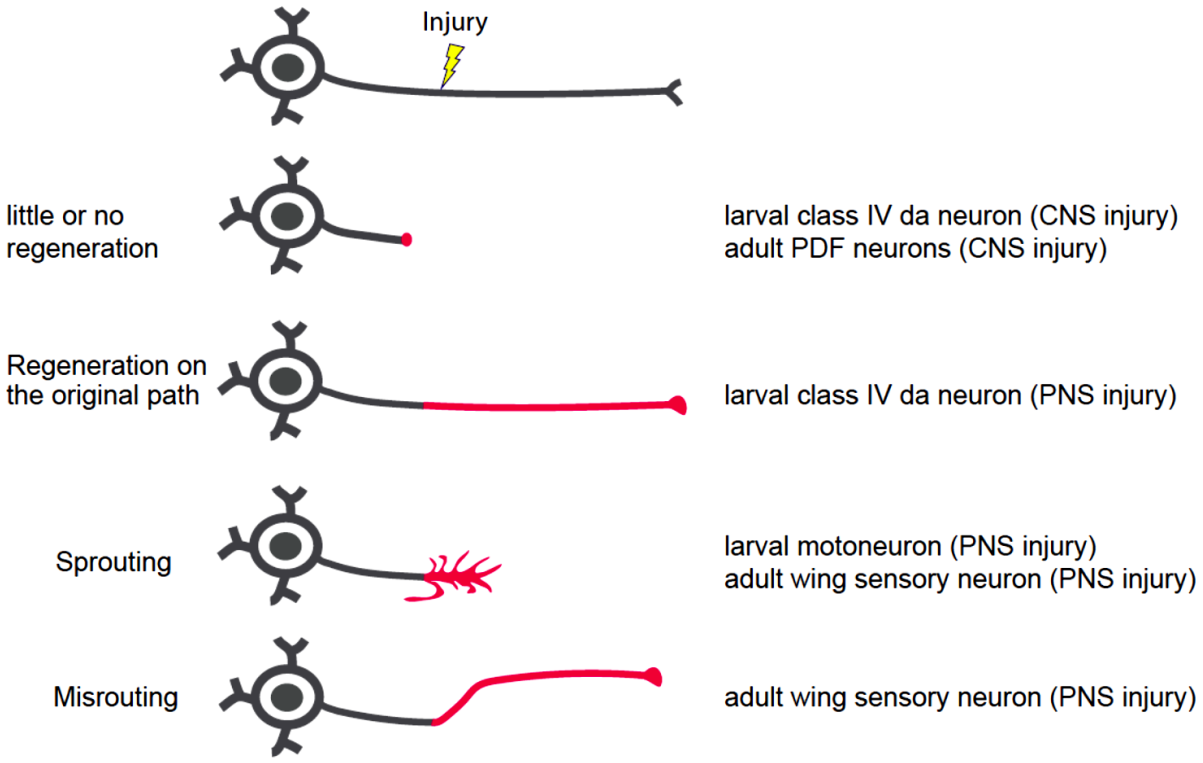

Figure 2.1Axons regenerate to varying extents in different Drosophila axon injury models

New axonal growth after injury, cartooned in pink, occurs to varying degrees after injuries in the adult and larval PNS. Some of the sensory neurons that line the larval body wall initiate remarkable regeneration along the original path of the lost axon (Song et al., 2012). Other injury models in the adult wing and larval peripheral nerves note extensive new axonal sprouting (Xiong et al., 2010, Soares et al., 2014). This undirected growth (‘sprouting’) may reflect an absence of salient cues to guide directed growth for the regenerating axon. In some cases, sensory neurons in the adult wing can initiate extensive directed growth, however this occurs along a new path that is distinct (‘misrouted’) from the original path (Soares et al., 2014). This may be a side effect of massive tissue damage and scar formation at the injury site that prevents the axon from finding its correct path.

In contrast to the PNS injuries, the two studies thus far that have injured axons in the CNS have noted very poor growth responses (Song et al., 2012, Ayaz et al., 2008). The contrast is particularly interesting for the Class IV da sensory neuron axons, since the axons grow robustly after injury in a PNS location but very poorly after injury in a CNS location (Song et al., 2012, Song et al., 2015).

Figure 2.2

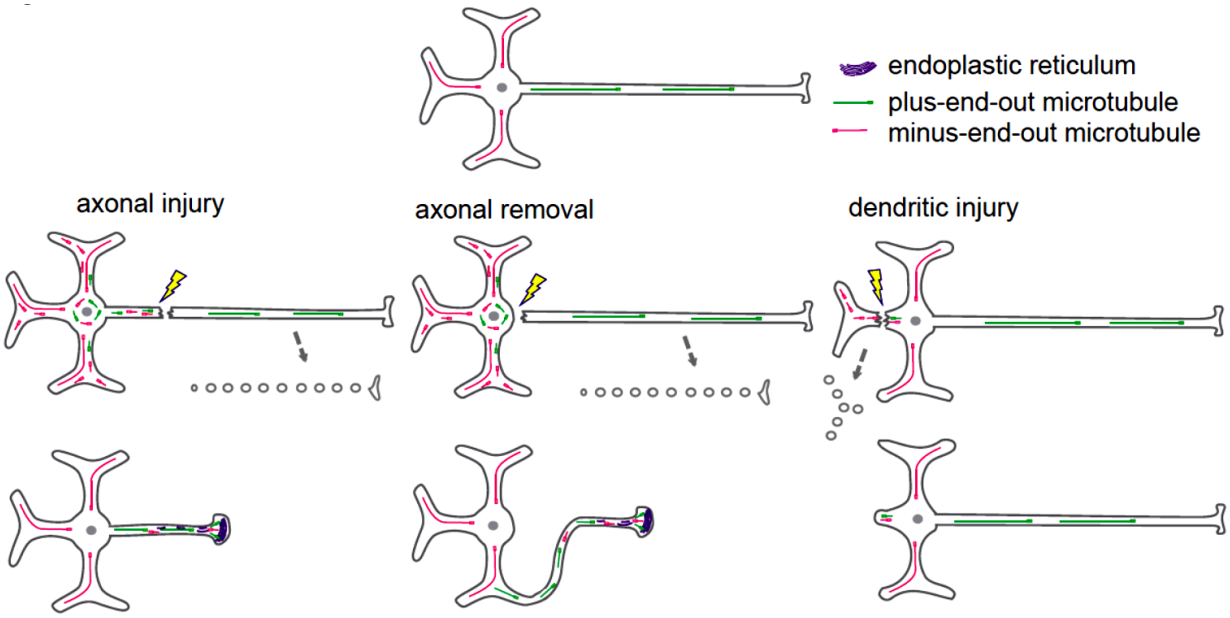

Figure 2.2 Injury triggered microtubule dynamics in neurons

Microtubules are organized in axons and dendrites with distinct orientations of their growing (plus) ends: in axons microtubules orient with plus ends facing away from the cell body (plus-end-out), colored green, while in dendrites microtubules orient with minus-ends-out, colored red. Axonal injury, but not dendritic injury, triggers a global increase in the number of growing microtubules. In contrast, increased microtubule is observed in distal dendrite stump, but not distal axon stump. Mixed-polarity microtubules are observed in both proximal axon stump and dendrite stump. ER and microtubules are accumulated in growing axon tips, but not in growing dendrite tips, 96 hours after axotomy (for class I da neuron). In the case of completely axonal removal, one dendrite can switch microtubule polarity and become a growing axon.

Chapter 3 :

An evolutionarily conserved mechanism for cAMP elicited axonal regeneration involves direct activation of the dual leucine zipper kinase DLK

3.1 Abstract

A broadly known method to stimulate the growth potential of axons is to elevate intracellular levels of cAMP, however the cellular pathway(s) that mediate this are not known. Here we identify the Dual Leucine-zipper Kinase (DLK, Wnd in *Drosophila*) as a critical target and effector of cAMP in injured axons. DLK/Wnd is thought to function as an injury 'sensor', as it becomes activated after axonal damage. Our findings in both *Drosophila* and mammalian neurons indicate that the cAMP effector kinase PKA is a conserved and direct upstream activator of Wnd/DLK. PKA is required for the induction of Wnd signaling in injured axons, and DLK is essential for the regenerative effects of cAMP in mammalian DRG neurons. These findings link two important mediators of responses to axonal injury, DLK/Wnd with cAMP/PKA, into a unified and evolutionarily conserved molecular pathway for stimulating the regenerative potential of injured axons.

3.2 Introduction

Repair of lost axonal connections generally fails to occur after neuronal injury in the adult mammalian central nervous system (CNS). This failure is not only a reflection of the

growth inhibitory nature of CNS tissue (Fawcett et al., 2012, Filbin, 2003, Silver et al., 2015), but also due to the lack of intrinsic capacity for neurons in the adult CNS to grow axons (Liu et al., 2011, Sun and He, 2010). However, landmark studies by Richardson and Issa have suggested that neurons indeed possess an innate ability to regenerate their axons in the adult mammalian CNS, and that this ability can be unlocked by a 'conditioning lesion' (Richardson and Issa, 1984). In adult DRG neurons, an injury to peripherally projecting axons, i.e. a compression injury to the sciatic nerve, unleashes growth programs within the DRG and allows for regeneration of its centrally projecting axons in the spinal cord (Neumann and Woolf, 1999, Richardson and Issa, 1984). This growth can be induced by a PNS lesion even after the CNS lesion has occurred (Ylera et al., 2009), hence it is of great interest from a therapeutic perspective to understand the molecular mechanisms that allow for the unlocking of such regenerative potential.

Previous studies have discovered that several signal transduction pathways are activated in DRG neurons upon a conditioning injury, including JAK-STAT3 (Qiu et al., 2005), ATF3 (Fagoe et al., 2015, Hollis and Zou, 2012), Smad1 (Zou et al., 2009), Activin (Omura et al., 2015), HIF-1alpha (Cho et al., 2015) and cAMP (Qiu et al., 2002, Neumann et al., 2002, Cai et al., 1999). Impressively, ectopic elevation of cAMP alone is sufficient to strongly enhance regeneration (Xiao et al., 2015b, Qiu et al., 2002, Neumann et al., 2002). Since this second messenger is commonly modulated by growth signals and neuronal activity, cAMP modulation has been suggested as a potential therapeutic inroad to stimulate the regenerative potential of neurons (Xiao et al., 2015b). However, the downstream pathways that are engaged by this broadly utilized second messenger to actually promote axonal regeneration are not known. Much attention has focused upon the cAMP-responsive element binding protein (CREB), since constitutive activation of CREB is sufficient to stimulate axonal regeneration in the presence of

CNS myelin *in vivo* (Gao et al., 2004). However, more recent studies indicate that endogenous CREB is not required for cAMP elicited axonal regeneration *in vitro* (Ma et al., 2014). Hence it remains elusive how cAMP elevation activates axonal regrowth programs in neurons.

A recent study has identified an essential role for the dual zipper-bearing kinase DLK in the pro-regenerative effect of a conditioning lesion in adult DRG neurons (Shin et al., 2012a). Similarly, the *Drosophila* homologue Wallenda (Wnd), mediates protective effects of a conditioning lesion in *Drosophila* motoneurons (Brace and DiAntonio, 2016, Xiong and Collins, 2012). This conserved axonal mitogen activated kinase kinase kinase (MAPKKK) is thought to function as a sensor of axonal damage, and therefore should become activated upon conditioning injury. In support of this, Wnd/DLK is transported in axons (Xiong et al., 2010) and is required acutely in injured axons for the generation of signals that are retrogradely transported to the cell body (Xiong et al., 2010, Shin et al., 2012a). DLK/Wnd is required for axonal regeneration in many types of neurons, including motoneurons in mammals, flies and worms, and CNS neurons where regeneration is ectopically induced by PTEN mutations (Yan et al., 2009, Hammarlund et al., 2009, Xiong et al., 2010, Shin et al., 2012a, Watkins et al., 2013). Conversely, in mammalian CNS neurons that do not regenerate (eg. retinal ganglion cells, RGCs), DLK activation after injury mediates cell death (Welsbie et al., 2013, Watkins et al., 2013).

Collectively, these findings support the model that a conserved function of the Wnd/DLK kinase is to ‘sense’ axonal damage. Through a yet unknown mechanism, axonal damage leads to activation of Wnd/DLK’s kinase function. Once activated, downstream signaling mediates both beneficial and deleterious outcomes in neurons, depending upon the context. The high stakes outcomes of regeneration or death, combined with additional findings that DLK mediates cell death in models for nerve growth factor withdrawal (Huntwork-Rodriguez et al., 2013, Ghosh et

al., 2011), glaucoma (Welsbie et al., 2013), MPTP toxicity (Mathiasen et al., 2004) and excitotoxicity (Pozniak et al., 2013), have inspired much interest in understanding the unknown pathways that lead to the activation of DLK/Wnd in injured axons.

Here we identify a direct upstream activator of DLK/Wnd in injured axons, in the form of the cAMP effector kinase PKA. We find that PKA phosphorylates evolutionarily conserved serines within the activation loop of DLK, which is sufficient to activate DLK independently of its downstream signaling mechanisms. In addition, our functional studies in both *Drosophila* motoneurons and adult mammalian DRG neurons indicate that the ability of cAMP and PKA to promote axonal regeneration depends entirely upon the ability of PKA to activate the DLK/Wnd kinase. These findings present a unified and evolutionarily conserved molecular pathway, from cAMP to PKA to DLK, which plays a central role in stimulating the ability of injured axons to regenerate.

3.3 Results

3.3.1 PKA regulates axonal regeneration via Wnd.

Previous studies in mammalian and *C. elegans* neurons suggest that cAMP signaling stimulates regenerative axonal growth (Qiu et al., 2002, Neumann et al., 2002, Cai et al., 1999, Ghosh-Roy et al., 2010). To study this axon regeneration pathway in *Drosophila*, we used previously developed axon injury assay in third instar larvae (Xiong et al., 2010), and found that knockdown of phosphodiesterase *dunce* (*dnc*) or activation of PKA by overexpression of the catalytic subunit (PKA^{CA}) (Li et al., 1995) led to an enhanced growth response of *Drosophila* motoneuron axons after nerve crush injury (Figure 3.1A). The new axonal growth from the injured proximal stump generally assumes a highly branched shape, characterized by a network

of small branches and a general thickening of the axon diameter. To assess the injury response, we quantified the total membrane volume within 100 μm of the axonal tip (indicated by the dash line in Figure 3.1A). In control animals, this total volume increases 3 fold, from 68.5 μm^3 to 200 μm^3 15 hours after injury. PKA activation led to a 1.5 fold increase in this volume compared to control (WT) axons (Figure 3.1B). The enhanced sprouting response stimulated by PKA was lost when DLK/Wnd function was inhibited by co-expression of RNAi targeting Wnd (but not a control RNAi) (Figure 3.1A and B). These observations are consistent with the previous finding in *C.elegans* that DLK is required for the regeneration that is induced by cAMP signaling (Ghosh-Roy et al., 2010). In addition, PKA alone is required for *Drosophila* motoneurons to initiate regenerative sprouting, as RNAi-mediated knockdown of the PKA catalytic subunit inhibited the sprouting response by 50% compared to control axons (Figure 3.1A and B). cAMP and PKA therefore play an influential role in the regenerative capacity of *Drosophila* motoneuron axons.

3.3.2 PKA modulates the levels of Wnd and downstream signaling in *Drosophila* motoneurons.

While the above and previously described genetic interactions (Ghosh-Roy et al., 2010) suggest a relationship, whether Wnd/DLK functions downstream of PKA or in a parallel pathway cannot be discerned from genetic epistasis alone. To probe the relationship between PKA and Wnd, we first utilized previously established tools in *Drosophila* for monitoring the activation of Wnd and downstream nuclear signaling. Wnd signaling induces expression of the c-Jun N-terminal Kinase (JNK) phosphatase puckered (*puc*), which can be measured as lacZ expression using fly lines that contain the *puc*-lacZ enhancer trap reporter (Xiong et al., 2010). *Puc*-lacZ is expressed at low levels in uninjured motoneurons, however it is induced by axonal

injury in a manner that requires both Wnd and JNK kinase function (Xiong et al., 2010). We found expression of either *dnc*-RNAi or PKA^{CA} induced the expression of *puc*-lacZ in motoneurons (Figure 2A). This induction is Wnd dependent, as RNAi knockdown of Wnd (but not a control RNAi) rescued the *puc*-lacZ elevation (Figure 3.2A).

Previous studies in multiple organisms suggest that Wnd/DLK is highly regulated at the level of protein turnover and increased levels of DLK correlate with the activation of downstream signaling (Xiong et al., 2010, Huntwork-Rodriguez et al., 2013, Welsbie et al., 2013, Collins et al., 2006, Nakata et al., 2005, Nihalani et al., 2000, Hammarlund et al., 2009). We therefore tested whether PKA activation altered Wnd levels. The total levels of endogenous Wnd within 2nd instar larval brains were significantly elevated (by 75%) when PKA^{CA} was expressed in neurons (Figure 3.2B).

To test whether the change of Wnd level is due to a posttranscriptional mechanism, we used the Gal4/UAS system to ectopically express a GFP tagged Wnd transgene in *Drosophila* motoneurons. Since overexpression of WT Wnd can cause lethality, we expressed an inactive (kinase dead) version (GFP-Wnd^{KD}) that contains a point mutation in the kinase domain (Collins et al., 2006). We found that expression of PKA^{CA} induced a 12-fold increase in the levels of GFP-Wnd^{KD} in motoneuron axons (Figure 3.2C). In contrast, GFP-Wnd^{KD} was not significantly altered in cell bodies (Figure 3.2C).

The increase in axonal GFP-Wnd^{KD} when PKA is induced has remarkable similarity to what occurs in axons after injury (Xiong et al., 2010, Huntwork-Rodriguez et al., 2013). We therefore tested whether PKA is required for the induction of Wnd in proximal axons after nerve crush injury. In control (WT) motoneurons, a significant increase in the mean intensity of the

GFP-Wnd^{KD} was observed 7 hours after injury (Figure 3.2D). This increase is abolished by co-expression of RNAi targeting PKA-C1, but not a control RNAi (Figure 3.2D). These observations suggest that PKA is required for the activation of Wnd signaling and the induction of Wnd protein levels downstream of axonal injury.

3.3.3 PKA activates DLK through phosphorylation of its activation loop.

To test whether the regulation of DLK by PKA is conserved in mammalian neurons, we examined endogenous DLK protein in cultured embryonic rat cortical neurons. Treatment with forskolin, which activates PKA via elevation of cAMP, led to a 2-fold increase in the level of endogenous DLK protein (Figure 3.3A). This effect of cAMP elevation requires PKA, since the increase in DLK levels was abolished by co-treatment with the PKA inhibitor H-89 (Figure 3.3A). In contrast, treatment with H-89 alone led to a significant reduction in the DLK levels (Figure 3.3B). Similar results were observed in HEK293 cells co-transfected with Flag-tagged DLK and either a control empty plasmid or PKA^{CA}. PKA^{CA} induces an approximately two-fold increase in DLK protein levels (Figure 3.3E and 3.4B). PKA^{CA} also stimulates a phosphatase-sensitive increase in DLK molecular weight, which is most visible when equal amounts of DLK protein are compared (Figure 3.3F).

Although the mechanism of DLK activation is unknown, phosphorylation of the activation loop is important for activation of other kinases in the mixed lineage kinase family that DLK belongs to (Durkin et al., 2004, Leung and Lassam, 2001). Notably, the activation loop contains a conserved consensus sequence for PKA (Figure 3.3C), and a recent study has demonstrated that the predicted phosphorylation site S302 is required for DLK to activate downstream kinases (Huntwork-Rodriguez et al., 2013).

To test whether PKA stimulates phosphorylation of DLK's activation loop (S302), we have generated phospho-specific antibodies against a phosphorylated peptide corresponding to activation loop of mouse DLK (KELSDKpSTKMpSFAGTV). The phospho-DLK antibodies detected a strong band in HEK293 cells that overexpress WT mouse DLK, but show no reactivity for mutant DLK^{S302A} (Figure 3.3D). Remarkably, expression of PKA^{CA} in HEK293 cells induces a dramatic increase of phospho-S302 DLK (Figure 3.3E), even when normalized to the levels of total DLK (Figure 3.4B). Similar results were observed when cells were treated with forskolin for 3 hours (Figure 3.4A).

Since the activation loop contains a conserved consensus sequence for PKA substrates, it should be capable of phosphorylating this site in DLK directly. Indeed, we found that purified PKA can strongly stimulate pS302 reactivity upon purified Flag-DLK *in vitro* (Figure 3.6).

A recent study in *Drosophila* has suggested that TORC1 may activate and phosphorylate Wnd (Wong et al., 2015), so we considered whether TORC1 plays a role in the activation of DLK by PKA. We used both torin1 and rapamycin to inhibit TORC1 in the presence or absence of PKA in HEK293 cells. However, in both cases we observed no effect upon DLK and pDLK levels (Figure 3.7). PKA therefore stimulates phosphorylation of DLK's activation loop independently of TORC1 function.

To confirm that S302 is a critical site for Wnd/DLK function in axonal regeneration, we conducted a rescue experiment in *Drosophila* motoneurons based on the requirement of Wnd for axonal sprouting after injury. We generated UAS-$Wnd^{S301A,S305A}$ transgenic flies expressing Wnd with mutations in two serines analogous to S298 and S302 in the activation loop of DLK. As shown in Figure 3G, all axons in *wnd* mutants fail to initiate a sprouting response, which can

be rescued by co-expression of WT Wnd , but not $Wnd^{S301A,S305A}$. In addition, overexpression of $Wnd^{S301A,S305A}$ does not give rise to any of the previous described gain-of-function phenotypes similar to wild type Wnd, suggesting that S302 is indeed required for DLK function.

3.3.4 PKA promotes DLK stability independently of downstream signaling.

A previous study has described a positive feedback loop for DLK stabilization that involves the action of DLK's downstream effector JNK (Huntwork-Rodriguez et al., 2013). JNK activation stimulates phosphorylation of DLK at sites outside of its activation loop (T43 and S533) and changes DLK's sensitivity to degradation via the ubiquitin proteasome system (UPS) (Huntwork-Rodriguez et al., 2013). This increase in protein stability leads to an increase in total levels of DLK. Since PKA stimulates an increase in ectopically expressed DLK, it most likely increases protein stability. We therefore tested whether this increased stability involves the previously described JNK-dependent feedback mechanism. If this is the case, the effects of PKA and forskolin should depend on the function of JNK. As shown in Figure 4A and B, treatment with JNK inhibitor VIII led to a 30% decrease in total DLK level, as expected for JNK's previously demonstrated role in promoting DLK stability. However, even in the presence of JNK inhibitor, treatment with forskolin (Fig 3.4A) or transfection with PKA^{CA} (Figure 3.4B) increases DLK level by 50%. Moreover, treatment with JNK inhibitor had very little effect upon the fraction of total DLK that is phosphorylated at S302 (Figure 3.4A and B). These results suggest that PKA stimulates DLK phosphorylation and stabilization independently of downstream JNK activation.

Previous biochemical studies suggest that DLK's activation mechanism is associated with dimerization and autophosphorylation (Nihalani et al., 2000, Mata et al., 1996, Merritt et al., 1999). We therefore further considered whether PKA could function either upstream or

downstream of DLK's own ability to function as a kinase. To test this, we utilized a kinase-dead version of DLK, DLK^{K185A} , which is unable to activate downstream signaling or undergo autophosphorylation (Nihalani et al., 2000, Mata et al., 1996, Merritt et al., 1999). Consistent with the previously described feedback mechanism (Huntwork-Rodriguez et al., 2013), the DLK^{K185A} mutant protein was less stable, and addition of JNK inhibitor had no further effect upon the levels of kinase dead DLK protein (Figure 3.4C). However, PKA^{CA} stimulated a strong increase of DLK^{K185A} levels and phosphorylation at S302 for DLK^{K185A}. The ability of PKA to increase DLK protein stability and activation loop phosphorylation independently of DLK's own signaling abilities places PKA firmly upstream of DLK, as an upstream regulator/activator.

Since PKA promotes DLK stability and directly phosphorylates S302, we wondered whether PKA stabilizes DLK by phosphorylation of S302. Previous work has shown that a decreased stability for DLK^{S302A} mutant protein is linked to the fact that it is inactive for kinase activity, and is therefore unable to activate the downstream stabilization mechanism via JNK (Huntwork-Rodriguez et al., 2013 and Figure 3.4D). Consistent with this, the reduced levels of DLK^{S302A} protein are not further reduced in the presence of JNK inhibitors (Figure 3.4D). However surprisingly, co-transfection with PKA^{CA} still caused an increase of the levels of DLK^{S302A} protein (Figure 3.4D). We interpret that PKA regulates DLK via an additional mechanism, in conjunction with phosphorylation of the critical activation loop S302. This additional mechanism may involve other sites of phosphorylation, or other modes of regulation (discussed further below).

3.3.5 PKA stimulates axonal regeneration via DLK in adult DRG neurons.

Our finding that PKA activates DLK, taken together with previous findings that DLK promotes axonal regeneration in different neuronal cell types (Shin et al., 2012a, Xiong et al.,

2010, Hammarlund et al., 2009, Yan et al., 2009), led to the hypothesis that DLK is an important downstream mediator of cAMP-stimulated axon regeneration. To test this hypothesis, we employed a recently described replating assay for DRG neurons cultured from adult mice, which allows for a controlled and quantitative measure of the induction of axonal regeneration by *in vitro* manipulations such as forskolin treatment (Frey et al., 2015, Valakh et al., 2015). In this assay (depicted in Figure 5A), DRG neurons removed from adult mice were first cultured for 4-5 days, which allowed for the regenerative response activated by the dissection to subside. Neurons were then treated with forskolin for 24 hours, and then replated onto a fresh dish. The replating process removes all existing neurites so that the neurites observed within the second culture period can be identified as new growth. As shown in Figure 5B and E, treatment with forskolin stimulates the regenerative response (Frey et al., 2015). In addition, co-treatment with H-89 abolished the effect of forskolin on neurite outgrowth (Figure 3.5B-D), suggesting that PKA is required.

To determine whether forskolin-induced neurite outgrowth requires DLK, we performed the same experiment in neurons from DLK conditional knockout (KO) mice and littermate controls. The effects of forskolin on neurite outgrowth were abolished in DLK conditional knockout DRG neurons (Figure 3.5E-G). These findings suggest that DLK and its downstream signaling pathway(s) are important mediators of the pro-regenerative effects of cAMP elevation in neurons.

3.4 Discussion

The Wnd/DLK kinase is likely to function as a sensor of axonal damage. Depending upon the context, its activation can lead to either axonal regeneration or cell death and

degeneration (Tedeschi and Bradke, 2013). The factors that determine beneficial versus detrimental outcomes, along with the general cellular mechanisms that lead to the activation of DLK, are poorly understood. In this study, we found that an immediate upstream activator of DLK is the cAMP regulated kinase PKA. Elevation of cAMP signaling, which is activated by pro-regenerative manipulations such as a conditioning lesion, is the most widely known pathway for promoting axonal regeneration (Hannila and Filbin, 2008). We found that an essential component of this regenerative pathway is the activation of Wnd/DLK by PKA. These findings delineate an evolutionarily conserved mechanism for the activation of the Wnd/DLK kinase. Taken together with previous findings that Wnd/DLK is an essential regulator of responses induced by a conditioning injury (Shin et al, 2012; Xiong and Collins 2012), the activation of Wnd/DLK by PKA in injured axons presents a unified molecular pathway for activating a regenerative response to axonal damage.

In contrast to a merging of cAMP and DLK pathways, some other studies have suggested that these pathways may act separately (Li et al., 2015, Chung et al., 2016). A recent study has noted that in certain sensory neuron types in *C. elegans*, PKA *gain-of-function* mutations can induce axonal outgrowth even in *dlk* mutants (Chung et al., 2016). Hence, multiple pathways for axonal regeneration may be inducible by PKA. However DLK is strongly required for cAMP stimulated regeneration in other neuron types in *C. elegans* (Ghosh-Roy et al., 2010), and, importantly, in axonal regeneration induced by a conditioning lesion in the mammalian PNS (Shin et al., 2012a). Our findings now indicate that DLK is an important molecular target and effector of cAMP-induced regeneration in mammalian neurons.

Previous biochemical studies indicate that DLK activation involves dimerization via its leucine zipper domains and autophosphorylation, at locations that are yet undefined (Nihalani et

al., 2000, Mata et al., 1996). Because ectopic elevation of DLK/Wnd protein is sufficient to activate its downstream signaling (Mata et al., 1996, Nihalani et al., 2000, Huntwork-Rodriguez et al., 2013), and DLK is known to be highly regulated at the level of protein turnover (Collins et al., 2006, Xiong et al., 2010, Huntwork-Rodriguez et al., 2013, Nakata et al., 2005, Hammarlund et al., 2009), a plausible mechanism for its regulation is to hold its levels and/or its ability to dimerize in check (Mata et al., 1996, Nihalani et al., 2000). The existence of a direct upstream activator of the kinase was not previously implied, and has thus far been unknown. Here we found that PKA stimulates the phosphorylation of the activation loop of DLK independently of DLK's kinase activity, and also independently of downstream JNK signaling. This defines PKA as an upstream activator of DLK.

A previous study in *C. elegans* has described a mechanism through which transient elevation of intracellular Ca^{2+} upon axonal injury leads to the activation of DLK-1(Yan and Jin, 2012, Cho et al., 2013, Ghosh-Roy et al., 2010, Spira et al., 2001). In addition, earlier studies have implicated calmodulin-regulated calcineurin in the regulation of mammalian DLK (Mata et al., 1996). However, the hexapeptide that mediates activation by Ca^{2+} in *C. elegans* is not present in mammalian or *Drosophila* DLK/Wnd, and mammalian DLK can be activated independently of Ca^{2+} elevation by cytoskeletal destabilizing agents (Valakh et al., 2015). In contrast, the consensus PKA phosphorylation site in the activation loop of Wnd/DLK is conserved in all phyla (Figure 3.3C), suggesting this pathway as a central (although not necessarily exclusive) mechanism for regulating DLK.

We note that in conjunction with phosphorylation of DLK's essential activation loop, PKA enhances DLK's stability via an additional mechanism (Figure 4E), since the mutated protein DLK^{S302A} can still be stabilized upon PKA activation. This mechanism may involve

additional phosphorylation sites on DLK, and indeed, multiple PKA consensus sequences are observed in Wnd/DLK's sequence (Figure 3.8). However, it is also possible that PKA regulates DLK's stability via other mechanisms, such as previously described ubiquitination (Nakata et al., 2005, Collins et al., 2006), palmitoylation modification (Holland et al., 2016), changes in DLK's interacting proteins or subcellular localization. Such aspects of regulation could involve additional molecular targets of PKA, which would provide some potential for context specificity in DLK's regulation. While PKA has many cellular targets, its specificity can be highly regulated at a subcellular level by interactions with AKAP scaffolding proteins and local changes in cAMP (Tasken and Aandahl, 2004, Wong and Scott, 2004). It will be interesting to identify the additional players in cAMP and PKA regulation of DLK through future work.

It is remarkable that PKA stimulates a specific increase in Wnd levels in axons but not cell bodies (Figure 3.2C), and inhibition of PKA strongly inhibits the induction of Wnd protein after axonal injury (Figure 3.2D). Does PKA act locally in axons to stimulate DLK? Other studies have suggested that Wnd/DLK can regulate retrograde signaling pathways that originate in axons (Xiong et al., 2010, Ghosh et al., 2011, Shin et al., 2012a, Huntwork-Rodriguez et al., 2013, Watkins et al., 2013, Yan et al., 2009, Holland et al., 2016). Intriguingly, the Hiw/Rpm-1/Phr1 ubiquitin ligase, which is previously known for its role in regulating Wnd/DLK's levels in axons (Collins et al., 2006, Nakata et al., 2005, Lewcock et al., 2007, Babetto et al., 2013), contains a RCC1-like domain that biochemically inhibits adenylate cyclase, and therefore may negatively regulate cAMP signaling (Pierre et al., 2004). Hiw/Rpm-1/Phr1 is previously known for its role in regulating synaptic arborization and growth via its regulation of Wnd/DLK (Nakata et al., 2005, Collins et al., 2006, Wang et al., 2013, Wan et al., 2000, Shin and DiAntonio, 2011). The addition of cAMP and PKA into this regulatory pathway suggests a mechanism that may be

broadly utilized to orchestrate structural changes within presynaptic terminals. We propose that the regulation of DLK by PKA may be generally important for neuronal plasticity as well as responses to axonal damage.

3.5 Material and methods

3.5.1 Fly genetics

The following fly strains were used in this study: Canton-S (WT), m12-Gal4 (Ritzenthaler et al., 2000), BG380-Gal4 (Budnik et al., 1996), OK6-Gal4 (Aberle et al., 2002), RRa(eve)-Gal4 (Fujioka et al., 2003), *puc*-lacZ^{E69}, *wnd*^{3} , UAS-Wnd, (Collins et al., 2006), UAS-GFP-Wnd^{KD} (Xiong et al., 2010), UAS-PKA^{CA} (Li et al., 1995). UAS-Wnd^{S301A,S305A} flies were generated from pUAST-Wnd^{S301A,S305A} plasmid for this study. UAS-*wnd*-RNAi (VDRC 13786) and UAS-*moody*-RNAi (VDRC 100674) were from the Vienna RNAi center. UAS-*pka-c1*-RNAis (31277, 35196) and UAS-*dnc*-RNAi (27250) were acquired from Bloomington stock center.

3.5.2 Nerve crush assay and immunocytochemistry in *Drosophila*

Peripheral nerve crush assays in 3rd instar larvae were performed according to (Xiong et al., 2010). Briefly, the segmental nerves of third instar larvae were pinched and crushed by a fine No.5 forceps while the larvae were anesthetized with CO_2 gas. After injury, larvae were transferred to a grape plate and kept in 25ºC incubator for specified time periods.

Drosophila third instar larva were dissected in ice-cold PBS and fixed in 4% paraformaldehyde for 25 minutes. Antibodies were used in PBS supplemented with 0.3% Triton and 5% normal goat serum. Anti-lacZ (40-1a, Developmental Studies Hybridoma Bank) was diluted 1:100. Anti-dsRed polyclonal antibody (632495, Clontech) was diluted 1:1000. For

secondary antibodies, A488- or Cy3-conjucated goat anti-mouse or rabbit (Invitrogen) were used at 1:1000.

3.5.3 Imaging and quantification

Confocal images were collected on an Improvision spinning disk confocal system, consisting of a Yokagawa Nipkow CSU10 scanner, and a Hamamatsu C1900-50 EMCCD camera, mounted on a Zeiss Axio Observer with 40X (1.3NA) oil objectives. Similar settings were used for imaging of all compared genotypes and conditions. Volocity software (Perkin Elmer) was used for intensity measurements and quantification of all confocal data.

A quantification of the sprouting response in injured motoneuron axons was measurement of the change of total volume of regenerating axonal membrane, labeled by mCD8-GFP, within a 100 µm distance from the injured tip. Pixels within the most distal 100 µm of the injured proximal stump were selected based on mCD8-GFP intensity criteria of >3 standard deviation above the mean, and then summed to measure total membrane volume. Figure 1 and 3F report the change in average volume at 15h after injury compared to T=0 (immediately after injury).

Puc-lacZ expression was quantified by measurement of mean intensity for lacZ staining in the nuclei of motoneurons located along the dorsal midline (segments A3-A7) of the nerve cord of third instar larva. The mean intensity of GFP-Wnd^{KD} within segmental nerves was quantified by measuring GFP intensity within 100 µm distance of each nerve at the site of exit from the ventral nerve cord.

3.5.4 Cell culture

HEK293 cells were cultured in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Invitrogen). For transient transfection, 1 µg of given vectors were transfected into 3.5 cm dish using Lipofectmine 2000 (Invitrogen). 20 h after transfection, cells were processed for Western blotting. Plasmids used for transfection were Flag-DLK (Huntwork-Rodriguez et al., 2013), Flag-DLK^{S302A} (Huntwork-Rodriguez et al., 2013), Flag-DLK^{K185A} (directly generated from Flag-DLK) and PKA^{CA} (Merrill et al., 2011).

3.5.5 Primary neuron culture and mouse model

Cortical neurons were dissected from E18 rat embryos. The cortex was digested by incubating with 0.5% trypsin-EDTA (Gibco) and DNAse I (Roche) at 37ºC for 10 min. Following digestion, neurons were washed twice in DMEM medium (Gibco) containing 10% FBS before resuspension in neuronal growth media which containing neurobasal (Gibco), Glucose (Sigma), Glutamax (Gibco), penicillin-streptomycin and B27 supplement (Gibco). All the plates were coated with 100 µg/mL ploy-D-lysine (P7886, Sigma) for 2 hours. Neurons were then triturated and plated at a final concentration 400,000 cells/mL.

For adult DRG experiments, DRG neurons were collected from either CD1(Charles River), or DLK F/F; Adv-Cre-/- (WT), or DLK F/F; Adv-Cre+/- (DLK KO) mice. WT and DLK KO mice were age-sex matched. Neurons were prepared as previously described (Frey et al., 2015). Briefly, DRG were digested for 15 minutes at 37ºC with 0.35 mg/mL liberase Blendzyme (Roche), 10mg/mL bovine serum albumin (sigma), and 0.6 mg/mL DNase (Sigma) followed by another 15 minutes digest at 37ºC with 0.05% trypsin. DRG were tritterated in culture media (DMEM containing 10% FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin) to dissociated cells. Cells were plated on PDL (10 mg/mL) and laminin (10 mg/mL) coated plates. On day in vitro (DIV)1, half of the media was removed and fresh media containing AraC (Sigma,

10 nM final) was added. Drug treatment and replating were performed as described previously (Frey et al., 2015). On DIV4, DMSO or forskolin (30 µM) were added to cells. 24 hours after drug application, drugs were washed out with DMEM. Neurons were lightly trypsinized with 0.025% trypsin for 5 minutes in the incubator (37ºC, 5%CO_2). Trypsin was removed and fresh culture media was added to the cells which were then gently pipetted and transferred to culture slides. 18 hours after replating, neurons were fixed (4% PFA) and stained for βIII-tubulin (Covance, mouse anti-Tuj1, 1:500). At least 100 neurons were imaged per group using either Leica DFC310 FX or DFC7000T color fluorescence cameras and longest neurite was traced using NeuronJ plugin for ImageJ. For replating experiments with PKA inhibitor (H-89, 5 µM), vehicle or inhibitor were added at the same time as DMSO and forskolin.

3.5.6 Immunochemistry, antibodies and chemicals

For detection of Wnd protein levels in larva nerve cords, the whole brains were carefully dissected from third instar larva. The two brain lobes were removed before they were frozen in liquid nitrogen and processed for western blotting (25 nerve cords per lane).

For western blots using HEK293 cells or cortical neurons, cells are lysed by incubating on ice for 10 min with RIPA buffer (Invitrogen) supplemented with Complete protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). Protein concentrations were measured by BCA assay kit (Thermo Scientific).

Equal amount of protein samples were loaded on each lane of NuPAGE 4-12% Bis-Tris gels (Invitrogen) and subject to electrophoresis separation in MOPS buffer (Invitrogen). Blots were visualized with SuperSignal chemiluminescent substrate (Thermo Scientific) and exposure

to either film or ChemiDoc (Bio-Rad). Bands intensities were determined using software ImageJ (NIH) using the gel analysis plug-in.

The following antibodies were used for Western blotting: anti-Wnd A3-1,2 at 1:700 (Collins et al., 2006) ; anti-DLK at 1:5000 (Huntwork-Rodriguez et al., 2013); anti-β-tubulin at 1:1000 (E7; Developmental Studies Hybridoma Bank); anti-Flag at 1:1000 (F1804, Sigma); anti-PKA C (catalytic subunit) antibody at 1:1000 (4782, Cell signaling); anti-phospho-cJun antibody at 1:1000 (3270, Cell signaling); anti-phospho-S6K antibody at 1:1000 (9234, Cell signaling) and anti-S6K antibody at 1:1000 (2708, Cell signaling).

Anti-phospho-DLK^{S302} antibodies were raised by immunization of rabbits with the peptide KELSDKpSTKMpSFAGTV and affinity purified before use at dilution 1:100. While the antibodies were raised against a dually phosphorylated peptide (pS298, pS302), no difference in reactivity was noticed for DLK S298A mutants (data not shown).

Forskolin (F6886, Sigma) was applied to either HEK293 cells or cortical neurons at the final concentration of 30 μM for 6 hours. H-89 (B1427, Sigma) was used at the final concentration of 5 μM for cortical neurons for 6 hours. JNK inhibitor VIII (420135, EMD Millipore) was used at the final concentration of 10 μM on HEK293 cells for 6 hours. Torin1 (4247, Tocris) or Rapamycin (LC laboratories) was applied to the cells at 1 μM (final concentration) for 2 hours before harvest.

3.5.7 Protein phosphatase assay

24 hours after transfection of the given constructs, HEK293 cells in a 6 cm dish were washed by ice-cold PBS and harvested in ice-cold RIPA buffer supplemented with EDTA-free protease inhibitor (Roche). The cell lysates were incubated on ice for 30 minutes and

centrifuged at 14,000 rpm at 4ºC for 10 minutes. The soluble lysate was incubated with 3 µg anti-Flag antibody pre-bound with 15 µl Dynabeads Protein G (Novex) for 1 hour at 4ºC. After removal of supernatants the beads were washed 3 times with RIPA buffer, and then incubated with 1X PMP buffer (NEB), 1X $MnCl_2$ (NEB) and either 1000 U lambda protein phosphatase (NEB) or equal amount of glycerol (control) in RIPA buffer for 30 minutes at 30ºC. Beads were then removed from the reaction buffer and FLAG-DLK was eluted by boiling in SDS sample buffer for 10 minutes. Equal amount of samples were analyzed by western blot.

3.5.8 *In vitro* PKA kinase assay

HEK293 cells transfected with Flag-DLK were washed by ice-cold PBS and harvested using ice-cold cell lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, Complete protease inhibitors (Roche) and 30 µM MG132 (Sigma)). Flag-tagged DLK was immunoprecipitated from HEK293 cell lysates using anti-Flag M2 antibody (Sigma) and Protein G Dynabeads (Novex). The DLK-bound beads were then washed with 3X lysis buffer and incubated with 2,000 U lambda protein phosphatase (NEB) for 30 min at 30ºC to remove all the phosphate groups. After incubation, the beads were washed with wash buffer containing cocktail phosphatase inhibitor (Roche) and split equally into two tubes containing kinase reaction buffer (50 mM Hepes, pH7.2, 10 mM $MgCl_2$, 0.01% Triton X-100, 2 mM DTT, and 30 µM ATP). 10,000 U recombinant human full length PKA catalytic subunit alpha (NED Millipore) was added to one of the tubes, while the control tube were added with glycerol. Both tubes were incubated at 30ºC for 90 min. Flag-DLK was eluted from beads by boiling in the SDS sample buffer. Equal amounts of samples were analyzed by western blotting with anti-DLK and anti-pDLK^{S302} antibodies.

3.5.9 Statistical analysis

For experiments in flies, we knew from previous work that a sample size of 10 animals per genotype was large enough to detect significant differences among genotypes (Xiong et al., 2010, Xiong et al., 2012). Therefore, at least 10 animals (≥ 50 axons) were examined and quantified in each genotype. Each experiment was repeated at least 3 times with independent biological replicates. For experiments in mice, we knew from previous work that measuring 100 neurons per genotype and an N=3-4 was sufficient to detect reproducible differences between the experimental groups (Frey et al., 2015, Valakh et al., 2015). Therefore, all the experiments were performed with at least 3 independent biological replicates.

One way ANOVA and multiple comparisons were conducted when more than two samples are compared. Tukey post-hoc test was used to correct for multiple comparisons. For binned DRG neurites length distribution, statistical significance was determined by two way ANOVA followed by Bonferroni post-hoc test. p values smaller than 0.05 were considered statistically significant. All p values are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ and **** $p<0.0001$. Data are presented as mean ± SEM.

3.6 Figures

Figure 3.1

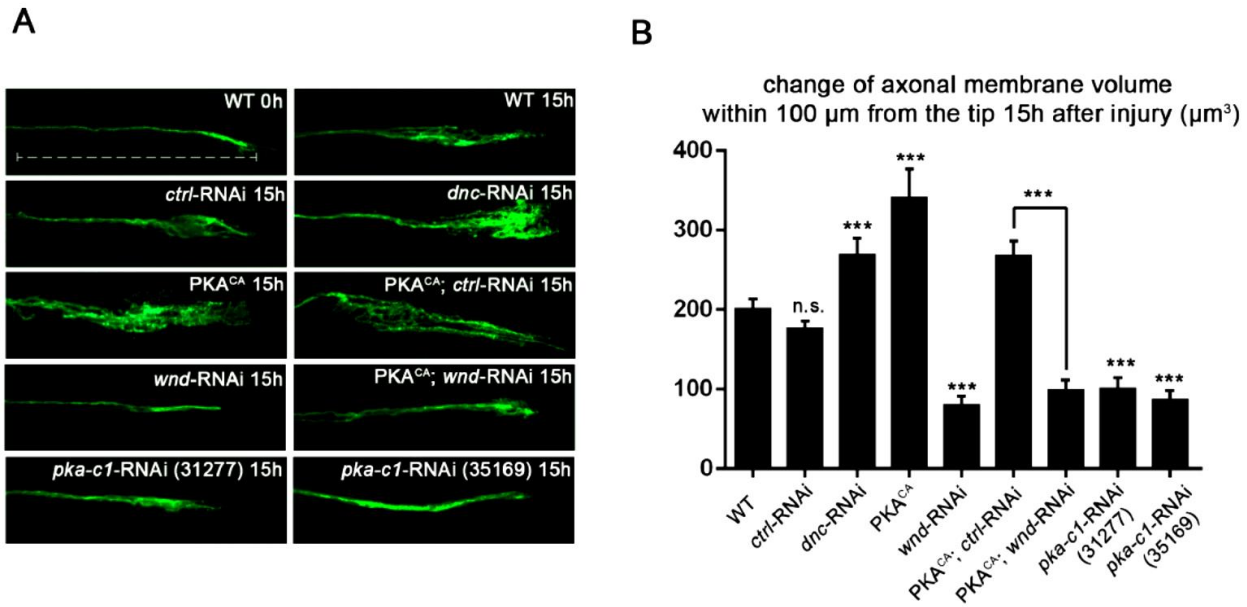

Figure 3.1 PKA stimulates and is required for axonal regeneration in Drosophila motoneurons.

Single motoneuron axons were labeled by expression of UAS-mCD8-GFP using the m12-Gal4 driver and imaged either 0 hour or 15 hours after nerve crush injury. Representative images are shown in (A), while (B) shows quantification of the increased volume in axonal membrane, which is measured within 100 µm of the proximal axon tip, indicated in dotted line. Genotypes used in (A): WT(;;m12-Gal4, UAS-mCD8GFP/+); *control*-RNAi (UAS-*dcr2*;; UAS-*moody*-RNAi/m12-Gal4, UAS-mCD8GFP); *dnc*-RNAi (UAS-*dcr2*;; UAS-*dnc*-RNAi/m12-Gal4,UAS-mCD8GFP); *wnd*-RNAi (UAS-*dcr2*;; UAS-*wnd*-RNAi/m12-Gal4,UAS-mCD8GFP); PKA^{CA}(; UAS-PKA^{CA}/+; UAS-mCD8GFP/+); PKA^{CA},*control*-RNAi (UAS-*dcr2*; UAS-PKA^{CA}/+; UAS-*moody*-RNAi (VDRC 100674)/m12-Gal4, UAS-mCD8GFP); PKA^{CA},*wnd*-RNAi (UAS-*dcr2*; UAS-PKA^{CA}/+; UAS-*wnd*-RNAi/m12-Gal4, UAS-mCD8GFP); *pka-c1*-RNAi (UAS-*dcr2*; UAS-*pka-c1*-RNAi/+; m12-Gal4, UAS-mCD8GFP (using two different lines, Bloomington 31277 and 35169)). All data are represented as mean ± SEM; At least 10 animals (≥50 axons) are examined per genotype; *** $P<0.001$; 'n.s.' indicates non-significant; scale bar, 100 µm.

Figure 3.2

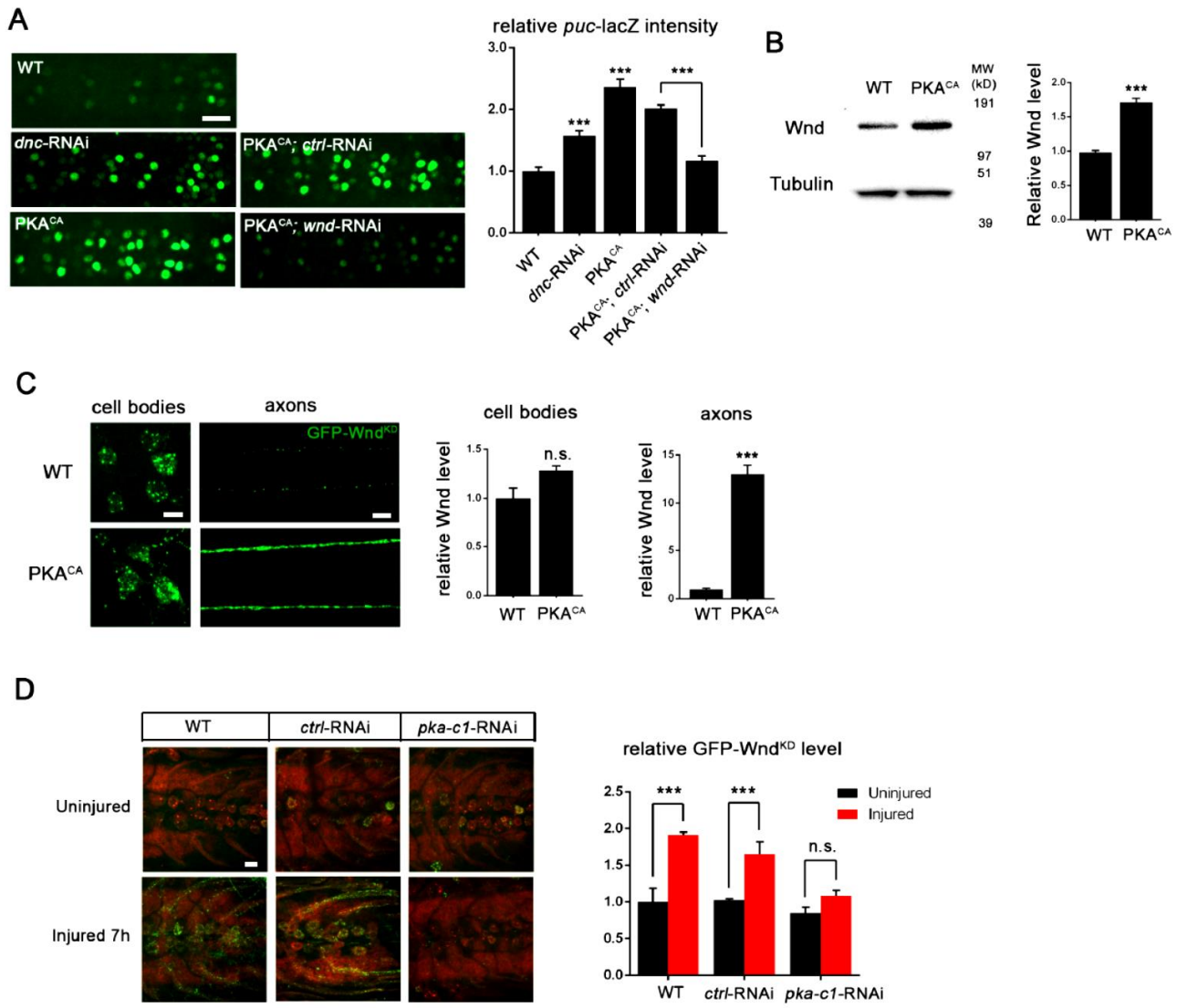

Figure 3.2 PKA modulates the levels of Wnd protein and downstream signaling in Drosophila motoneurons.

(A) The *puc*-lacZ transcriptional reporter for Wnd/JNK signaling indicates that activated PKA stimulates Wnd signaling. A pan-neuronal driver (BG380-Gal4) is used to express UAS-*dnc*-RNAi, UAS-PKA^{CA} or UAS-PKA^{CA} together with UAS-*wnd*-RNAi or a *control*-RNAi. Example images are shown of cell bodies in the dorsal midline of the ventral nerve cord; (all but two of these neurons are motoneurons). Quantification (described in methods) was carried out for 10 animals per genotype.

(B) Endogenous Wnd protein levels are increased in PKA^{CA} expressing neurons. Ventral nerve cords were dissected from third instar larvae (BG380-Gal4 [WT control] and BG380-Gal4; UAS-PKA^{CA}/+) and processed for Western blotting with anti-Wnd and anti-tubulin antibodies. The quantification shows Wnd/tubulin ratios (normalized to WT control) averaged from 3 independent experiments (25 nerve cords per experiment).

(C) PKA increases DLK levels via a posttranscriptional mechanism. GFP-tagged kinase dead Wnd (GFP-Wnd^{KD}) was ectopically expressed using m12-Gal4 driver (WT control) or co-expressed with UAS-PKA^{CA} and imaged directly after fixation. Example images and quantification of GFP-Wnd^{KD} intensity in cell bodies and axons within segmental nerves. n>10 animals for each condition.

(D) PKA-C1 is required for induction of Wnd protein after axonal injury. Example images and quantification of GFP-Wnd^{KD} in nerve cords and segmental nerves before and after (8 hours) injury. UAS-GFP-Wnd^{KD} was expressed in motoneurons by OK6-Gal4. WT or together with UAS-*pka-c1*-RNAi or UAS-*moody*-RNAi (control) and imaged similarly to Figure 2C. The quantification method for GFP intensity is described in materials and methods. n>10 animals for each condition.

All data are represented as mean ± SEM; *** P<0.001, ** P<0.01, *P<0.05, 'n.s.' indicates non-significant; scale bars, 10 μm.

Figure 3.3

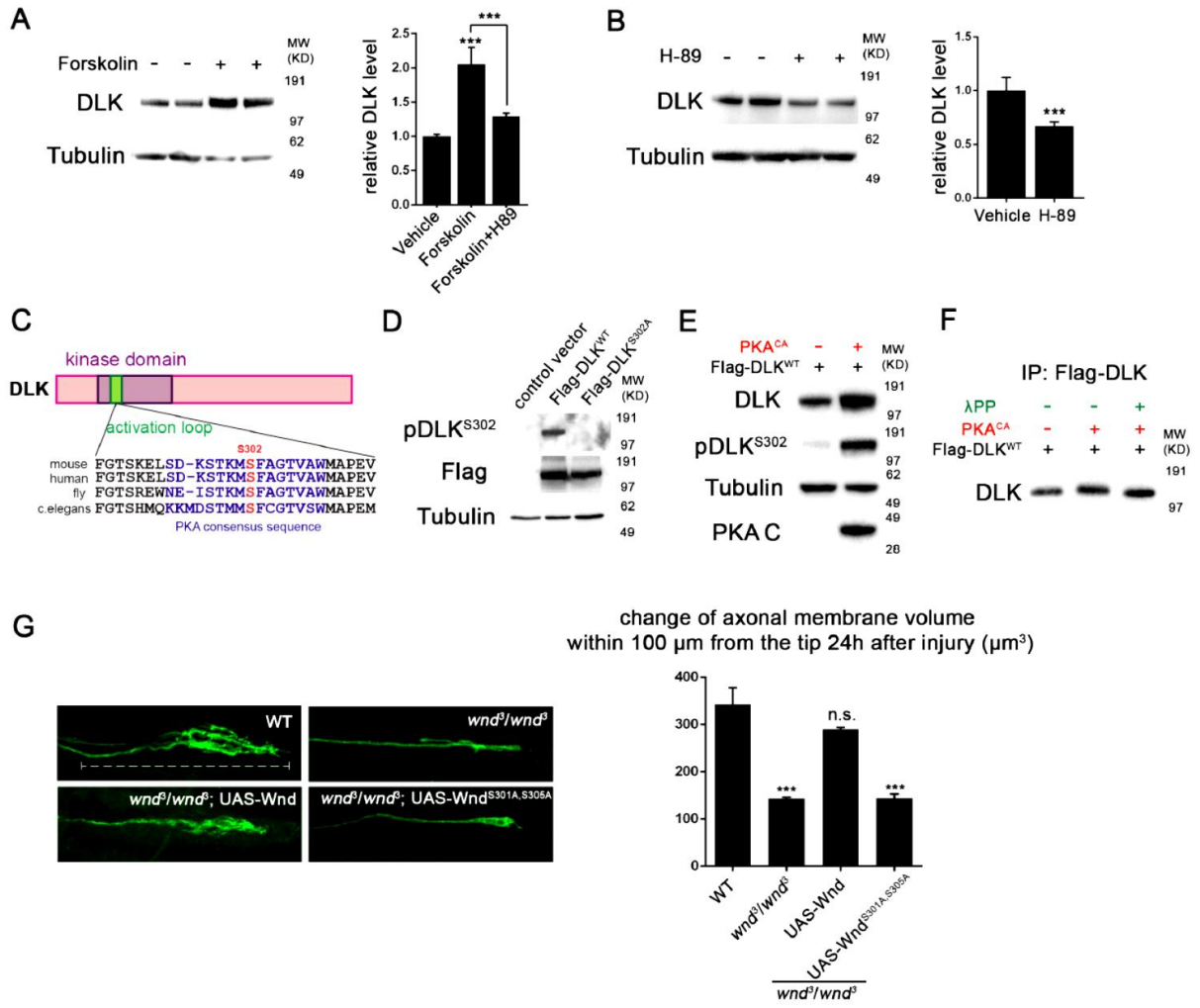

Figure 3.3 PKA activates DLK via phosphorylation of its activation loop.

(A-B) Changes in endogenous DLK abundance in response to treatment with forskolin (30 μM) (A) or the PKA inhibitor H-89 (5 μM) (B) for 6 hours in cultured rat embryonic cortical neurons. Quantification shows relative DLK/Tubulin levels in western blots.

(C) Alignment of activation loop sequences in different species.

(D) The anti-pDLK^{S302} antibodies recognize transfected Flag-DLK^{WT}, but not activation loop mutation Flag-DLK^{S301A,S305A}. Both proteins were transiently expressed in HEK293 cells. Western blots were probed with anti-pDLK^{S302} antibody, anti-Flag antibody to detect the total DLK expression levels, and anti-Tubulin (which remains similar in all manipulations) for normalization.

(E) PKA^{CA} stimulates phosphorylation of DLK S302 in HEK293 cells. HEK293 cells were co-transfected with Flag-tagged DLK^{WT} and an empty control plasmid or PKA^{CA}. Cell lysates were

probed with anti-DLK antibody, anti-pDLK^{S302} antibody, anti-PKA C antibody and anti-tubulin antibody.

(F) PKA^{CA} stimulates an increase in DLK molecular weight. FLAG-DLK protein was immunoprecipitated from HEK293 cells co-transfected with DLK and either Flag-tagged DLK^{WT} and an empty control plasmid or PKA^{CA}. The immunoprecipitated FLAG-DLK was then incubated with either glycerol (control) or lambda protein phosphatase (λPP). PKA^{CA} induced a upward shift in DLK molecular weight, which was lost upon phosphatase treatment.

(G) The activation loop is required for axonal regeneration in *Drosophila* neurons. Single axons in *Drosophila* third instar larva are labeled by mCD8RFP using eve-Gal4 driver. 24 hours after injury, these neurons in animals heterozygyous for *wnd* (*wnd*^{3}/+) show robust axonal sprouting. However, sprouting fails to occur in *wnd*^{3}/*wnd*^{3} animals. Expression of UAS-Wnd (WT) can restore axonal regeneration in *wnd* mutant background (UAS-Wnd, *wnd*^{3}; *wnd*^{3},eve-Gal4, UAS-mCD8RFP). However, expression of activation loop mutant UAS-Wnd^{S301A,S305A} failed to rescue the sprouting defect in *wnd* mutant animals (UAS-Wnd^{S301A,S305A}, *wnd*^{3}; *wnd*^{3}, eve-Gal4,m12-mCD8RFP). Quantification of the volume of axonal membrane within 100 μm of the distal ending of the proximal stump. n> 50 axons for each genotype. Data are presented as mean ± SEM for 3 independent experiments; *** P<0.001; scale bar, 100 μm.

Figure 3.4

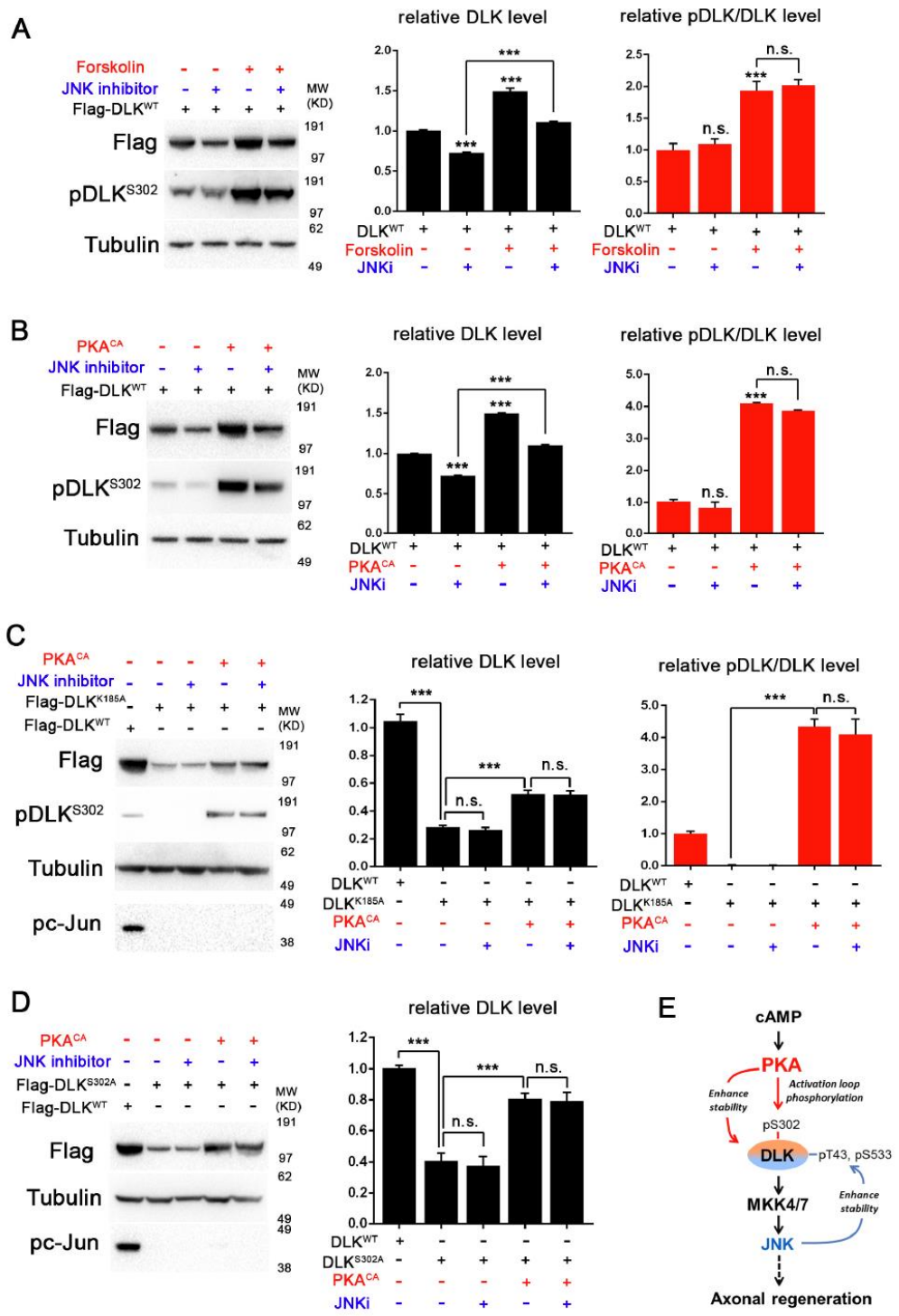

Figure 3.4 PKA promotes the stability of DLK independently of DLK downstream signaling.

(A-B) Activation of PKA promotes DLK stability independently of JNK. HEK293 cells were transiently transfected with Flag-DLK^{WT}, and (A) treated with forskolin (6 hours, 30 μM) or (B) co-transfected with either PKA^{CA} or empty vector (control). In both cases, co-treatment with JNK inhibitor VIII (10 μM, 6 hours) led to a decrease in total Flag-DLK levels. However, both forskolin and PKA^{CA} induced an increase in DLK levels even in the presence of JNK inhibitor. Quantification shows average total DLK/Tubulin intensities and average $pDLK^{S302}$/total DLK ratios (where total DLK is detected using anti-Flag antibody). All data are represented as mean ± SEM; quantifications of relative intensity from Western Blots were averaged from 3 independent experiments; *** $P<0.001$, ** $P<0.01$, *$P<0.05$, 'n.s.' indicates non-significant.

(C-D) Activation of PKA increases the stability of kinase dead DLK mutants, DLK^{K185A} (C) and DLK^{S302A} (D). HEK293 cells were transiently transfected with Flag-DLK^{K185A} or Flag-DLK^{S302A} together with PKA^{CA} or empty plasmid. Treatment JNK inhibitor VIII (10 μM) for 6 hours had no effect upon the PKA^{CA} induced levels of DLK^{K185A} and DLK^{S302A} mutant protein. Quantifications are similar to Figure 4A-B. Western bands intensity were averaged from 4 independent experiments; data are shown as mean±SEM; *** $P<0.001$, ** $P<0.01$, *$P<0.05$, 'n.s.' indicates non-significant.

(E) Proposed model for the activation and stabilization of DLK by cAMP and PKA. cAMP elevation and PKA activation leads to the phosphorylation of S302 on DLK, which activates its kinase activity. Indicated in the blue arrow, downstream signaling via JNK leads to enhanced DLK stability and phosphorylation of DLK at other sites (Huntwork-Rodriguez et al., 2013). PKA also enhances DLK's stability via an additional mechanism that is independent of S302 (red arrow).

Figure 3.5

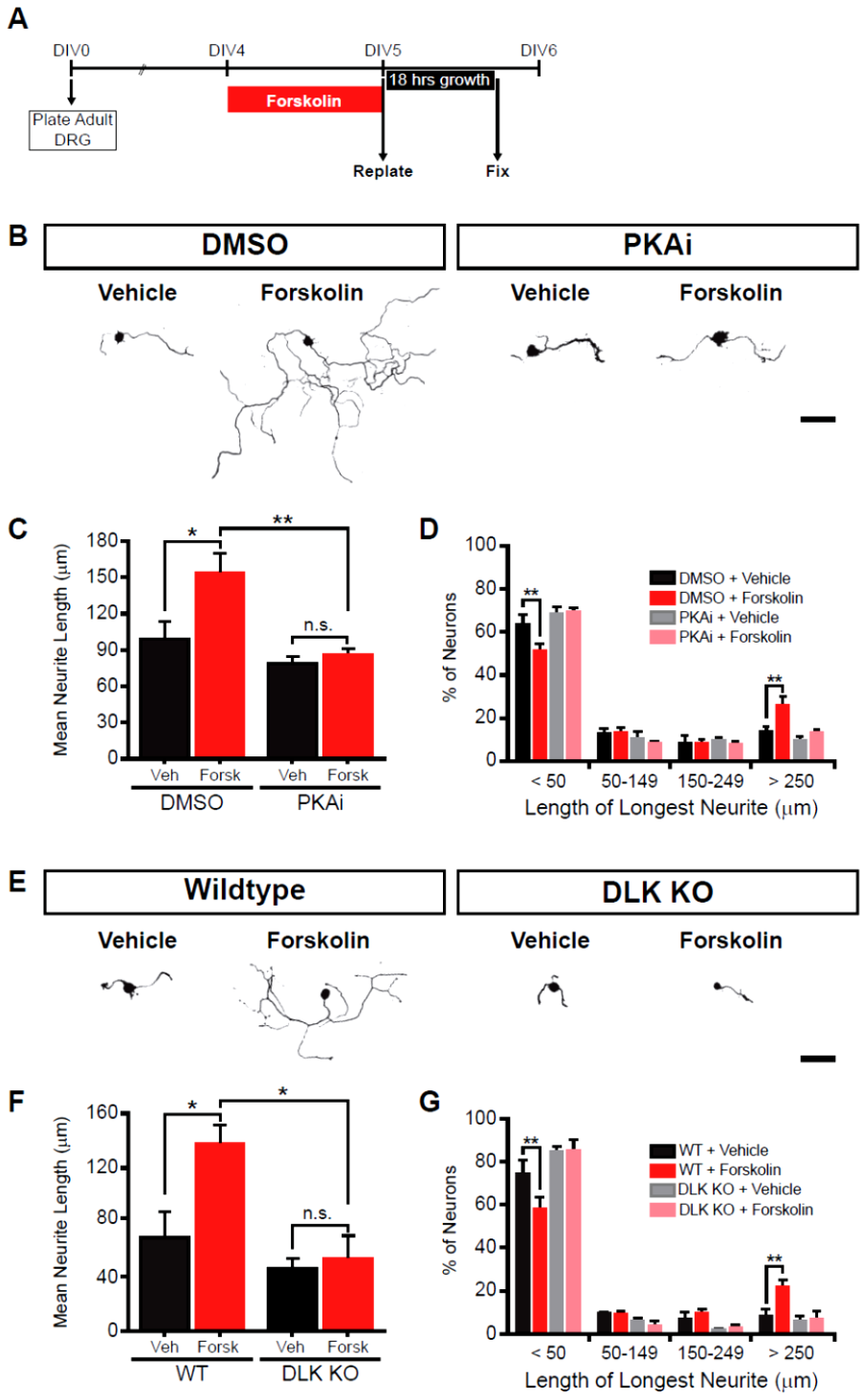

Figure 3.5 PKA stimulates axonal regeneration via DLK in adult DRG neurons.

(A-D) Induction of regeneration by forskolin requires PKA. Experimental design (A and also see Materials and Methods). To demonstrate that forskolin-induced neurite outgrowth is mediated by PKA, we assessed whether PKA signaling was required using the PKA inhibitor H-89 (PKAi, 5 μM). Representative neurons are shown in (B). Neurite outgrowth was assessed by quantifying mean neurite length (C) and distribution of longest neurite (D). Data are mean ± SEM for 4 independent experiments.

(E-G) Induction of regeneration by forskolin requires DLK. WT and DLK KO neurons were treated with DMSO or forskolin (30 μM) as described in (A). Representative neurons are shown in (E). Neurite outgrowth was assessed by quantifying mean neurite length (F) and distribution of the longest neurite (G). Data are mean ± SEM for 3 independent experiments.

*** $P<0.001$, ** $P<0.01$, *$P<0.05$, 'n.s.' indicates non-significant; scale bars, 100 μm.

Figure 3.6

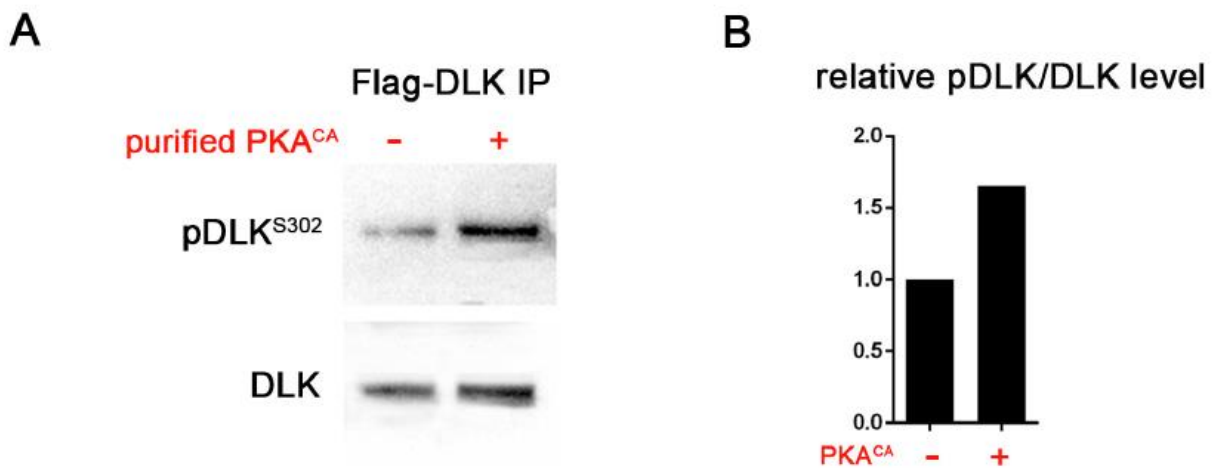

Figure 3.6 PKA can directly phosphorylate DLK at S302.

(A) PKA can induce DLK S302 phosphorylation *in vitro*. Flag-DLK was purified from HEK293 cells by anti-Flag immunoprecipitation and used for an *in vitro* kinase activity with purified PKA catalytic subunit. 5 µg of purified DLK was incubated with or without 10,000 U PKA catalytic subunit. Equal amounts of DLK in both samples (as demonstrated by probing with anti-DLK antibody) were analyzed by western blotting with anti-pDLK^{S302} antibody. (B) Quantification shows relative pDLK^{S302}/total DLK levels.

Figure 3.7

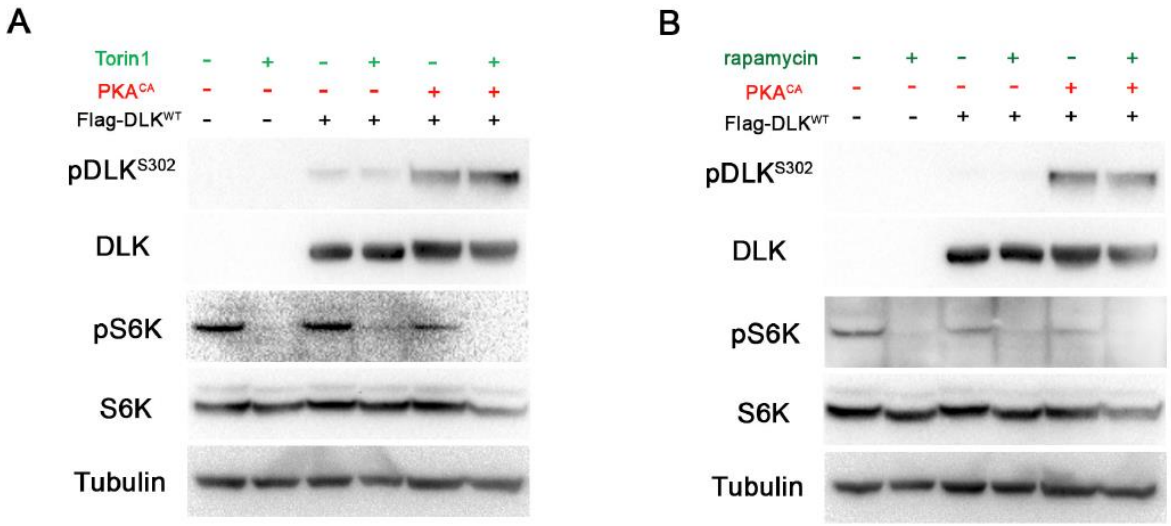

Figure 3.7 DLK activation by PKA does not require TORC1.

(A-B) The phosphorylation level of DLK S302 is not sensitive to treatments of TORC1 inhibitors. HEK293 cells were either untransfected or transfected with Flag-DLK^{WT} + empty plasmid or Flag-DLK^{WT} + PKA^{CA}. Cells were treated with torin1 (A) or rapamycin (B) for 2 hours. The efficiency of the drugs were demonstrated by probing with anti-phospho-S6K antibody.

Figure 3.8

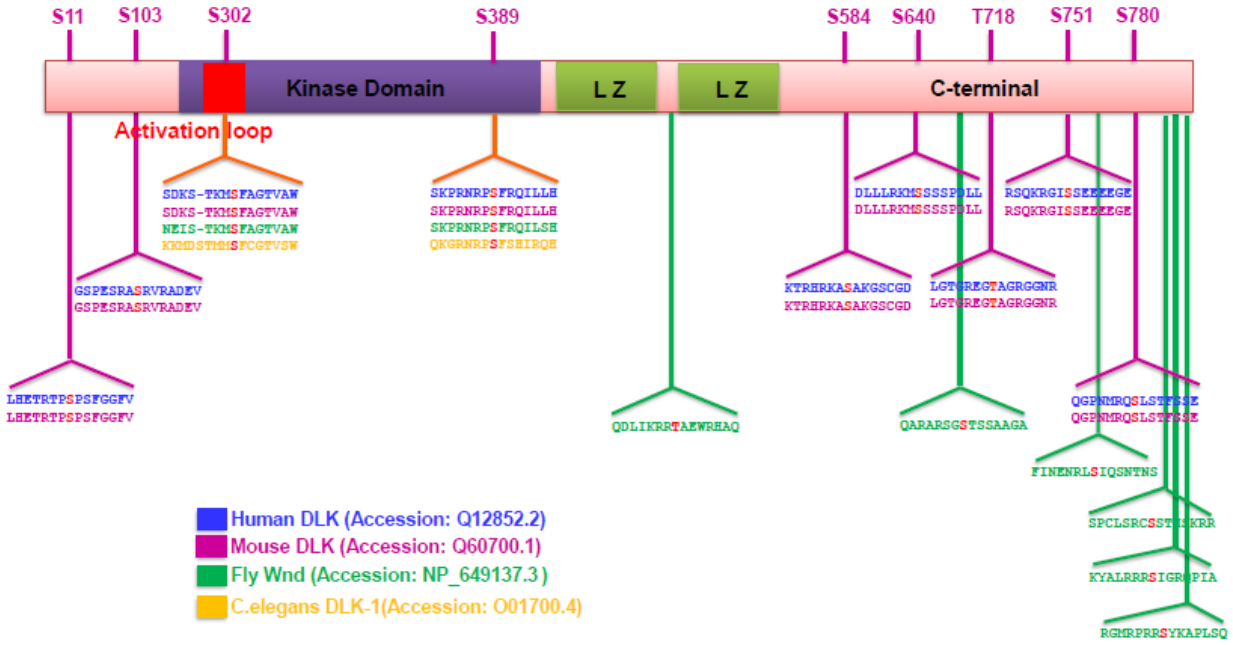

Figure 3.8 Summary of predicted PKA phosphorylation sites on DLK/Wnd in different species.

The sequence of DLK homologues in different species (human, mouse, *Drosophila* and *C. elegans*) were analyzed by Group-based Prediction System (GPS) to computationally predict PKA phosphorylation sites (Xue et al., 2008, Xue et al., 2005). Identified potential PKA^{CA} phosphorylation motifs in different species are shown, with the phosphorylation site highlighted in red. Numbering is shown for mouse DLK. Two sites, S302 and S389, are conserved among all the species. Other sites are conserved in mammals, while fly DLK has a similar number of sites but in distinct locations. Not shown, *C. elegans* DLK also has 11 additional predicted sites, but at distinct locations from mammalian and fly DLK.

Chapter 4 :

Raw is required for axonal degeneration after injury

4.1 Abstract

Axonal degeneration after injury is self-destruction process that results in widespread axon breakdown and functional loss. Despite great interest, this process is still poorly understood. In this study, we have identified the *Drosophila* transmembrane protein Raw as an important mediator of axonal and synapses degeneration. In raw mutants, axons and synapses of both motoneurons and sensory neurons remain intact and fail to degenerate after peripheral nerve injury. We show that Raw functions cell autonomously to regulate susceptibility to degeneration by antagonizing the function of the AP-1 transcription factor. Although the NAD+ synthetic enzyme nicotinamide mononucleotide adenylyl transferase (Nmnat) has previously been implicated in axonal degeneration, knockdown of *nmnat* in motoneurons in raw mutant background only very modestly affects the protective effect. Thus, our study suggests a novel mechanism through which axonal degeneration is regulated.

4.2 Introduction

Axonal degeneration is morphologically characterized by catastrophic fragmentation of the entire axonal cytoskeleton and membrane. Acute axonal transection (also termed ‘axotomy’) represents a severe axonal damage and results in the degeneration of the axon stump distal to the injury site, called Wallerian degeneration. Wallerian degeneration is not only morphologically

similar to axon degeneration in many neurodegenerative diseases and neuropathies, but also shares similar molecular mechanisms (reviewed in (Wang et al., 2012a, Coleman and Freeman, 2010)). Wallerian degeneration occurs via a cell-autonomous 'self-destruction' pathway, whose molecular components and their associated cellular functions are still in the process of being identified. Hence the cellular mechanism by which injured axons and synapses degenerate is still poorly understood.

Previous studies have implicated a role for the E3 ubiquitin ligase Highwire (Hiw) in Wallerian degeneration: axonal degeneration is significantly delayed in hiw mutant animals (Xiong et al., 2012, Neukomm et al., 2014, Babetto et al., 2013). Hiw modulates axon degeneration via down-regulating the NAD+ synthetic enzyme nicotinamide mononucleotide adenylyl transferase (Nmnat). Nmnat has been demonstrated to play a central role in axon degeneration. Studies in multiple organisms suggest that Nmnat strongly protects axons from degeneration after injury (Sasaki et al., 2009, Gilley and Coleman, 2010, Fang et al., 2012, Milde et al., 2013, Sasaki et al., 2016). Nmnat is considered to be a 'survival factor' in axons, since knockdown of *nmnat* leads to spontaneous axon degeneration (Gilley and Coleman, 2010). How does Nmnat protect axon from degeneration? A recent study suggest that it can inhibit the function of Sarm1 (Sasaki et al., 2016), which was identified as a crucial mediator of axonal degeneration (Osterloh et al., 2012). However, it is not clear that whether this is the only pathway that regulates axonal degeneration.

Studies using the model organism *Drosophila* have identified several new genes important for axon degeneration, illustrating the power of genetics and significantly influencing our understanding of this degeneration process. To study axonal injury signaling, we have previously established a *Drosophila* larval nerve crush assay, in which injured axons undergo a

highly stereotyped degeneration process (Xiong and Collins, 2012). Using this model, we have identified a mutation on the second chromosome that strongly inhibits axon degeneration in vivo, and have mapped this mutation via whole genome sequencing to the *Drosophila* gene *raw*. Raw is previously known as a negative regulator of JNK signaling via the AP-1 transcription factor during embryonic dorsal closure and gonad development (Jack and Myette, 1997, Byars et al., 1999, Bates et al., 2008, Jemc et al., 2012). Here we find AP-1 is an important mediator of the regulation of axonal degeneration by Raw. Therefore, Raw not only functions during development, but also appears to be a crucial component of the axonal degeneration machinery.

4.3 Results

4.3.1 Identification of an unexpected mutation that strongly protects injured axons from degeneration in *Drosophila*

Caspases, which are important mediators programmed cell death, are suggested to be involved in axonal pruning during development (Simon et al., 2012). Previous studies suggest that Wallerian degeneration involves mechanisms that are molecularly distinct from apoptosis (Finn et al., 2000), so we were surprised to notice that in null mutant animals for the dcp-1 caspase, ($dcp\text{-}1^{Prev1}$), axonal degeneration was strongly inhibited. To assess the degeneration of motoneuron axons, we used the m12-Gal4 driver to label only two neurons per hemi-segment with membrane-bound mCD8-GFP. Within 20 hours after nerve crush injury, axons in wild type (WT) animals were completely fragmented (Figure 4.1A and B). In contrast, injured motoneuron axons in dcp-1Prev1 animals remained completely intact even 2 days after injury (Figure 1A and B). Degeneration at the synaptic neuromuscular junction (NMJ) terminal was also inhibited in the $dcp\text{-}1^{Prev1}$mutant (Figure 4.1B). In WT animals, the axonal membrane, labeled by anti-HRP antibodies, becomes fragmented within 20 hours after injury, and the

microtubule binding protein MAP1B homologue Futsch, disappears completely from the NMJ boutons (Figure 4.1B). In contrast, in *dcp-1*^{Prev1} line, both markers remained intact 20 hours after injury (Figure 4.1B). We also observed that class IV Da sensory neuron axons, which were also injured in the segmental nerve crush assay and could be labeled ppk-Gal4 driven membrane-bound GFP, failed to degenerate in *dcp-1*^{Prev1} mutants (Figure 4.1C).

Importantly, further genetic analysis of the *dcp-1*^{Prev1} line led to the finding that the degeneration phenotype was caused by a new mutation in the dcp-1Prev1 strain background. First we noticed that other alleles of *dcp-1* (Etchegaray et al. 2012) had no effect on larval NMJ degeneration (Figure 4.6A and B). Then we noticed that the *dcp-1*^{Prev1} mutation was not linked to the degeneration phenotype: out of 13 recombinant lines that contained the *dcp-1*^{Prev1} mutation, none were able to elicit the degeneration phenotype of the founder strain (Figure 4.6A and B).

4.3.2 Axonal degeneration in *dcp-1*^{Prev1} is caused by a mutation in *raw*

The *dcp-1*^{Prev1} line was originally generated by an imprecise P-element excision of a P-element insertion (k05606), which is inserted within the first exon in the *dcp-1* gene (Laundrie et al., 2003). The parental line k05606, which is also a *dcp-1* null mutant, does not have an axon degeneration phenotype (Figure 4.6A and B). We therefore inferred that the new mutation could be identified as a variant between k05606 line and *dcp-1*^{Prev1} line. We used next-generation sequencing technology to sequence the whole genome of *dcp-1*^{Prev1}, the parental line k05606, and compared variants with three *dcp-1*^{Prev1} recombinant lines which did not have the degeneration phenotype. Candidate mutations that inhibit degeneration should only be present in the sequence of *dcp-1*^{Prev1}, but not in the parental or recombinant lines. Based on these criteria, we identified 332 variants. After annotation of these variants to genes, we focused on non-synonymous variants and identified 6 candidate genes (Table 4.1). We obtained small

chromosome deficiencies for each and tested by complementation whether the deficiency could unveil the degeneration phenotype as a transheterozygote with *dcp-1*^{Prev1} (Table 4.1). This was the case for one of the deficiencies Df(2L)BSC204 (from 29D5 to 29F8) which uncovers mutations in the candidate gene *raw* (29E4-29E6) (Figure 2A and B).

To test the role of Raw in axon degeneration, we tested two previously identified raw mutants (*raw*$^{134.47}$ and *raw*$^{155.27}$) (Jemc et al., 2012). As both of these EMS-generated mutations are homozygous lethal, we examined the trans-heterozygous *raw*$^{134.47}$/ *dcp-1*^{Prev1} and *raw*$^{155.27}$/ *dcp-1*^{Prev1} and found that NMJ degeneration is strongly inhibited (Figure 4.2A and B). Together with rescue data described below, we infer that the mutation of interest in the *dcp-1*^{Prev1} mutant background is a mutation in raw, for further reference we named this mutation *raw*$^{dcp-1}$.

4.3.3 Raw promotes axonal degeneration in *Drosophila* neurons

Based on the sequencing data, we noted that the *raw*$^{dcp-1}$ allele contains a missense mutation in raw open reading frame. Interestingly, this point mutation A532D is within the predicted EF hand of Raw (Figure 4.2C). Raw is known to play multiple important roles during development. It regulates embryo dorsal closure and gonad morphogenesis (Jemc et al., 2012, Byars et al., 1999, Jack and Myette, 1997). Therefore, most of the raw mutants are embryonic lethal. Since *raw*$^{dcp-1}$ mutants are viable and fertile, and the degeneration phenotype is recessive, we suspect that the *raw*$^{dcp-1}$ mutations confer a partial loss in raw function. To test this and to determine of the cellular locus of Raw's function in degeneration, we carried out both RNAi knockdown and rescue experiments. Using the Gal4/UAS system, we drove expression of UAS-Raw-RA and Raw-RB (two different isoform of Raw). Expression of either isoform via the single motoneuron m12-Gal4 driver was sufficient to fully revert axon degeneration phenotype of *raw*$^{dcp-1}$ mutants (Figure 4.2D and E). Moreover, expression of Raw using a motoneuron

driver D42-Gal4 can revert the NMJ degeneration phenotype of *raw*$^{dcp-1}$ mutants (Figure 4.2A and B). In contrast, expression of Raw in glia using repo-Gal4 failed to rescue the phenotype (Figure 4.2A and B), arguing that Raw is play a cell-autonomous role in neurons to promote axon degeneration. In further support of this, knockdown of Raw by UAS-Raw-RNAi lines using m12-Gal4 also strongly inhibits axon degeneration (Figure 4.2D and E). Together, these data indicate that Raw is required in larval neurons to drive axon degeneration after injury.

4.3.4 Knockdown of *nmnat* only modestly affects the axonal protective effect of Raw

The strong raw loss-of-function phenotype strikingly resembles the loss-of-function phenotype of Hiw in their ability to delay axonal degeneration (Xiong et al., 2012). Previous studies suggest that Hiw promotes axonal degeneration by promoting the turnover of Nmnat, a key protective factor in axons (Xiong et al., 2012, Babetto et al., 2013). We therefore wondered whether Raw regulates axon degeneration by promoting an increase in Nmnat levels and/or function. To test this, we used UAS-*nmnat*-RNAi to knockdown *nmnat* in motoneurons. This knockdown is effective as demonstrated by the immunostaining with anti-Nmnat antibodies in our previous studies (Xiong et al., 2012). *nmnat*-RNAi mediated knockdown of *nmnat* using m12-Gal4 driver did not affect development, but leads to a modest enhancement to the rate of axonal degeneration after injury (Figure 4.3B). As it is shown in Figure 3B and our previous study (Xiong et al., 2012), knockdown of *nmnat* in motoneurons significantly rescued the axon degeneration phenotype in *hiw* mutants. In contrast, knockdown of *nmnat* using the same driver m12-Gal4 only very modestly affects the suppression of axon degeneration in *raw*$^{dcp-1}$ mutant (Figure 4.3A and B). Consistent with this, while knockdown of *nmnat* in motoneurons suppressed the NMJ protective phenotype in *hiw* mutant (Figure 4.3F and (Xiong et al., 2012)), it has little effect on the NMJ degeneration phenotype in *raw* mutant (Figure 4.3F).

As a survival factor in axons, the axonal protective effect of Nmnat is associated with its level in axons (Xiong et al., 2012, Gilley and Coleman, 2010, Fang et al., 2012). Endogenous Nmnat level is not detectable in neurite-rich neuropil in WT animals, but it increased dramatically *hiw* mutant animals (Figure 4.3C and D). However, we cannot detect any increase of Nmnat level in the neuropil of $raw^{dcp\text{-}1}$ animals (Figure 4.3C and D). Surprisingly, we observed dramatic increase of Nmnat level in glia wrapping segmental nerves (Figure 4.3E), which made us wonder whether Nmnat in glia affected axon degeneration. To test this, we use the glia specific driver repo-Gal4 to knockdown Nmnat in the glia. However, we found that even when Nmnat level is significantly reduced in glia, NMJ degeneration in $raw^{dcp\text{-}1}$ animals is still strongly protected from degeneration (Figure 4.3F and G). Thus, the elevated Nmnat level in glia in $raw^{dcp\text{-}1}$ mutant is not crucial for the delay of axon degeneration phenotype. Together, we conclude that Raw might mediate axonal degeneration independently of Nmnat.

4.3.5 Raw regulates axonal degeneration via AP-1

Previous studies suggest that Raw regulates dorsal closure and gonad morphogenesis through down-regulation of the c-Jun N-terminal kinase (JNK) signaling cascade (Jemc et al., 2012). Consistent with these studies, we found that the Puckered reporter *puc*-lacZ, which contains lacZ enhancer trap for the JNK downstream phosphatase Puckered, is strongly activated in neurons when UAS-raw-RNAi was expressed using a pan-neuronal driver BG380-Gal4 (Figure 4.4A), suggesting that Raw is a negative regulator of JNK signaling in neurons.

Importantly, JNK signaling has also been intensively studied as an axonal injury signaling pathway (Yang et al., 2015, Shin et al., 2012b, Miller et al., 2009, Xiong and Collins, 2012). In fact, our previous study suggested that the *puc*-lacZ can be used as a reporter for injury signaling. In WT animals, *puc*-lacZ expression level increased by 3-fold 24 hours after injury

(Figure A and (Xiong et al., 2010)). Interestingly, comparing to uninjured *raw*$^{dcp-1}$ animals, which have a higher basal *puc*-lacZ expression level, injury also induced a 3-fold increase of *puc*-lacZ expression (Figure 4.4 A and B). Therefore, it seems that the increased basal JNK signaling in *raw*$^{dcp-1}$ does not affect the axonal injury response reported by *puc*-lacZ. We then wondered whether the elevated JNK signaling contributed to the delayed axon degeneration in *raw*$^{dcp-1}$ animals. To directly test this, we expressed a dominant negative form of JNK (JNK^{DN}) using the UAS/Gal4 system in motoneurons in *raw* mutants. As reported previously, JNK^{DN} also causes an inhibition to degeneration, however this phenotype was not as strong as the *raw* mutant phenotype. Strikingly, even while JNK^{DN} inhibits degeneration on its own, it caused a modest increase in degeneration in the raw mutants and the appearance of varicosities within axons which typically occurs as a precursor to complete degeneration (Figure 4.4C and D). This suggests that JNK might play dual roles in axonal degeneration: it promotes axonal degeneration in WT animals, whereas it also plays a protective role in Raw mediated axonal degeneration. Indeed, previous studies suggest that JNK is required both for axonal degeneration and maintenance of axonal integrity (reviewed in (Coffey, 2014)).

Raw is also suggested as a negative regulator of transcription factor Fos/Jun (activator protein 1, AP-1) (Bates et al., 2008). Intriguingly, we and others have previously demonstrated that Fos is involved in axonal injury signaling and required for the axonal protection effects by a conditioning lesion (Xiong et al., 2010, Xiong and Collins, 2012). We therefore tested whether Fos and Jun are required for the protection by Raw. Neither expression of a dominant negative form of Fos (Fos^{DN}) or Jun (Jun^{DN}) in motoneurons in a WT background affect axonal degeneration (Figure 4D). However, expression of either Fos^{DN} or Jun^{DN} in the *raw*$^{dcp-1}$ background significantly rescued the degeneration phenotype (Figure 4.4C and D). We noticed

that neither Fos^{DN} or Jun^{DN} can fully revert the protection in raw^{dcp-1} animals. We think this is likely due to the inefficiency of expression Fos^{DN} or Jun^{DN} to completely inhibit AP-1. It is also possible that Raw also regulates axonal degeneration via additional mechanisms. We therefore conclude that Raw protects axon degeneration, at least partially, through the downstream transcription factor AP-1.

4.4 Discussion

Since the discovery of the gain-of-function mutation WldS which delays Wallerian degeneration for weeks in mammals, many studies have been focused on identifying loss-of-function mutants to better understand the molecular mechanisms of this self-destruction axon death program. In this study, we identified Raw as a novel regulator of axon degeneration in *Drosophila*. We have demonstrated that a hypermorph allele of *raw* strongly suppressed Wallerian degeneration in *Drosophila* larvae for days. We have further showed that Raw promotes axonal degeneration via a pathway that involves transcription factor AP-1. Interestingly, although Nmnat has been thought as a central regulator of axonal degeneration, knockdown of Nmnat appears only very modestly affect the axonal protective phenotypes in *raw* mutants. Therefore, our studies provide a potential novel mechanism of axonal degeneration, which might function in parallel with Nmnat signaling. Identification of additional Raw interacting partners would provide more insights into the relationships of these signaling pathways.

Raw and its ortholog olrn-1 in *C.elegans* are both neuronal expressed proteins which show localization in axons, dendrites and cell bodies (Bauer Huang et al., 2007, Lee et al., 2015). The *C.elegans* homologue olrn-1 contains multiple transmembrane domains and genetically

interacts with calcium signaling pathways (Bauer Huang et al., 2007). In *Drosophila*, a recent study characterized Raw as a single transmembrane protein with a large extracellular domain which contains a predicted EF hand (Lee et al., 2015), but the multiple transmembrane domains in olrn-1 are not present in Raw. Interestingly, the raw^{dcp-1} allele that we have identified has a point mutation within the EF hand (Figure 4.2). As calcium influx upon axotomy plays a critical role in axonal degeneration (reviewed in (Wang et al., 2012a)), it would be interesting know whether Raw is regulated by calcium signaling in modulating axonal degeneration.

Previous studies suggest that Raw regulates cell-cell interactions in several developmental events, including dorsal closure, gonad morphogenesis and dendrite patterning (Jack and Myette, 1997, Byars et al., 1999, Bauer Huang et al., 2007, Bates et al., 2008, Jemc et al., 2012, Lee et al., 2015). It is possible that Raw mediates axon degeneration via interaction with an extracellular signal, which might from glia, since glia have been suggested to play a role in axon degeneration (Purice et al., 2016). Indeed, a recent study demonstrated disrupted glia-axon interaction leads to axon degeneration (Takagishi et al., 2016, Xiao et al., 2015a).

Although Raw does not have an obvious homolog in mammals, its downstream molecules JNK and transcription factor AP-1 are both evolutionarily conserved. JNK and its upstream kinase Wallenda (Wnd, DLK in mammals) are activated by axonal injury and actively transported in axons to mediate a retrograde signal to cell bodies (Xiong et al., 2010, Zrouri et al., 2004, Lindwall and Kanje, 2005). Our previous studies suggest that an axonal conditioning lesion can protect axons from degeneration after further injury, and the protective effect is Wnd/DLK and Fos dependent (Xiong and Collins, 2012). As an important downstream kinase of Wnd and upstream kinase of Fos, it is very likely that JNK is also involved in this axonal protection. However, how JNK and Fos mediate this protective effect is not clear. In the present

study, we have identified Raw functions upstream of JNK and Fos to regulate axonal degeneration, suggesting that loss-of-function of *raw* could mimic the conditioning lesion effects. Dissection of the molecular mechanism by which Raw modulates JNK and Fos might enrich our understanding of the protective effect of the conditioning lesion.

Studies in both *Drosophila* and mammals suggested that transcription factor AP-1 is regulated by neuronal activity (Vonhoff et al., 2013, Tuvikene et al., 2016, Morgan et al., 1987). Intriguingly, we have previously suggested that neuronal silencing delays axonal degeneration (Mishra et al., 2013). It is therefore possible that Raw regulates axonal degeneration in a pathway that involves neuronal activity. Since the basic molecular mechanisms of Wallerian degeneration is highly conserved between *Drosophila* and mammals (reviewed in (Rooney and Freeman, 2014, Neukomm and Freeman, 2014)), understanding the mechanisms for Raw would shed light on our understating and treatment for nerve damage in vertebrates.

4.5 Material and methods

4.5.1 Fly Stocks

The following strains were used in this study: Canton-S (WT), *dcp-1*^{prev1} (*raw*$^{dcp-1}$) originally from (Laundrie et al., 2003), k05606 (10390), repo-Gal4 (7415) and second chromosome deficiencies from Bloomington fly stock center, *raw*$^{134.47}$, *raw*$^{155.27}$, UAS-Raw-RA and UAS-Raw-RB from (Jemc et al., 2012), *hiw*^{ND8} and *hiw*$^{\Delta N}$ from (Xiong et al., 2012) m12-Gal4 (P(Gal4)^{5053A}) (Ritzenthaler et al., 2000), BG380-Gal4 (Budnik et al., 1996), ppk-Gal4 (Kuo et al., 2005), D42-Gal4 (Sanyal, 2009), UAS–dcr2 (a gift from Stephen Thor), UAS-JNK^{DN} (Weber et al., 2000), UAS-Fos^{DN} (Eresh et al., 1997), UAS-Jun^{DN} (Eresh et al., 1997). UAS-

nmnat-RNAi (32255), UAS-raw-RNAi (KK) (101255) and UAS-raw-RNAi (GD) (7727) were from the Vienna RNAi center.

4.5.2 Larval nerve crush assay and immunocytochemistry

The nerve crush assay was previously described in details in (Xiong et al., 2010, Xiong et al., 2012, Hao et al., 2016). Briefly, wandering 3rd instar larvae were anesthetized with CO_2 gas, and the segmental nerves were pinched and crushed by a shape No.5 forceps. After the crush, larvae were kept on a grape plate in 25°C incubator for specified time periods.

Drosophila larvae were dissected in ice-cold PBS and then fixed in 4% paraformaldehyde for 25 minutes. After fixation, the samples were incubated in blocking buffer (PBS with 0.3% Triton and 5% normal goat serum) for 30 minutes at room temperature. Primary antibodies were used at the following concentrations: ms anti-Futsch (22c10, developmental studies hybridoma bank (DSHB)) 1:100, ms anti-lacZ (40-1a, DSHB) 1:100, guinea pig (gp) anti-Nmnat (gift from Grace Zhai) 1:1000. For secondary antibodies, Cy3-Gt anti-HRP (from Jackson labs) were used at 1:1000, A488-Gt anti-mouse or A488-Gt anti-gp (Invitrogen) were used at 1:1000.

4.5.3 Analysis of whole genome sequencing data

The sequences were aligned to reference *Drosophila* melanogaster genome (DM3) using Burrows-Wheerler Alignment tool (BMA). Reads that map to multiple locations were removed. Samtools was used to call variants. There are 332 variants that fit the criteria (homozygous mutation in raw^{dcp-1}, heterozygous mutation in $raw^{dcp-1}/k05606$ and homozygous wild-type in all three recombinant lines). These variants were then annotated to genes using software SnpEff and only non-synonymous mutations were kept as candidates. The 6 candidate genes were *raw, eya, msp-300, CG10874, CG9525 and CG17211* (summarized in Table 4.1).

4.5.4 Imaging

All the confocal images were collected on an Improvision spinning disk confocal microscope, consisting of a Hamamatsu C9100-50 EMCCD camera, a Yokagawa Nipkow CSU10 scanner and a Zeiss Axio Observer. All the images were taken using the 40X (1.3NA) oil objective. Similar settings were used to collect compared genotypes and conditions.

4.5.5 Quantifications

To quantify axon degeneration, we scored the m12-Gal4, UAS-mCD8-GFP labeled axons within the segmental nerves according to one of the four categories between 0 to 3 (with 3 meaning completely fragmented) (Figure 4.5). For each specific genotype and condition, more than 50 axons were quantified.

To quantify the degeneration of NMJ, dissected larvae were stained for the MAP1B homologue Futsch and axonal membrane marker HRP. NMJs were scored to one of the three categories: complete degeneration (complete loss of Futsch staining and fragmentation of synaptic membrane), partial degeneration (partial loss of Futsch staining and partial fragmentation of synaptic membrane) and no degeneration (both Futsch and synaptic membrane are intact). For each specific genotype and condition, more than 20 NMJs from multiple animals were quantified. Percentages of NMJs in each category are shown.

For *puc*-lacZ intensity quantification, the mean intensities for lacZ staining in the nuclei of motoneurons located along the dorsal midline of the larval nerve cord were measured using the Volocity software (Perkin Elmer). More than 6 animals were measured for each genotype and condition.

For measurement of Nmnat staining intensity in the neuropil, the neuropil area in each image was selected. We used auto-selection function of the Volocity software to select objects that meet the intensity criteria of >1 standard deviations above the mean within the neuropil region. The mean Nmant intensity was calculated by total intensity divided by total area in each animal. For each genotype, more than 5 animals were measured.

4.6 Figures

Figure 4.1

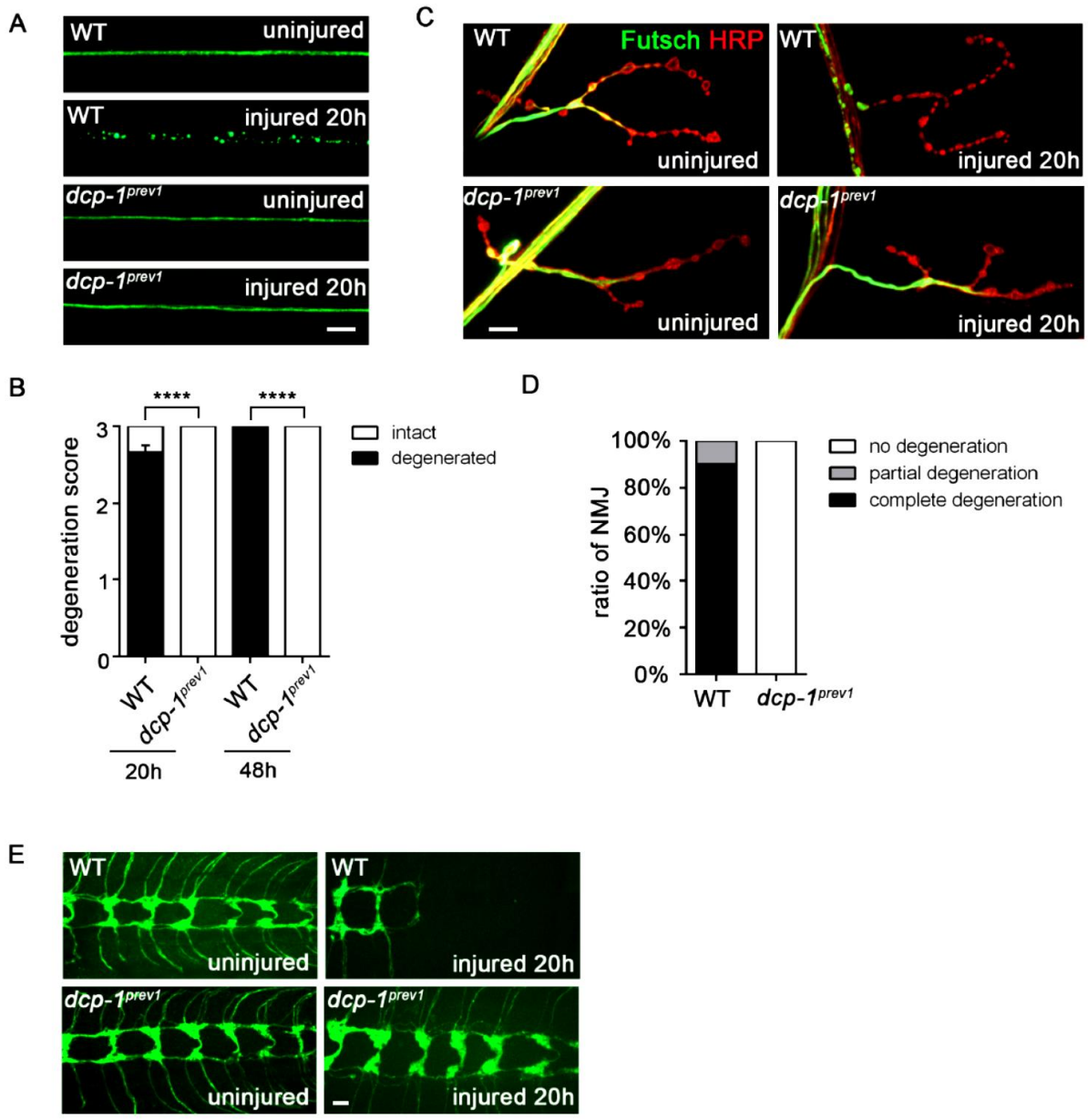

Figure 4.1 Wallerian degeneration of motoneuron and sensory neuron axons and synapses is strongly delayed in *dcp-1*^{prev1} animals.

(A) Single motoneuron axons in third instar larvae are labeled with membrane bound mCD8GFP with m12-Gal4 driver. In WT animals, axons that distal to the injury sites have completely fragmented within 20 hours after nerve crush injury. However, axons remain completely intact in *dcp-1*^{prev1} mutant background.

(B) Quantification of the degree of axonal degeneration in different genotypes and different time points (See Figure 4.5A for scoring criteria). Black bars show the degeneration scores, whereas the white bars show the complementary intact scores.

(C) Representative images of muscle 4 NMJs in WT and *dcp-1*^{prev1} animals 20 hours after injury. In WT animals, the cytoskeleton marker Futsch (labeled as green) disappear from the NMJ, while the neuronal membrane (labeled with antibodies to HRP as red) become completely fragmented. In contrast, NMJs in the *dcp-1*^{prev1} animals show no sign of degeneration.

(D) Quantification of NMJ degeneration. Black bars represent the percentage of NMJs that completely degenerated, grey bars represent percentage of NMJs that partially degenerated, and white bars represent the percentage of intact NMJs. The quantification criteria are described in Figure 4.5.

(E) The axons and nerve terminals of class IV sensory neurons in larval ventral nerve cord are labeled by membrane bound mCD8GFP with the ppk-Gal4 driver. In WT animals, these structures are completely fragmented and cleared within 20 hours after injury (the remaining parts are the axons that haven't been injured). However, these injured axons remain intact in *dcp-1*^{prev1} animals.

Scale bars=20μm , error bars represent SEM; **** represents $p<0.0001$ in t-test.

Figure 4.2

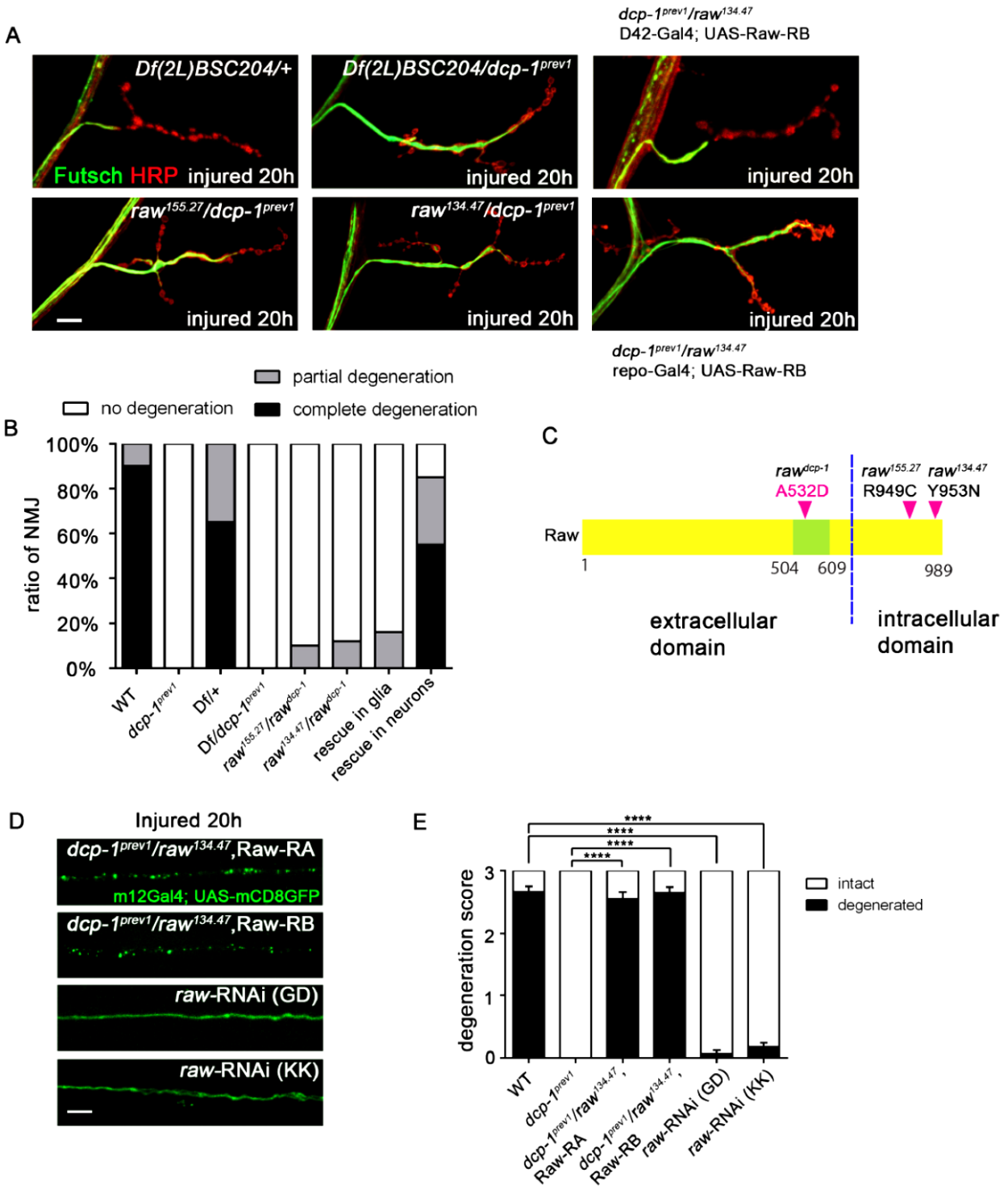

Figure 4.2 The mutation that affects axonal degeneration is a loss-of-function mutation on gene *raw*.

(A) Representative images of muscle 4 NMJs 20 hours after injury in deficiency line BSC204 (Df/+), deficiency and *dcp-1*^{prev1} heterozygous (Df/*dcp-1*^{prev1}), *raw* mutants and *dcp-1*^{prev1} heterozygous (*raw*$^{155.27}$/ *dcp-1*^{prev1} and *raw*$^{134.47}$/ *dcp-1*^{prev1}). While NMJs have degenerated in Df/+, they are largely preserved in animals with the following genotypes, *Df/dcp-1*^{prev1}, *raw*$^{155.27}$/ *dcp-1*^{prev1} and *raw*$^{134.47}$/ *dcp-1*^{prev1}. Expression of UAS-Raw in *raw*$^{134.47}$/ *dcp-1*^{prev1} background using a motoneuron driver D42-Gal4 significantly rescues the protective phenotype, whereas expression of UAS-Raw using a pan-glia driver repo-Gal4 fails to rescue.

(B) Quantification of the degeneration of NMJs the genotypes shown in (A).

(C) Schematic representation of the protein structure of Raw. It has a relatively large extracellular domain with a putative EF hand region labeled as green. *dcp-1*^{prev1} has a missense mutation within the EF hand. Both *raw*$^{134.47}$ and *raw*$^{155.27}$ has a missense mutation within the intracellular domain.

(D) Expression of either Raw-RA or Raw-RB in *raw*$^{134.47}$/ *dcp-1*^{prev1} background using a motoneuron driver m12-Gal4 rescues the protective effect of axon degeneration in *raw*$^{134.47}$/ *dcp-1*^{prev1} background animals. Moreover, depletion of Raw in motoneurons using *raw*-RNAi lines also strongly delays axonal degeneration.

(E) Quantification of axonal degeneration of the animals with genotypes shown in (D).

Scale bars=20μm, error bars represent SEM; **** represents $p<0.0001$ in t-test.

Figure 4.3

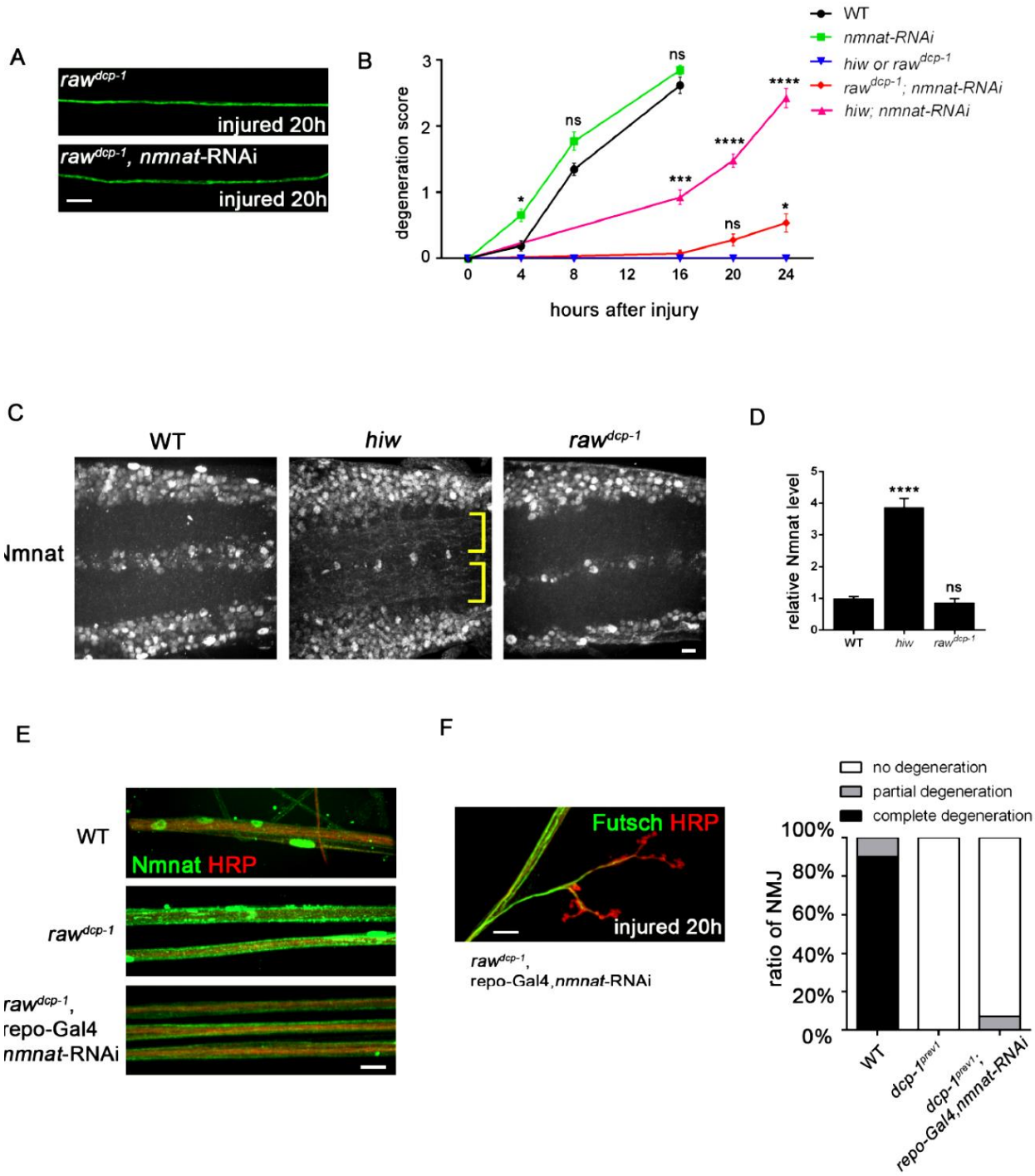

Figure 4.3 Knockdown of *nmnat* modestly affects the protective effects of Raw.

(A) 20 hours after injury, axons in either *raw*$^{dcp-1}$ animals or *raw*$^{dcp-1}$ background with depletion of Nmnat in motoneuorns (*raw*$^{dcp-1}$; m12-Gal4,UAS-*nmnat*-RNAi) remain intact.

(B) Quantification of axonal degeneration in different time points for the noted genotypes. Comparing to WT animals (black line), knockdown of *nmnat* by RNAi (green line) modestly promotes axonal degeneration. While knockdown of *nmnat* in the hiw mutant background significantly rescues the axonal protection phenotype in *hiw* mutant (compare pink line to blue line), knockdown of nmnat in *raw*$^{dcp-1}$ mutant background very modestly rescues the axonal protective effect of *raw*$^{dcp-1}$ (compare red line to blue line).

(C) Hiw up-regulates endogenous Nmnat level in neuropil (noted with yellow brackets), whereas Nmnat level is not affected in raw mutant (*raw*$^{dcp-1}$).

(D) Quantification of relative level of Nmnat in the neuropil regions of WT, hiw$^{\Delta N}$ and *raw*$^{dcp-1}$ animals.

(E) Endogenous Nmnat level is up-regulated in glia that sheathing the segmental nerves in *raw*$^{dcp-1}$ animals. Knockdown of nmnat using pan-glia driver repo-Gal4 can rescue the elevated Nmnat level in *raw*$^{dcp-1}$ animals.

(F) Knockdown of nmnat either in glia or in motoneuron fails to rescue the NMJ protective effects in *raw*$^{dcp-1}$ animals.

(G) Quantification of NMJ degeneration in different genotypes.

Scale bar=20μm, error bars represent SEM; **** represents $p<0.0001$, ***= $p<0.001$, *=$p<0.05$, ns= not significant in t-test.

Figure 4.4

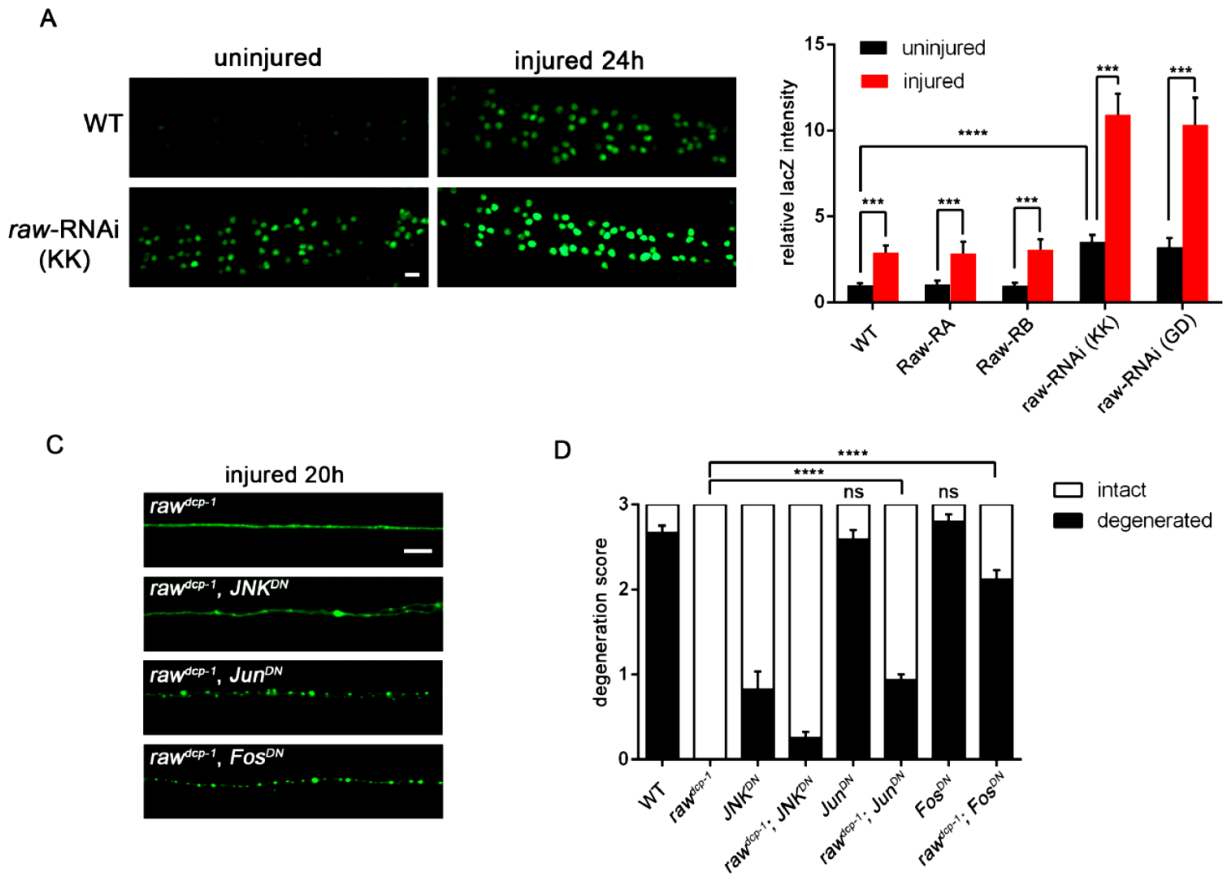

Figure 4.4 Raw regulates axonal degeneration via transcription factor AP-1.

(A) Comparing to uninjured WT animals, the *puc*-lacZ transcriptional reporter for JNK signaling is elevated in motoneurons depleted for Raw by expression of UAS-*raw*-RNAi using a pan-neuronal driver BG380-Gal4. Nerve crush injury activates *puc*-lacZ reporter in WT animals, and in Raw depleted neurons, this reporter can be further activated by injury. (B) Quantification of relative puc-lacZ intensities (described in methods) in different genotypes. (C) Single motoneuron axons are labeled by membrane bound mCD8GFP. As shown in Figure 4.1, axonal degeneration is strongly delayed in raw^{dcp-1} animals. Expression of dominant negative form JNK (JNK^{DN}) using motoneuron driver m12-Gal4 has very little effect axonal degeneration, whereas expression of Jun^{DN} or Fos^{DN} can partially rescue the protective effect in raw^{dcp-1} animals. (E) Quantification of axon degeneration scores in indicated genotypes. Scale bar=20μm, error bars represent SEM; **** represents p<0.0001, ***= p<0.001, ns= not significant in t-test.

Figure 4.5

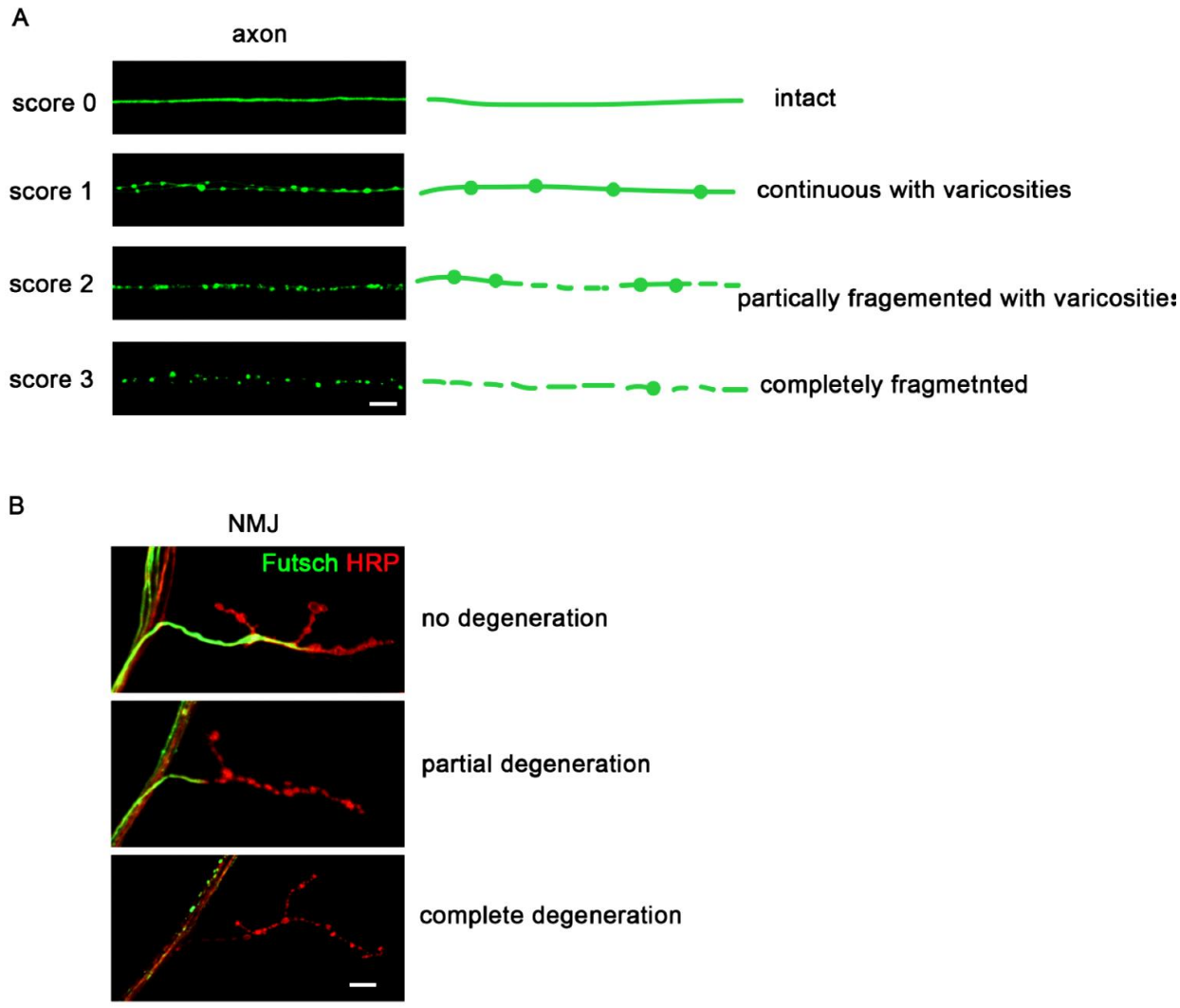

Figure 4.5 The quantification standards for axon degeneration and NMJ degeneration.

(A) Axons that are completely intact and smooth after injury are scored as 0. Axons that continuous but with varicosities are score as 1. Axons that partially fragmented, but partially continuous are scored as 2. Axons that are completely fragmented are scored as 3.

(B) NMJs that innervating larval muscle 4 are labeled by cytoskeleton marker Futsch (green) and HRP antibodies against neuronal membrane (red). Partial degeneration is defined by partially loss of Futsch and partially fragmentation of neuronal membrane. Completely degeneration is characterized by completely loss of Futsch in the NMJ and completely fragmentation of neuronal membrane.

Scale bar=20μm

Figure 4.6

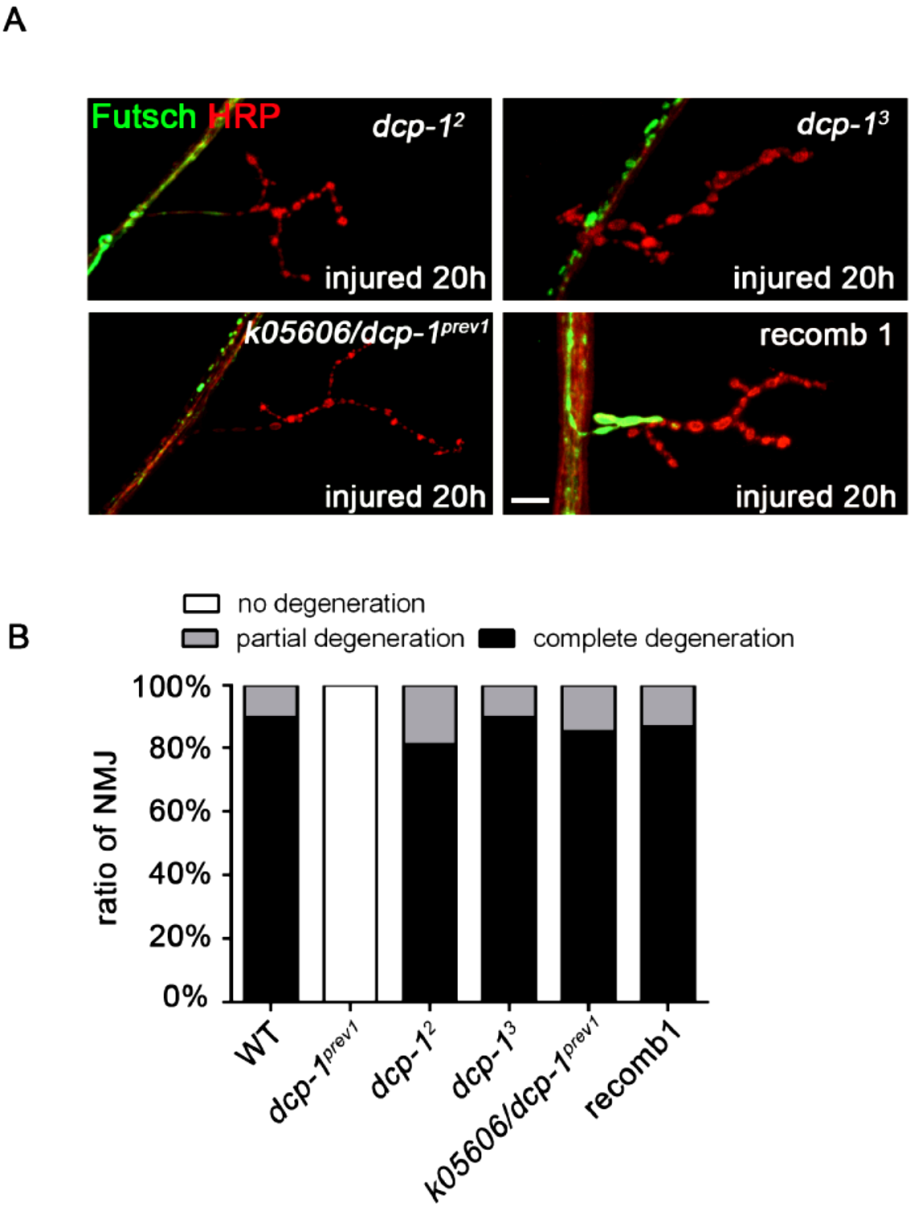

Figure 4.6 The axonal degeneration phenotype in *dcp-1*^{prev1} animals are not linked to the mutation on gene *dcp-1*.

(A) Representative images of muscle 4 NMJs in *dcp-1*^{2}, *dcp-1*^{3}, *k05606/dcp-1*^{prev1} and one recombinant line (mutation on dcp-1 in *dcp-1*^{prev} + WT chromosome). At 20 hours after injury,

NMJs are completely degenerated in WT animals, but largely preserved in *dcp-1*^{prev} animals (Figure 4.1). The protective effects are not observed in all the genotypes shown here.

(B) Quantifications of the percentages of NMJs that are completely degenerated, partially degenerated or intact for the genotypes shown.

Scale bar=20μm

Table 4.1

candidate genes (chromosome location)	complementary deficiency line (deleted region)	*dcp-1*^{Prev1}/Df axon degeneration phenotype?
CG10874 (22D1)	Df(2L)BSC688 (22B1-22D6)	No
Msp-300 (25C6-25C10)	Df(2L)Exel6011 (25C8-25D5)	No
	Df(2L)BSC109 (25C4-25C8)	No
Eya (26E1-26E2)	Df(2L)BSC354 (26D7-26E3)	No
Raw (29E4-29E6)	Df(2L)BSC204 (29D5-29F8)	Yes
CG9525 (29F5-29F6)	Df(2L)BSC204 (29D5-29F8)	No
	Df(2L)ED678 (29F5-30B12)	No
CG17211 (33D2)	Df(2L)ED775 (33B8-34A3)	No

Table 4.1 complementation test for candidate genes with deficiencies.

Small deficiencies that have the candidate genes deleted were crossed with *dcp-1*^{prev1} animals. Heterozygous df/ *dcp-1*^{prev1} animals were injured and tested for NMJ degeneration phenotype. Only the deficiency line that has gene *raw* deleted recapitulated the NMJ protective phenotype in homozygous *dcp-1*^{prev1} animals, suggesting the mutation on gene *raw* causes the axonal protective phenotype.

Chapter 5 :

Discussion and future directions

Neurons need to maintain their function for a lifetime once they are mature. Therefore the ability to cope with stress and environmental insults is fundamental. Neurons are also unique because of their highly polarized structure (axons and dendrites) for information transmission. Axons connect neurons over long distances in the brain and body, and thus are vulnerable to damage and injury. In some cases damaged axons can initiate new growth and reconnect with their targets. However in many cases, including in the mammalian central nervous system (CNS), damaged axons fail to regrow and the disconnected neurons undergo cell death. Although it is now widely acknowledged that both intrinsic and extrinsic factors contribute to axonal regenerative capacity, the molecular mechanisms that regulate axonal regeneration are still not fully understood. For neurons that can regenerate axons after injury, a series of responses are involved. These include retrograde transport of injury signaling molecules, large scale transcriptional responses, initiation of new axonal growth from the proximal axonal stumps, and degeneration and clearance of the disconnected distal axonal stumps to clear the path for axonal regeneration. In this thesis, I used *Drosophila* as a model organism to study the molecular and cellular mechanisms of these processes. I have described new mechanisms for both axonal regeneration and axonal degeneration. Some of my studies have extended from *Drosophila* to the mammalian nervous system, suggesting the regulatory mechanisms are highly evolutionarily conserved. While these studies have enriched our understanding of the axonal injury response,

they also lead to new questions. In this chapter, I will focus on summarizing and discussing my current findings as well as future research directions.

5.1 Wnd/DLK 'senses' axonal damage via cAMP/PKA

In order to promote new axonal growth after injury, neurons respond to axonal injury by initiating a large-scale transcriptional responses. However, in most cases, the axonal injury site is far away from the cell body. Then how does the cell body 'know' that a distant site in the axon is injured? Previous work from our lab and others have identified the conserved axonal MAPKKK Wnd/DLK, which appears to function as an acute 'sensor' of axonal damage and mediate a retrograde injury signaling in neurons (Xiong et al., 2010, Hammarlund et al., 2009, Yan et al., 2009, Holland et al., 2016). The levels of this protein appear to be tightly regulated in axons (via a post-transcriptional mechanism), and rise within hours in damaged axons (Xiong et al., 2010, Huntwork-Rodriguez et al., 2013). Wnd/DLK is required for axonal regeneration in many types neurons in the PNS. Conversely, in mammalian CNS neurons that do not regenerate, DLK activation after injury mediates cell death (Watkins et al., 2013, Welsbie et al., 2013). The dramatic and dichotomous outcomes of DLK have attracted great recent interest in understanding how this kinase is regulated. My studies in Chapter 3 have identified cAMP/PKA as a direct upstream activator of DLK in the context of axonal regeneration after acute axonal injury. Although the activation loop of DLK is known to be critical for DLK's function, my study provided the first evidence that it is directly phosphorylated and activated by a highly evolutionarily conserved kinase PKA. Importantly, cAMP has been previously demonstrated to play a central role in axon regeneration: up-regulation of cAMP level is sufficient to enhance axon regeneration in both the PNS and CNS (Cai et al., 1999, Neumann and Woolf, 1999, Cai et al., 2001, Qiu et al., 2002). Therefore, my study has unified two important regeneration signaling

pathways by providing a direct molecular link, suggesting that axonal regeneration is regulated in one common signaling pathway. However, this novel signaling pathway is still not fully understood. For example, it is not clear that how this pathway is activated and whether it has other functional implications. I this section, I will discuss these outstanding questions derived from my studies.

5.1.1 Does PKA activate DLK in other contexts?

As discussed above, DLK is not only activated during axonal regeneration, it is also activated in other contexts, including nerve growth factor (NGF) withdrawal induced DRG neuron death (Huntwork-Rodriguez et al., 2013) and retinal ganglion cell (RGC) death in glaucoma model (Welsbie et al., 2013). Moreover, a recent unpublished study suggest that DLK is also activated in Amyotrophic lateral sclerosis (ALS) models. It would be interesting to test whether PKA mediates DLK's activation in these models. Although I found that PKA directly phosphorylates DLK at its activation loop (which is critical for DLK to function as a kinase), I cannot exclude the possibility that the activation loop can also be phosphorylated by other kinases. Therefore, it is entirely possible that DLK is activated via different mechanisms in different models. Indeed, a recent study in *Drosophila* suggested that mTORC1 also phosphorylates DLK at its activation loop in mediating synaptic growth (Wong et al., 2015). My data suggest that PKA regulates DLK independently of mTORC1 (Figure 3.7).

5.1.2 How does PKA stabilize DLK?

DLK protein level strongly correlates with DLK activation and function. Axonal injury stabilizes DLK protein in axons and activates its downstream signaling (Huntwork-Rodriguez et al., 2013, Xiong et al., 2010). In cultured DRG neurons, NGF withdrawal also promotes DLK level and activates DLK signaling (Huntwork-Rodriguez et al., 2013). Moreover, increased

DLK level is sufficient to activate the DLK signaling pathway (Mata et al., 1996, Huntwork-Rodriguez et al., 2013). Therefore, understanding how DLK level is regulated is of great interest. Previous study suggested that DLK level can be stabilized via a feedback mechanism through which a downstream kinase JNK directly phosphorylated DLK outside of its activation loop (Huntwork-Rodriguez et al., 2013). My findings described in Chapter 3 suggests that activation of PKA also stabilizes DLK (Figure 3.4). Interestingly, further studies demonstrated that PKA stabilizes DLK via a mechanism that is independent of the phosphorylation of DLK's activation loop or the phosphorylation of DLK mediated by JNK (Figure 3.4). However, it is not clear that how PKA stabilizes DLK.

A recent study suggests that DLK is also palmitoylated and palmitoylation is important for DLK's function (Holland et al., 2016). I therefore wonder whether PKA stabilizes DLK in a palmitoylation dependent manner. Since DLK is palmitoylated in HEK293 cells (Holland et al., 2016), I tested this idea by expressing a mutant DLK with the palmitoylation site mutated in HEK293 cells. I found that either Forskolin treatment or PKA co-expression can stabilize the non-palmitoylation DLK mutant (Figure 5.1A and B). Therefore, I conclude that PKA stabilizes DLK independent of DLK palmitoylation.

In seeking to understand the mechanisms by which PKA regulates DLK, we have performed a proteomic analysis of DLK upon PKA transfection or forskolin treatment. In this study, we have identified several putative PKA phosphorylation sites (Figure 5.2). Therefore, it is possible that phosphorylation of DLK by PKA at sites other than S302 stabilizes DLK. Indeed, some of the potential sites, such as S584 and S640, are located within PKA consensus sequences (discussed in Chapter 3).

Moreover, previous studies suggest that DLK level is tightly regulated through the ubiquitin proteasome system (UPS) (Huntwork-Rodriguez et al., 2013, Nakata et al., 2005). Therefore, it is also possible that PKA regulates DLK stability indirectly through modulation of the UPS. This possibility can be tested in cultured cells by either pharmacologically inhibiting the proteasome or manipulating DLK ubiquitination through expression of the de-ubiqutinating enzyme USP9X.

5.1.3 Does cAMP level elevation correlate with axon regeneration and DLK activation?

A central feature of axonal regeneration is that axons can regenerate after injuries in the PNS, but generally fail to re-grow after injuries in the CNS. Studies have been focusing on understanding the differences in underlying molecular mechanisms between the CNS and the PNS in response to axonal injuries. Previous studies demonstrated that DLK protein level is stabilized by axonal injury in both the PNS (sciatic nerve injury model) and the CNS (optical nerve injury model)(Xiong et al., 2010, Huntwork-Rodriguez et al., 2013, Watkins et al., 2013, Shin et al., 2012a), suggesting that DLK's activation is not always associated with axonal regeneration. Instead, activation of DLK in the CNS promotes cell death (Watkins et al., 2013, Welsbie et al., 2013). Therefore, understanding the mechanisms of DLK activation in the PNS and the CNS after axonal injury is of great interest.

It has been documented that after sciatic nerve crush injury in mice, cAMP level increases two-fold within 24 hours in DRG neurons, and this effect is correlated with enhanced axonal re-growth capacity (Qiu et al., 2002). My study in Chapter 3 suggested that increased cAMP level in the PNS is responsible for the activation of DLK and through which axonal regeneration is stimulated. I therefore wonder whether that is the mechanism specific for axonal

regeneration in the PNS, but not the CNS, which could potentially explain the different outcomes. However, it is not clear whether cAMP level changes after axonal injury in the CNS.

As I have discussed in Chapter 2, *Drosophila* nervous system also share the CNS and PNS dichotomy in axonal regeneration. Therefore, this hypothesis can be pursued using *Drosophila* as a model. An attractive approach would be utilizing genetic encoded sensors of cAMP levels in live neurons, in combination with laser axotomy and live imaging techniques.

5.1.4 Which adenylyl cyclase (AC) is required for regulating cAMP level after injury?

Intracellular cAMP level in tightly controlled by adenylyl cyclases (ACs), which catalyze the conversion of ATP to cAMP. As there are several different types of ACs in neurons, identifying the specific type of AC that regulates injury induced cAMP change would shed light on our understanding of injury signaling. There are totally 13 AC genes in *Drosophila* genome. One could test which AC is required for the cAMP or Wnd level changes in axons after axotomy by using RNAi mediated knock-down for each of them.

It is well documented that in both cultured neurons in vitro and live animals in vivo that intra-axonal calcium level increases within seconds after axotomy (Adalbert et al., 2012, Wolf et al., 2001, Avery et al., 2012). Moreover, studies in *C.elegans* suggested that calcium promotes axon regeneration in a DLK dependent manner (Ghosh-Roy et al., 2010, Yan and Jin, 2012). Since some ACs are directly modulated by calcium, a logical hypothesis is that a Ca2+-sensitive AC connects axotomy to cAMP changes. In Drosophila genome, the gene called *rutabaga* is identified as the single homolog of calcium-sensitive AC. Therefore, Rutabaga is an interesting candidate to test.

5.1.5 Are A-Kinase Anchoring Proteins (AKAPs) involved in mediating axonal injury induced Wnd/DLK activation?

AKAPs are regulators of cAMP-PKA signaling. AKAPs have the ability to form multi-protein complexes allowing for a coordination of different enzymes affecting the spatial and temporal aspects of the signaling (Wong and Scott, 2004, Dell'Acqua et al., 2006). It would be interesting to know that whether AKAPs play a role in the regulation of DLK/Wnd by forming a stabilizing complex when PKA activates DLK/Wnd. Since Wnd protein becomes rapidly up-regulated in axons after axonal injury, an axonally localized AKAP scaffold could provide an attractive mechanism for its induction in injured axons.

There are 5 conserved AKAPs in *Drosophila*: Nervy, Spoonbill, Rugose (Akap550), Akap200 and Pkaap (Han et al., 1997, Lu et al., 2007, Jackson and Berg, 2002, Shamloula et al., 2002, Terman and Kolodkin, 2004). In order to test the role of AKAPs in Wnd's induction after injury, we can knockdown individual AKAPs using RNAi lines in *Drosophila* larvae *in vivo*. Since Wnd protein becomes induced after axon injury, one can compare the relative levels of Wnd within motoneurons lacking individual AKAPs to wild-type controls at time points before and after injury. One can also examine whether AKAP knockdown affects the activation of the puckered reporter (*puc*-lacZ), which is a downstream reporter for Wnd signaling. The information obtained from these experiments would give insight into mechanisms through which Wnd/DLK is regulated.

5.1.6 Does Hiw regulate Wnd/DLK via modulating cAMP level?

As I have discussed in Chapter 1, the E3 ubiquitin ligase Hiw serves as an important regulator of Wnd in many circumstances. One study in *C. elegans* suggested that Hiw's homologue Rpm-1 down-regulates DLK by directly promoting its ubiquitination (Nakata et al.,

2005). Nakata et al. compared the ubiquitin levels of immunoprecipitated Flag-tagged DLK from HA-ubiquitin, HA-Rpm-1 and Flag-DLK co-transfected HEK cells to that from HA-ubiquitin and Flag-DLK co-transfected cells by probing the western blot with HA antibody. They showed that the immunoprecipitated DLK from the cells that co-transfected with HA-Rpm-1 has higher level of co-immunoprecipitated HA-ubiquitin. However, the increased HA antibody signal could be co-immunoprecitated HA-Rpm-1. Therefore the data for such direct regulation in vivo is not satisfying and ubiquitination could not be the only mechanism that Hiw regulates Wnd/DLK.

Intriguingly, a previous study suggested that Hiw contains a RCC-1 like domain that can biochemically inhibit ACs and therefore negatively regulate cAMP level (Pierre et al., 2004). Since the relationship between cAMP and DLK has been established, it is possible that Hiw regulates Wnd/DLK through cAMP (this model is depicted in Figure 5.3). An interesting future direction would be to test whether Hiw regulates cAMP levels at presynaptic terminals, where it localizes, and whether this regulation mediates Hiw's regulation of Wnd.

5.2 PKA regulates Wallerian degeneration via Wnd

Previous studies from our lab and others have suggested that Wnd/DLK signaling can influence Wallerian degeneration (Xiong and Collins, 2012, Miller et al., 2009, Yang et al., 2015). Since PKA functions upstream of Wnd/DLK, I wonder whether PKA regulates Wallerian degeneration. To test this, I expressed membrane-bound mCD8GFP using the motoneuron driver m12-Gal4, which labels single axons in *Drosophila*. I found that overexpression of the constitutively active form of PKA (PKA^{CA}) strongly protected axons from degeneration after nerve crush injury (Figure 5.4). Furthermore, knockdown of Wnd using RNAi can partially rescue the protective effects of PKA (Figure 5.4). There are two possible reasons that can

explain why *wnd*-RNAi cannot completely rescue the phenotype. First, wnd-RNAi may only partially disrupt Wnd expression in neurons. Alternatively, PKA may influence Wallerian degeneration via additional mechanisms that are independent of Wnd.

While cAMP and PKA are known for their important roles in axonal regeneration, here I report for the first time that PKA also regulates axonal degeneration. As Nmnat has been thought as a central regulator of axon degeneration, I wondered whether Nmnat level changed when PKA^{CA} was expressed in neurons. To test this, I co-expressed UAS-nmnat-RFP in neurons. However, I found overexpressed Nmnat level was not affected by PKA (Figure 5.5). In the future, it would interesting to test whether knockdown of *nmnat* rescues the axonal protective effect mediated by PKA.

5.3 PKA regulates mitochondrial morphology via Wnd

Mitochondria are highly dynamic organelles which play important roles including ATP generation, biosynthesis, calcium buffering and apoptotic signaling. In neurons, mitochondria are transported into axons to maintain normal axonal function. Mitochondrial fission and fusion are dynamic are critical for mitochondrial transport, mitophagy, apoptosis and necrosis (Shutt and McBride, 2013, Sheridan and Martin, 2010, Wang et al., 2012b). Deficiency in mitochondrial fission and fusion machinery is associated with a broad array of neurodegenerative diseases (Reilly et al., 2011, Waterham et al., 2007, Ishihara et al., 2009). Therefore, understanding of the regulatory machinery of mitochondrial fission and fusion in neurons is of great importance.

I have found that expression of PKA^{CA} in neurons induced elongated mitochondria in axons (Figure 5.4A and B). This finding is consistent with a previous study which suggests that PKA regulates mitochondrial elongation in cultured neurons (Merrill et al., 2011). Moreover, I

found that this effect is Wnd dependent, as knockdown of Wnd by *wnd*-RNAi rescues the elongated mitochondrial phenotype (Figure 5.6A and B). These findings suggest that PKA functions upstream of Wnd/DLK in regulating mitochondrial morphology. In the future, it would be interesting to study the functional consequences of these elongated mitochondria. For example, one could investigate whether they play a role in axonal regeneration and degeneration.

5.4 PKA regulates synaptic morphology during development via Wnd

Normal synaptic growth during development is essential for the nervous system to mediate complex behavior such as learning and memory. The *Drosophila* NMJ has been demonstrated as a powerful system to study the underlying molecular mechanisms of synaptic development and plasticity (Collins and DiAntonio, 2007). Since both PKA and Wnd have been previously suggested to function in regulating synaptic growth in *Drosophila* (Yoshihara et al., 2005, Chen and Ganetzky, 2012, Collins et al., 2006), I wondered whether they function in a common signaling pathway to regulate synaptic development. To test this, I expressed membrane bound mCD8GFP in motoneurons using m12-Gal4 driver and observed the morphology of NMJs that innervating muscle 12. I found that expression of PKA^{CA} or Wnd alone induced filopodia-like structures along the boutons (Figure 5.7). Moreover, the effect of PKA^{CA} on synaptic morphology is Wnd dependent as knockdown of Wnd rescued this phenotype, whereas expression of control RNAi (moody-RNAi) failed to rescue (Figure 5.7). This finding suggested that PKA functions upstream of Wnd to regulate synaptic morphology during development. Previous studies suggested that PKA signaling is required for synaptic plasticity in memory and learning (reviewed by (Lee, 2015)), my finding here suggested an intriguing possibility that Wnd/DLK might also contribute to these mechanisms.

5.5 Figures

Figure 5.1

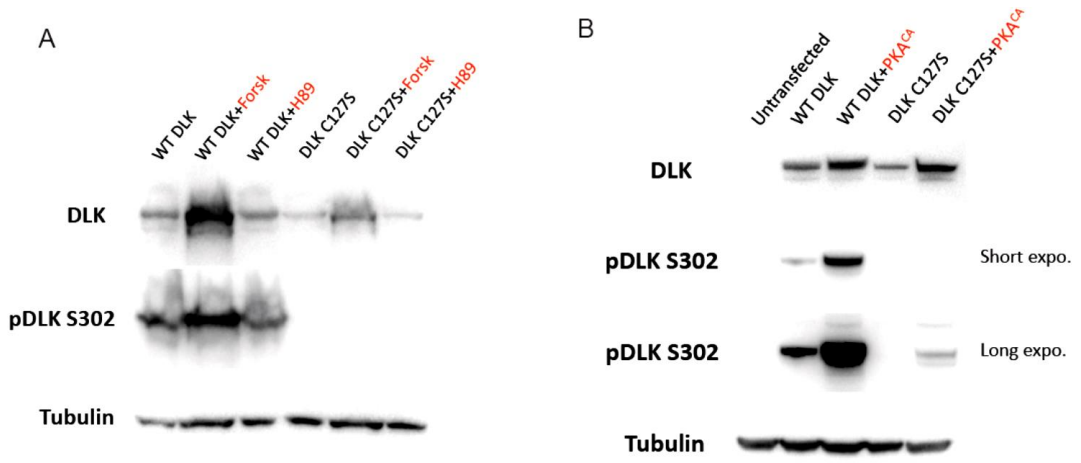

Figure 5.1 PKA regulates DLK stability independent of DLK palmitoylation.

(A) HEK293 cells were either transfected with WT DLK or DLK-C127S, a point mutation that prevents DLK from palmitoylation. Treatment with Forskolin stabilizes both WT DLK and DLK-C127S.

(B) Co-transfection of PKA^{CA} either with WT DLK or DLK-C127S stabilizes their levels in HEK293 cells. Moreover, phosphorylation of Ser302 was strongly reduced in DLK-C127S mutant.

Figure 5.2

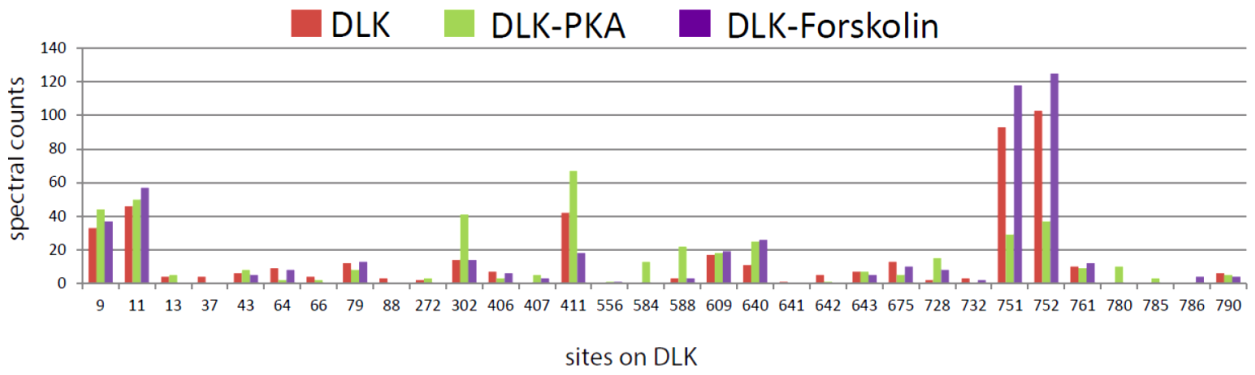

Figure 5.2 proteomic analysis on DLK upon Forskolin treatment or PKA co-transfection.

HEK 293 cells were transfected with Flag-DLK alone (either treated with control vehicle or Forskolin) or transfected with both Flag-DLK and PKA. Flag-DLK was immunoprecipitated by anti-Flag antibodies. 5 µg purified (via SDS-PAGE gel) DLK from each group was subjected to mass spec analysis. Spectral count for each identified site in each experiment group were plotted in the graph.

Figure 5.3

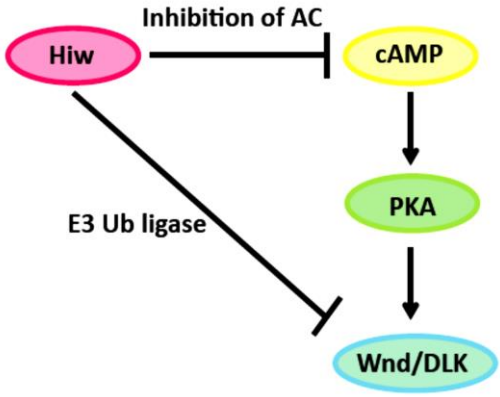

Figure 5.3 Proposed model of the mechanisms for regulation of Wnd/DLK by Hiw.

Previous studies suggested that Hiw down-regulates Wnd/DLK level through promoting its ubiquitination. Here I propose that hiw might regulate Wnd/DLK via an alternative mechanism by which Hiw modulates cAMP level via its RCC-1 domain.

Figure 5.4

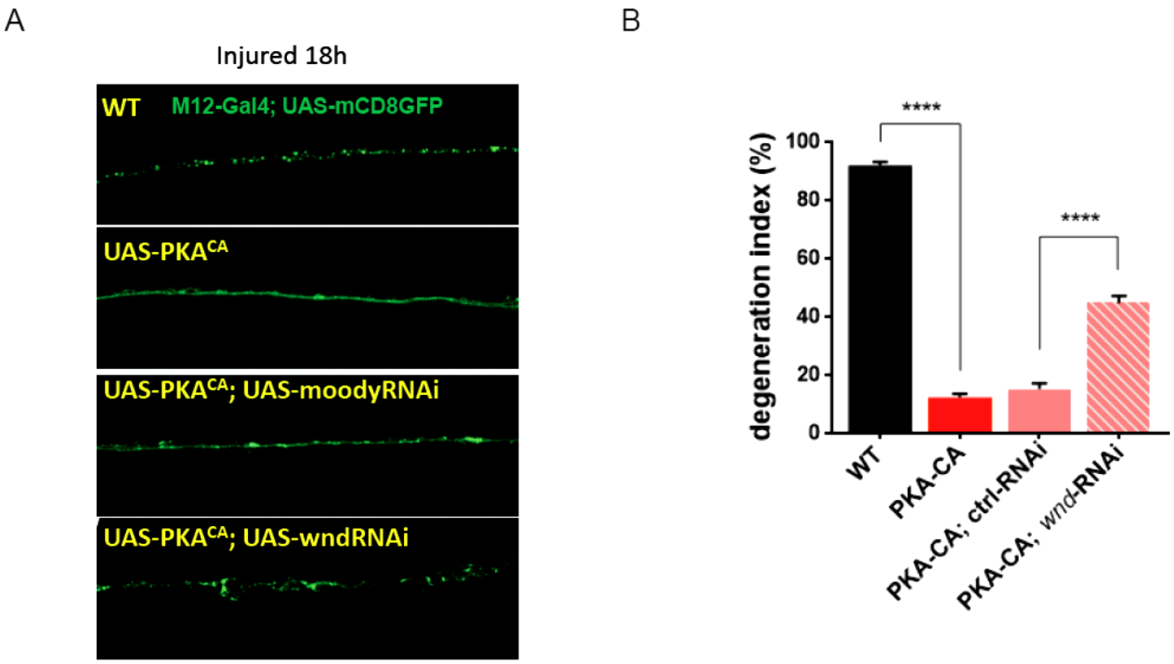

Figure 5.4 PKA regulates Wallerian degeneration via Wnd.

Expression of PKA^{CA} in motoneurons strongly suppressed axon degeneration after nerve crush assay comparing to WT axons. Co-expression of wnd-RNAi with PKA^{CA} can partially rescue axon degeneration, whereas co-expression of a control RNAi cannot.

Figure 5.5

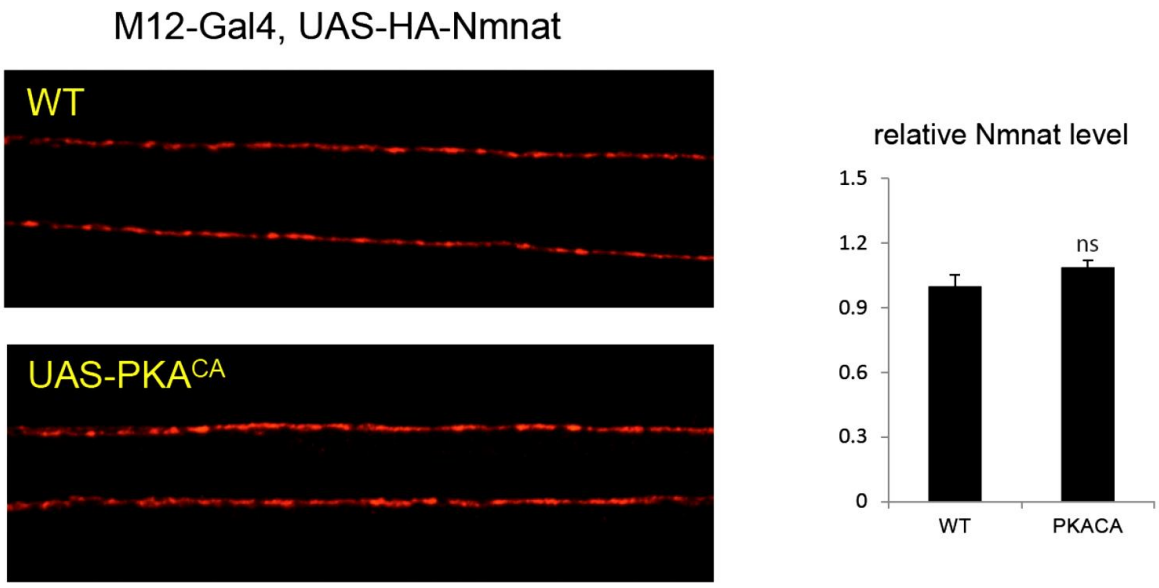

Figure 5.5 Nmnat level does not change significantly when PKA^{CA} is expressed in neurons.

HA-tagged Nmnat is expressed in motoneurons using m12-Gal4 driver. Ectopically expression of PKA^{CA} does not significantly change Nmnat level in axons. ns, not significant.

Figure 5.6

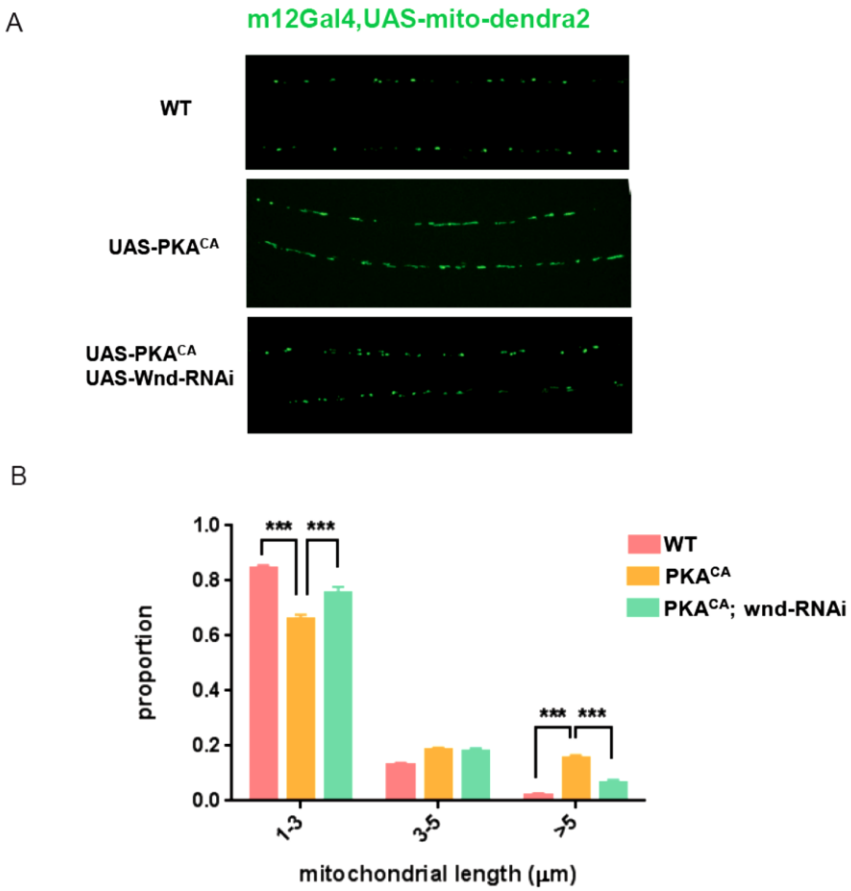

Figure 5.6 PKA promotes elongated mitochondria in axons via Wnd.

(A) Overexpression of PKA^{CA} induces elongated mitochondria in motoneuron axons. This phenotype is Wnd dependent as knockdown of Wnd rescues this phenotype.

(B) In each genotype, mitochondria were grouped based their length. The proportion of mitochondria in each group was quantified.

Figure 5.7

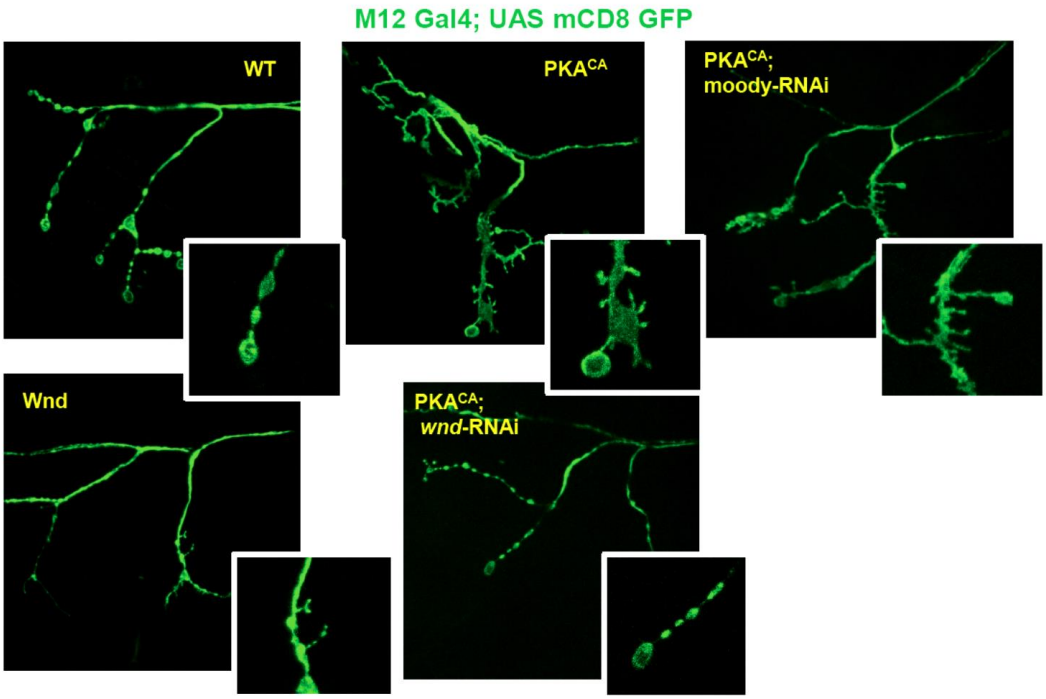

Figure 5.7 PKA regulates synaptic morphology via Wnd.

Overexpression of PKA^{CA} or Wnd in motoneurons induced filopodia-like protrusions on the bontons of NMJ. The phenotype caused by PKA^{CA} is Wnd dependent as knockdown of Wnd completely rescues this phenotype, whereas it cannot be rescued by a control RNAi.

REFERENCES

ABERLE, H., HAGHIGHI, A. P., FETTER, R. D., MCCABE, B. D., MAGALHAES, T. R. & GOODMAN, C. S. 2002. wishful thinking encodes a BMP type II receptor that regulates synaptic growth in Drosophila. *Neuron,* 33**,** 545-58.

ADALBERT, R., MORREALE, G., PAIZS, M., CONFORTI, L., WALKER, S. A., RODERICK, H. L., BOOTMAN, M. D., SIKLOS, L. & COLEMAN, M. P. 2012. Intra-axonal calcium changes after axotomy in wild-type and slow Wallerian degeneration axons. *Neuroscience,* 225**,** 44-54.

ALI, Y. O., ALLEN, H. M., YU, L., LI-KROEGER, D., BAKHSHIZADEHMAHMOUDI, D., HATCHER, A., MCCABE, C., XU, J., BJORKLUND, N., TAGLIALATELA, G., BENNETT, D. A., DE JAGER, P. L., SHULMAN, J. M., BELLEN, H. J. & LU, H. C. 2016. NMNAT2:HSP90 Complex Mediates Proteostasis in Proteinopathies. *PLoS Biol,* 14**,** e1002472.

ALI, Y. O., MCCORMACK, R., DARR, A. & ZHAI, R. G. 2011. Nicotinamide mononucleotide adenylyltransferase is a stress response protein regulated by the heat shock factor/hypoxia-inducible factor 1alpha pathway. *J Biol Chem,* 286**,** 19089-99.

AVERY, M. A., ROONEY, T. M., PANDYA, J. D., WISHART, T. M., GILLINGWATER, T. H., GEDDES, J. W., SULLIVAN, P. G. & FREEMAN, M. R. 2012. WldS prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca2+ buffering. *Curr Biol,* 22**,** 596-600.

AYAZ, D., LEYSSEN, M., KOCH, M., YAN, J., SRAHNA, M., SHEEBA, V., FOGLE, K. J., HOLMES, T. C. & HASSAN, B. A. 2008. Axonal injury and regeneration in the adult brain of Drosophila. *J Neurosci,* 28**,** 6010-21.

BABETTO, E., BEIROWSKI, B., RUSSLER, E. V., MILBRANDT, J. & DIANTONIO, A. 2013. The Phr1 ubiquitin ligase promotes injury-induced axon self-destruction. *Cell Rep,* 3**,** 1422-9.

BAKER, B. J., AKHTAR, L. N. & BENVENISTE, E. N. 2009. SOCS1 and SOCS3 in the control of CNS immunity. *Trends Immunol,* 30**,** 392-400.

BATES, K. L., HIGLEY, M. & LETSOU, A. 2008. Raw mediates antagonism of AP-1 activity in Drosophila. *Genetics,* 178**,** 1989-2002.

BAUER HUANG, S. L., SAHEKI, Y., VANHOVEN, M. K., TORAYAMA, I., ISHIHARA, T., KATSURA, I., VAN DER LINDEN, A., SENGUPTA, P. & BARGMANN, C. I. 2007. Left-right olfactory asymmetry results from antagonistic functions of voltage-activated calcium channels and the Raw repeat protein OLRN-1 in C. elegans. *Neural Dev,* 2**,** 24.

BENOWITZ, L. I. & POPOVICH, P. G. 2011. Inflammation and axon regeneration. *Curr Opin Neurol,* 24**,** 577-83.

BERGER, F., LAU, C., DAHLMANN, M. & ZIEGLER, M. 2005. Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. *J Biol Chem,* 280**,** 36334-41.

BLACKMORE, M. G. 2012. Molecular control of axon growth: insights from comparative gene profiling and high-throughput screening. *Int Rev Neurobiol,* 105**,** 39-70.
BLOOM, A. J., MILLER, B. R., SANES, J. R. & DIANTONIO, A. 2007. The requirement for Phr1 in CNS axon tract formation reveals the corticostriatal boundary as a choice point for cortical axons. *Genes Dev,* 21**,** 2593-606.
BOUNOUTAS, A., KRATZ, J., EMTAGE, L., MA, C., NGUYEN, K. C. & CHALFIE, M. 2011. Microtubule depolymerization in Caenorhabditis elegans touch receptor neurons reduces gene expression through a p38 MAPK pathway. *Proc Natl Acad Sci U S A,* 108**,** 3982-7.
BRACE, E. J. & DIANTONIO, A. 2016. Models of axon regeneration in Drosophila. *Exp Neurol*.
BUDNIK, V., KOH, Y. H., GUAN, B., HARTMANN, B., HOUGH, C., WOODS, D. & GORCZYCA, M. 1996. Regulation of synapse structure and function by the Drosophila tumor suppressor gene dlg. *Neuron,* 17**,** 627-40.
BYARS, C. L., BATES, K. L. & LETSOU, A. 1999. The dorsal-open group gene raw is required for restricted DJNK signaling during closure. *Development,* 126**,** 4913-23.
CAFFERTY, W. B., GARDINER, N. J., DAS, P., QIU, J., MCMAHON, S. B. & THOMPSON, S. W. 2004. Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. *J Neurosci,* 24**,** 4432-43.
CAI, D., QIU, J., CAO, Z., MCATEE, M., BREGMAN, B. S. & FILBIN, M. T. 2001. Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. *J Neurosci,* 21**,** 4731-9.
CAI, D., SHEN, Y., DE BELLARD, M., TANG, S. & FILBIN, M. T. 1999. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron,* 22**,** 89-101.
CAVALLI, V., KUJALA, P., KLUMPERMAN, J. & GOLDSTEIN, L. S. 2005. Sunday Driver links axonal transport to damage signaling. *J Cell Biol,* 168**,** 775-87.
CHEN, L., STONE, M. C., TAO, J. & ROLLS, M. M. 2012. Axon injury and stress trigger a microtubule-based neuroprotective pathway. *Proc Natl Acad Sci U S A,* 109**,** 11842-7.
CHEN, X. & GANETZKY, B. 2012. A neuropeptide signaling pathway regulates synaptic growth in Drosophila. *J Cell Biol,* 196**,** 529-43.
CHO, Y., SHIN, J. E., EWAN, E. E., OH, Y. M., PITA-THOMAS, W. & CAVALLI, V. 2015. Activating Injury-Responsive Genes with Hypoxia Enhances Axon Regeneration through Neuronal HIF-1alpha. *Neuron,* 88**,** 720-734.
CHO, Y., SLOUTSKY, R., NAEGLE, K. M. & CAVALLI, V. 2013. Injury-induced HDAC5 nuclear export is essential for axon regeneration. *Cell,* 155**,** 894-908.
CHUNG, S. H., AWAL, M. R., SHAY, J., MCLOED, M. M., MAZUR, E. & GABEL, C. V. 2016. Novel DLK-independent neuronal regeneration in Caenorhabditis elegans shares links with activity-dependent ectopic outgrowth. *Proc Natl Acad Sci U S A*.
COFFEY, E. T. 2014. Nuclear and cytosolic JNK signalling in neurons. *Nat Rev Neurosci,* 15**,** 285-99.
COLEMAN, M. P. & FREEMAN, M. R. 2010. Wallerian degeneration, wld(s), and nmnat. *Annu Rev Neurosci,* 33**,** 245-67.
COLLINS, C. A. & DIANTONIO, A. 2007. Synaptic development: insights from Drosophila. *Curr Opin Neurobiol,* 17**,** 35-42.
COLLINS, C. A., WAIRKAR, Y. P., JOHNSON, S. L. & DIANTONIO, A. 2006. Highwire restrains synaptic growth by attenuating a MAP kinase signal. *Neuron,* 51**,** 57-69.

CONFORTI, L., GILLEY, J. & COLEMAN, M. P. 2014. Wallerian degeneration: an emerging axon death pathway linking injury and disease. *Nat Rev Neurosci,* 15**,** 394-409.

CONFORTI, L., WILBREY, A., MORREALE, G., JANECKOVA, L., BEIROWSKI, B., ADALBERT, R., MAZZOLA, F., DI STEFANO, M., HARTLEY, R., BABETTO, E., SMITH, T., GILLEY, J., BILLINGTON, R. A., GENAZZANI, A. A., RIBCHESTER, R. R., MAGNI, G. & COLEMAN, M. 2009. Wld S protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice. *J Cell Biol,* 184**,** 491-500.

D'SOUZA, J., HENDRICKS, M., LE GUYADER, S., SUBBURAJU, S., GRUNEWALD, B., SCHOLICH, K. & JESUTHASAN, S. 2005. Formation of the retinotectal projection requires Esrom, an ortholog of PAM (protein associated with Myc). *Development,* 132**,** 247-56.

DEL CASTILLO, U., WINDING, M., LU, W. & GELFAND, V. I. 2015. Interplay between kinesin-1 and cortical dynein during axonal outgrowth and microtubule organization in Drosophila neurons. *Elife,* 4**,** e10140.

DELL'ACQUA, M. L., SMITH, K. E., GORSKI, J. A., HORNE, E. A., GIBSON, E. S. & GOMEZ, L. L. 2006. Regulation of neuronal PKA signaling through AKAP targeting dynamics. *Eur J Cell Biol,* 85**,** 627-33.

DIANTONIO, A., HAGHIGHI, A. P., PORTMAN, S. L., LEE, J. D., AMARANTO, A. M. & GOODMAN, C. S. 2001. Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature,* 412**,** 449-52.

DICKENDESHER, T. L., BALDWIN, K. T., MIRONOVA, Y. A., KORIYAMA, Y., RAIKER, S. J., ASKEW, K. L., WOOD, A., GEOFFROY, C. G., ZHENG, B., LIEPMANN, C. D., KATAGIRI, Y., BENOWITZ, L. I., GELLER, H. M. & GIGER, R. J. 2012. NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. *Nat Neurosci,* 15**,** 703-12.

DOMENICONI, M., ZAMPIERI, N., SPENCER, T., HILAIRE, M., MELLADO, W., CHAO, M. V. & FILBIN, M. T. 2005. MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron,* 46**,** 849-55.

DONNELLY, D. J. & POPOVICH, P. G. 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol,* 209**,** 378-88.

DURKIN, J. T., HOLSKIN, B. P., KOPEC, K. K., REED, M. S., SPAIS, C. M., STEFFY, B. M., GESSNER, G., ANGELES, T. S., POHL, J., ATOR, M. A. & MEYER, S. L. 2004. Phosphoregulation of mixed-lineage kinase 1 activity by multiple phosphorylation in the activation loop. *Biochemistry,* 43**,** 16348-55.

ERESH, S., RIESE, J., JACKSON, D. B., BOHMANN, D. & BIENZ, M. 1997. A CREB-binding site as a target for decapentaplegic signalling during Drosophila endoderm induction. *EMBO J,* 16**,** 2014-22.

FAGOE, N. D., ATTWELL, C. L., KOUWENHOVEN, D., VERHAAGEN, J. & MASON, M. R. 2015. Overexpression of ATF3 or the combination of ATF3, c-Jun, STAT3 and Smad1 promotes regeneration of the central axon branch of sensory neurons but without synergistic effects. *Hum Mol Genet,* 24**,** 6788-800.

FANG, Y. & BONINI, N. M. 2012. Axon degeneration and regeneration: insights from Drosophila models of nerve injury. *Annu Rev Cell Dev Biol,* 28**,** 575-97.

FANG, Y., SOARES, L., TENG, X., GEARY, M. & BONINI, N. M. 2012. A novel Drosophila model of nerve injury reveals an essential role of Nmnat in maintaining axonal integrity. *Curr Biol,* 22**,** 590-5.

FAWCETT, J. W. & ASHER, R. A. 1999. The glial scar and central nervous system repair. *Brain Res Bull,* 49**,** 377-91.
FAWCETT, J. W., SCHWAB, M. E., MONTANI, L., BRAZDA, N. & MULLER, H. W. 2012. Defeating inhibition of regeneration by scar and myelin components. *Handb Clin Neurol,* 109**,** 503-22.
FILBIN, M. T. 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci,* 4**,** 703-13.
FINN, J. T., WEIL, M., ARCHER, F., SIMAN, R., SRINIVASAN, A. & RAFF, M. C. 2000. Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. *J Neurosci,* 20**,** 1333-41.
FISHER, D., XING, B., DILL, J., LI, H., HOANG, H. H., ZHAO, Z., YANG, X. L., BACHOO, R., CANNON, S., LONGO, F. M., SHENG, M., SILVER, J. & LI, S. 2011. Leukocyte common antigen-related phosphatase is a functional receptor for chondroitin sulfate proteoglycan axon growth inhibitors. *J Neurosci,* 31**,** 14051-66.
FREY, E., VALAKH, V., KARNEY-GROBE, S., SHI, Y., MILBRANDT, J. & DIANTONIO, A. 2015. An in vitro assay to study induction of the regenerative state in sensory neurons. *Exp Neurol,* 263**,** 350-63.
FUJIOKA, M., LEAR, B. C., LANDGRAF, M., YUSIBOVA, G. L., ZHOU, J., RILEY, K. M., PATEL, N. H. & JAYNES, J. B. 2003. Even-skipped, acting as a repressor, regulates axonal projections in Drosophila. *Development,* 130**,** 5385-400.
GALTREY, C. M. & FAWCETT, J. W. 2007. The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Res Rev,* 54**,** 1-18.
GAO, Y., DENG, K., HOU, J., BRYSON, J. B., BARCO, A., NIKULINA, E., SPENCER, T., MELLADO, W., KANDEL, E. R. & FILBIN, M. T. 2004. Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. *Neuron,* 44**,** 609-21.
GAO, Y., NIKULINA, E., MELLADO, W. & FILBIN, M. T. 2003. Neurotrophins elevate cAMP to reach a threshold required to overcome inhibition by MAG through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase. *J Neurosci,* 23**,** 11770-7.
GEEVEN, G., MACGILLAVRY, H. D., EGGERS, R., SASSEN, M. M., VERHAAGEN, J., SMIT, A. B., DE GUNST, M. C. & VAN KESTEREN, R. E. 2011. LLM3D: a log-linear modeling-based method to predict functional gene regulatory interactions from genome-wide expression data. *Nucleic Acids Res,* 39**,** 5313-27.
GEOFFROY, C. G. & ZHENG, B. 2014. Myelin-associated inhibitors in axonal growth after CNS injury. *Curr Opin Neurobiol,* 27**,** 31-8.
GERDTS, J., BRACE, E. J., SASAKI, Y., DIANTONIO, A. & MILBRANDT, J. 2015. SARM1 activation triggers axon degeneration locally via NAD(+) destruction. *Science,* 348**,** 453-7.
GERDTS, J., SUMMERS, D. W., SASAKI, Y., DIANTONIO, A. & MILBRANDT, J. 2013. Sarm1-mediated axon degeneration requires both SAM and TIR interactions. *J Neurosci,* 33**,** 13569-80.
GHOSH-ROY, A., GONCHAROV, A., JIN, Y. & CHISHOLM, A. D. 2012. Kinesin-13 and tubulin posttranslational modifications regulate microtubule growth in axon regeneration. *Dev Cell,* 23**,** 716-28.

GHOSH-ROY, A., WU, Z., GONCHAROV, A., JIN, Y. & CHISHOLM, A. D. 2010. Calcium and cyclic AMP promote axonal regeneration in Caenorhabditis elegans and require DLK-1 kinase. *J Neurosci,* 30**,** 3175-83.

GHOSH, A. S., WANG, B., POZNIAK, C. D., CHEN, M., WATTS, R. J. & LEWCOCK, J. W. 2011. DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity. *J Cell Biol,* 194**,** 751-64.

GILLEY, J., ADALBERT, R., YU, G. & COLEMAN, M. P. 2013. Rescue of peripheral and CNS axon defects in mice lacking NMNAT2. *J Neurosci,* 33**,** 13410-24.

GILLEY, J. & COLEMAN, M. P. 2010. Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. *PLoS Biol,* 8**,** e1000300.

GILLEY, J., ORSOMANDO, G., NASCIMENTO-FERREIRA, I. & COLEMAN, M. P. 2015. Absence of SARM1 rescues development and survival of NMNAT2-deficient axons. *Cell Rep,* 10**,** 1974-81.

GORDON, T. 2009. The role of neurotrophic factors in nerve regeneration. *Neurosurg Focus,* 26**,** E3.

GRUEBER, W. B., YE, B., MOORE, A. W., JAN, L. Y. & JAN, Y. N. 2003. Dendrites of distinct classes of Drosophila sensory neurons show different capacities for homotypic repulsion. *Curr Biol,* 13**,** 618-26.

HAMMARLUND, M., NIX, P., HAUTH, L., JORGENSEN, E. M. & BASTIANI, M. 2009. Axon regeneration requires a conserved MAP kinase pathway. *Science,* 323**,** 802-6.

HAN, J. D., BAKER, N. E. & RUBIN, C. S. 1997. Molecular characterization of a novel A kinase anchor protein from Drosophila melanogaster. *J Biol Chem,* 272**,** 26611-9.

HANNILA, S. S. & FILBIN, M. T. 2008. The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. *Exp Neurol,* 209**,** 321-32.

HAO, Y., FREY, E., YOON, C., WONG, H., NESTOROVSKI, D., HOLZMAN, L. B., GIGER, R. J., DIANTONIO, A. & COLLINS, C. 2016. An evolutionarily conserved mechanism for cAMP elicited axonal regeneration involves direct activation of the dual leucine zipper kinase DLK. *Elife,* 5.

HARVEY, A. R., OOI, J. W. & RODGER, J. 2012. Neurotrophic factors and the regeneration of adult retinal ganglion cell axons. *Int Rev Neurobiol,* 106**,** 1-33.

HILL, S. E., PARMAR, M., GHERES, K. W., GUIGNET, M. A., HUANG, Y., JACKSON, F. R. & ROLLS, M. M. 2012. Development of dendrite polarity in Drosophila neurons. *Neural Dev,* 7**,** 34.

HIRAI, S., CUI DE, F., MIYATA, T., OGAWA, M., KIYONARI, H., SUDA, Y., AIZAWA, S., BANBA, Y. & OHNO, S. 2006. The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. *J Neurosci,* 26**,** 11992-2002.

HOLLAND, S. M., COLLURA, K. M., KETSCHEK, A., NOMA, K., FERGUSON, T. A., JIN, Y., GALLO, G. & THOMAS, G. M. 2016. Palmitoylation controls DLK localization, interactions and activity to ensure effective axonal injury signaling. *Proc Natl Acad Sci U S A,* 113**,** 763-8.

HOLLIS, E. R., 2ND & ZOU, Y. 2012. Reinduced Wnt signaling limits regenerative potential of sensory axons in the spinal cord following conditioning lesion. *Proc Natl Acad Sci U S A,* 109**,** 14663-8.

HOLZMAN, L. B., MERRITT, S. E. & FAN, G. 1994. Identification, molecular cloning, and characterization of dual leucine zipper bearing kinase. A novel serine/threonine protein

kinase that defines a second subfamily of mixed lineage kinases. *J Biol Chem,* 269**,** 30808-17.

HU, Y., PARK, K. K., YANG, L., WEI, X., YANG, Q., CHO, K. S., THIELEN, P., LEE, A. H., CARTONI, R., GLIMCHER, L. H., CHEN, D. F. & HE, Z. 2012. Differential effects of unfolded protein response pathways on axon injury-induced death of retinal ganglion cells. *Neuron,* 73**,** 445-52.

HUBER, A. B., WEINMANN, O., BROSAMLE, C., OERTLE, T. & SCHWAB, M. E. 2002. Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. *J Neurosci,* 22**,** 3553-67.

HUNTWORK-RODRIGUEZ, S., WANG, B., WATKINS, T., GHOSH, A. S., POZNIAK, C. D., BUSTOS, D., NEWTON, K., KIRKPATRICK, D. S. & LEWCOCK, J. W. 2013. JNK-mediated phosphorylation of DLK suppresses its ubiquitination to promote neuronal apoptosis. *J Cell Biol,* 202**,** 747-63.

ISHIHARA, N., NOMURA, M., JOFUKU, A., KATO, H., SUZUKI, S. O., MASUDA, K., OTERA, H., NAKANISHI, Y., NONAKA, I., GOTO, Y., TAGUCHI, N., MORINAGA, H., MAEDA, M., TAKAYANAGI, R., YOKOTA, S. & MIHARA, K. 2009. Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. *Nat Cell Biol,* 11**,** 958-66.

ITOH, A., HORIUCHI, M., WAKAYAMA, K., XU, J., BANNERMAN, P., PLEASURE, D. & ITOH, T. 2011. ZPK/DLK, a mitogen-activated protein kinase kinase kinase, is a critical mediator of programmed cell death of motoneurons. *J Neurosci,* 31**,** 7223-8.

JACK, J. & MYETTE, G. 1997. The genes raw and ribbon are required for proper shape of tubular epithelial tissues in Drosophila. *Genetics,* 147**,** 243-53.

JACKSON, S. M. & BERG, C. A. 2002. An A-kinase anchoring protein is required for protein kinase A regulatory subunit localization and morphology of actin structures during oogenesis in Drosophila. *Development,* 129**,** 4423-33.

JEMC, J. C., MILUTINOVICH, A. B., WEYERS, J. J., TAKEDA, Y. & VAN DOREN, M. 2012. raw Functions through JNK signaling and cadherin-based adhesion to regulate Drosophila gonad morphogenesis. *Dev Biol,* 367**,** 114-25.

KOSMACZEWSKI, S. G., EDWARDS, T. J., HAN, S. M., ECKWAHL, M. J., MEYER, B. I., PEACH, S., HESSELBERTH, J. R., WOLIN, S. L. & HAMMARLUND, M. 2014. The RtcB RNA ligase is an essential component of the metazoan unfolded protein response. *EMBO Rep,* 15**,** 1278-85.

KOSMACZEWSKI, S. G., HAN, S. M., HAN, B., IRVING MEYER, B., BAIG, H. S., ATHAR, W., LIN-MOORE, A. T., KOELLE, M. R. & HAMMARLUND, M. 2015. RNA ligation in neurons by RtcB inhibits axon regeneration. *Proc Natl Acad Sci U S A,* 112**,** 8451-6.

KUO, C. T., JAN, L. Y. & JAN, Y. N. 2005. Dendrite-specific remodeling of Drosophila sensory neurons requires matrix metalloproteases, ubiquitin-proteasome, and ecdysone signaling. *Proc Natl Acad Sci U S A,* 102**,** 15230-5.

KURUP, N., YAN, D., GONCHAROV, A. & JIN, Y. 2015. Dynamic microtubules drive circuit rewiring in the absence of neurite remodeling. *Curr Biol,* 25**,** 1594-605.

KWON, M. J., SHIN, H. Y., CUI, Y., KIM, H., THI, A. H., CHOI, J. Y., KIM, E. Y., HWANG, D. H. & KIM, B. G. 2015. CCL2 Mediates Neuron-Macrophage Interactions to Drive Proregenerative Macrophage Activation Following Preconditioning Injury. *J Neurosci,* 35**,** 15934-47.

LAUNDRIE, B., PETERSON, J. S., BAUM, J. S., CHANG, J. C., FILEPPO, D., THOMPSON, S. R. & MCCALL, K. 2003. Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in Drosophila. *Genetics,* 165**,** 1881-8.
LEE, D. 2015. Global and local missions of cAMP signaling in neural plasticity, learning, and memory. *Front Pharmacol,* 6**,** 161.
LEE, J., PENG, Y., LIN, W. Y. & PARRISH, J. Z. 2015. Coordinate control of terminal dendrite patterning and dynamics by the membrane protein Raw. *Development,* 142**,** 162-73.
LEE, J. K. & ZHENG, B. 2012. Role of myelin-associated inhibitors in axonal repair after spinal cord injury. *Exp Neurol,* 235**,** 33-42.
LEUNG, I. W. & LASSAM, N. 2001. The kinase activation loop is the key to mixed lineage kinase-3 activation via both autophosphorylation and hematopoietic progenitor kinase 1 phosphorylation. *J Biol Chem,* 276**,** 1961-7.
LEWCOCK, J. W., GENOUD, N., LETTIERI, K. & PFAFF, S. L. 2007. The ubiquitin ligase Phr1 regulates axon outgrowth through modulation of microtubule dynamics. *Neuron,* 56**,** 604-20.
LI, C., HISAMOTO, N. & MATSUMOTO, K. 2015. Axon Regeneration Is Regulated by Ets-C/EBP Transcription Complexes Generated by Activation of the cAMP/Ca2+ Signaling Pathways. *PLoS Genet,* 11**,** e1005603.
LI, C., HISAMOTO, N., NIX, P., KANAO, S., MIZUNO, T., BASTIANI, M. & MATSUMOTO, K. 2012. The growth factor SVH-1 regulates axon regeneration in C. elegans via the JNK MAPK cascade. *Nat Neurosci,* 15**,** 551-7.
LI, W., OHLMEYER, J. T., LANE, M. E. & KALDERON, D. 1995. Function of protein kinase A in hedgehog signal transduction and Drosophila imaginal disc development. *Cell,* 80**,** 553-62.
LINDWALL, C. & KANJE, M. 2005. Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. *Mol Cell Neurosci,* 29**,** 269-82.
LIU, K., TEDESCHI, A., PARK, K. K. & HE, Z. 2011. Neuronal intrinsic mechanisms of axon regeneration. *Annu Rev Neurosci,* 34**,** 131-52.
LU, W., LAKONISHOK, M. & GELFAND, V. I. 2015. Kinesin-1-powered microtubule sliding initiates axonal regeneration in Drosophila cultured neurons. *Mol Biol Cell,* 26**,** 1296-307.
LU, Y., LU, Y. S., SHUAI, Y., FENG, C., TULLY, T., XIE, Z., ZHONG, Y. & ZHOU, H. M. 2007. The AKAP Yu is required for olfactory long-term memory formation in Drosophila. *Proc Natl Acad Sci U S A,* 104**,** 13792-7.
MA, T. C., BARCO, A., RATAN, R. R. & WILLIS, D. E. 2014. cAMP-responsive element-binding protein (CREB) and cAMP co-regulate activator protein 1 (AP1)-dependent regeneration-associated gene expression and neurite growth. *J Biol Chem,* 289**,** 32914-25.
MA, T. C. & WILLIS, D. E. 2015. What makes a RAG regeneration associated? *Front Mol Neurosci,* 8**,** 43.
MASON, M. R., LIEBERMAN, A. R., GRENNINGLOH, G. & ANDERSON, P. N. 2002. Transcriptional upregulation of SCG10 and CAP-23 is correlated with regeneration of the axons of peripheral and central neurons in vivo. *Mol Cell Neurosci,* 20**,** 595-615.
MASSARO, C. M., PIELAGE, J. & DAVIS, G. W. 2009. Molecular mechanisms that enhance synapse stability despite persistent disruption of the spectrin/ankyrin/microtubule cytoskeleton. *J Cell Biol,* 187**,** 101-17.

MATA, M., MERRITT, S. E., FAN, G., YU, G. G. & HOLZMAN, L. B. 1996. Characterization of dual leucine zipper-bearing kinase, a mixed lineage kinase present in synaptic terminals whose phosphorylation state is regulated by membrane depolarization via calcineurin. *Journal of Biological Chemistry,* 271**,** 16888-16896.

MATHIASEN, J. R., MCKENNA, B. A., SAPORITO, M. S., GHADGE, G. D., ROOS, R. P., HOLSKIN, B. P., WU, Z. L., TRUSKO, S. P., CONNORS, T. C., MARONEY, A. C., THOMAS, B. A., THOMAS, J. C. & BOZYCZKO-COYNE, D. 2004. Inhibition of mixed lineage kinase 3 attenuates MPP+-induced neurotoxicity in SH-SY5Y cells. *Brain Res,* 1003**,** 86-97.

MERRILL, R. A., DAGDA, R. K., DICKEY, A. S., CRIBBS, J. T., GREEN, S. H., USACHEV, Y. M. & STRACK, S. 2011. Mechanism of neuroprotective mitochondrial remodeling by PKA/AKAP1. *PLoS Biol,* 9**,** e1000612.

MERRITT, S. E., MATA, M., NIHALANI, D., ZHU, C., HU, X. & HOLZMAN, L. B. 1999. The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate. *J Biol Chem,* 274**,** 10195-202.

MILDE, S., GILLEY, J. & COLEMAN, M. P. 2013. Subcellular localization determines the stability and axon protective capacity of axon survival factor Nmnat2. *PLoS Biol,* 11**,** e1001539.

MILLER, B. R., PRESS, C., DANIELS, R. W., SASAKI, Y., MILBRANDT, J. & DIANTONIO, A. 2009. A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. *Nat Neurosci,* 12**,** 387-9.

MISHRA, B., CARSON, R., HUME, R. I. & COLLINS, C. A. 2013. Sodium and potassium currents influence Wallerian degeneration of injured Drosophila axons. *J Neurosci,* 33**,** 18728-39.

MORGAN, J. I., COHEN, D. R., HEMPSTEAD, J. L. & CURRAN, T. 1987. Mapping patterns of c-fos expression in the central nervous system after seizure. *Science,* 237**,** 192-7.

MUKHOPADHYAY, G., DOHERTY, P., WALSH, F. S., CROCKER, P. R. & FILBIN, M. T. 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron,* 13**,** 757-67.

NAKATA, K., ABRAMS, B., GRILL, B., GONCHAROV, A., HUANG, X., CHISHOLM, A. D. & JIN, Y. 2005. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell,* 120**,** 407-20.

NEUKOMM, L. J., BURDETT, T. C., GONZALEZ, M. A., ZUCHNER, S. & FREEMAN, M. R. 2014. Rapid in vivo forward genetic approach for identifying axon death genes in Drosophila. *Proc Natl Acad Sci U S A,* 111**,** 9965-70.

NEUKOMM, L. J. & FREEMAN, M. R. 2014. Diverse cellular and molecular modes of axon degeneration. *Trends Cell Biol,* 24**,** 515-23.

NEUMANN, S., BRADKE, F., TESSIER-LAVIGNE, M. & BASBAUM, A. I. 2002. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron,* 34**,** 885-93.

NEUMANN, S. & WOOLF, C. J. 1999. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron,* 23**,** 83-91.

NIEMI, J. P., DEFRANCESCO-LISOWITZ, A., CREGG, J. M., HOWARTH, M. & ZIGMOND, R. E. 2016. Overexpression of the monocyte chemokine CCL2 in dorsal root ganglion neurons causes a conditioning-like increase in neurite outgrowth and does so via a STAT3 dependent mechanism. *Exp Neurol,* 275 Pt 1**,** 25-37.

NIHALANI, D., MERRITT, S. & HOLZMAN, L. B. 2000. Identification of structural and functional domains in mixed lineage kinase dual leucine zipper-bearing kinase required for complex formation and stress-activated protein kinase activation. *J Biol Chem,* 275**,** 7273-9.

OHTAKE, Y. & LI, S. 2015. Molecular mechanisms of scar-sourced axon growth inhibitors. *Brain Res,* 1619**,** 22-35.

OMURA, T., OMURA, K., TEDESCHI, A., RIVA, P., PAINTER, M. W., ROJAS, L., MARTIN, J., LISI, V., HUEBNER, E. A., LATREMOLIERE, A., YIN, Y., BARRETT, L. B., SINGH, B., LEE, S., CRISMAN, T., GAO, F., LI, S., KAPUR, K., GESCHWIND, D. H., KOSIK, K. S., COPPOLA, G., HE, Z., CARMICHAEL, S. T., BENOWITZ, L. I., COSTIGAN, M. & WOOLF, C. J. 2015. Robust Axonal Regeneration Occurs in the Injured CAST/Ei Mouse CNS. *Neuron,* 86**,** 1215-27.

ONATE, M., CATENACCIO, A., MARTINEZ, G., ARMENTANO, D., PARSONS, G., KERR, B., HETZ, C. & COURT, F. A. 2016. Activation of the unfolded protein response promotes axonal regeneration after peripheral nerve injury. *Sci Rep,* 6**,** 21709.

OSTERLOH, J. M., YANG, J., ROONEY, T. M., FOX, A. N., ADALBERT, R., POWELL, E. H., SHEEHAN, A. E., AVERY, M. A., HACKETT, R., LOGAN, M. A., MACDONALD, J. M., ZIEGENFUSS, J. S., MILDE, S., HOU, Y. J., NATHAN, C., DING, A., BROWN, R. H., JR., CONFORTI, L., COLEMAN, M., TESSIER-LAVIGNE, M., ZUCHNER, S. & FREEMAN, M. R. 2012. dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. *Science,* 337**,** 481-4.

PARK, K. K., LIU, K., HU, Y., SMITH, P. D., WANG, C., CAI, B., XU, B., CONNOLLY, L., KRAMVIS, I., SAHIN, M. & HE, Z. 2008. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science,* 322**,** 963-6.

PIERRE, S. C., HAUSLER, J., BIROD, K., GEISSLINGER, G. & SCHOLICH, K. 2004. PAM mediates sustained inhibition of cAMP signaling by sphingosine-1-phosphate. *EMBO J,* 23**,** 3031-40.

POZNIAK, C. D., SENGUPTA GHOSH, A., GOGINENI, A., HANSON, J. E., LEE, S. H., LARSON, J. L., SOLANOY, H., BUSTOS, D., LI, H., NGU, H., JUBB, A. M., AYALON, G., WU, J., SCEARCE-LEVIE, K., ZHOU, Q., WEIMER, R. M., KIRKPATRICK, D. S. & LEWCOCK, J. W. 2013. Dual leucine zipper kinase is required for excitotoxicity-induced neuronal degeneration. *J Exp Med,* 210**,** 2553-67.

PURICE, M. D., SPEESE, S. D. & LOGAN, M. A. 2016. Delayed glial clearance of degenerating axons in aged Drosophila is due to reduced PI3K/Draper activity. *Nat Commun,* 7**,** 12871.

QIU, J., CAFFERTY, W. B., MCMAHON, S. B. & THOMPSON, S. W. 2005. Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. *J Neurosci,* 25**,** 1645-53.

QIU, J., CAI, D., DAI, H., MCATEE, M., HOFFMAN, P. N., BREGMAN, B. S. & FILBIN, M. T. 2002. Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron,* 34**,** 895-903.

RAIKER, S. J., LEE, H., BALDWIN, K. T., DUAN, Y., SHRAGER, P. & GIGER, R. J. 2010. Oligodendrocyte-myelin glycoprotein and Nogo negatively regulate activity-dependent synaptic plasticity. *J Neurosci,* 30**,** 12432-45.

RAIVICH, G., BOHATSCHEK, M., DA COSTA, C., IWATA, O., GALIANO, M., HRISTOVA, M., NATERI, A. S., MAKWANA, M., RIERA-SANS, L., WOLFER, D. P., LIPP, H. P.,

AGUZZI, A., WAGNER, E. F. & BEHRENS, A. 2004. The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron,* 43**,** 57-67.
RAO, K., STONE, M. C., WEINER, A. T., GHERES, K. W., ZHOU, C., DEITCHER, D. L., LEVITAN, E. S. & ROLLS, M. M. 2016. Spastin, atlastin and ER relocalization are involved in axon, but not dendrite, regeneration. *Mol Biol Cell*.
REILLY, M. M., MURPHY, S. M. & LAURA, M. 2011. Charcot-Marie-Tooth disease. *J Peripher Nerv Syst,* 16**,** 1-14.
RICHARDSON, P. M. & ISSA, V. M. 1984. Peripheral injury enhances central regeneration of primary sensory neurones. *Nature,* 309**,** 791-3.
RICHARDSON, P. M. & LU, X. 1994. Inflammation and axonal regeneration. *J Neurol,* 242**,** S57-60.
RITZENTHALER, S., SUZUKI, E. & CHIBA, A. 2000. Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. *Nat Neurosci,* 3**,** 1012-7.
ROONEY, T. M. & FREEMAN, M. R. 2014. Drosophila models of neuronal injury. *ILAR J,* 54**,** 291-5.
SANDVIG, A., BERRY, M., BARRETT, L. B., BUTT, A. & LOGAN, A. 2004. Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. *Glia,* 46**,** 225-51.
SANYAL, S. 2009. Genomic mapping and expression patterns of C380, OK6 and D42 enhancer trap lines in the larval nervous system of Drosophila. *Gene Expr Patterns,* 9**,** 371-80.
SASAKI, Y., NAKAGAWA, T., MAO, X., DIANTONIO, A. & MILBRANDT, J. 2016. NMNAT1 inhibits axon degeneration via blockade of SARM1-mediated NAD+ depletion. *Elife,* 5.
SASAKI, Y., VOHRA, B. P., BALOH, R. H. & MILBRANDT, J. 2009. Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. *J Neurosci,* 29**,** 6526-34.
SCHREYER, D. J. & SKENE, J. H. 1993. Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *J Neurobiol,* 24**,** 959-70.
SCHWEIGER, M., HENNIG, K., LERNER, F., NIERE, M., HIRSCH-KAUFFMANN, M., SPECHT, T., WEISE, C., OEI, S. L. & ZIEGLER, M. 2001. Characterization of recombinant human nicotinamide mononucleotide adenylyl transferase (NMNAT), a nuclear enzyme essential for NAD synthesis. *FEBS Lett,* 492**,** 95-100.
SHAMLOULA, H. K., MBOGHO, M. P., PIMENTEL, A. C., CHRZANOWSKA-LIGHTOWLERS, Z. M., HYATT, V., OKANO, H. & VENKATESH, T. R. 2002. rugose (rg), a Drosophila A kinase anchor protein, is required for retinal pattern formation and interacts genetically with multiple signaling pathways. *Genetics,* 161**,** 693-710.
SHERIDAN, C. & MARTIN, S. J. 2010. Mitochondrial fission/fusion dynamics and apoptosis. *Mitochondrion,* 10**,** 640-8.
SHIN, J. E., CHO, Y., BEIROWSKI, B., MILBRANDT, J., CAVALLI, V. & DIANTONIO, A. 2012a. Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. *Neuron,* 74**,** 1015-22.
SHIN, J. E. & DIANTONIO, A. 2011. Highwire regulates guidance of sister axons in the Drosophila mushroom body. *J Neurosci,* 31**,** 17689-700.

SHIN, J. E., MILLER, B. R., BABETTO, E., CHO, Y., SASAKI, Y., QAYUM, S., RUSSLER, E. V., CAVALLI, V., MILBRANDT, J. & DIANTONIO, A. 2012b. SCG10 is a JNK target in the axonal degeneration pathway. *Proc Natl Acad Sci U S A,* 109**,** E3696-705.
SHUAI, K. & LIU, B. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol,* 3**,** 900-11.
SHUTT, T. E. & MCBRIDE, H. M. 2013. Staying cool in difficult times: mitochondrial dynamics, quality control and the stress response. *Biochim Biophys Acta,* 1833**,** 417-24.
SILVER, J., SCHWAB, M. E. & POPOVICH, P. G. 2015. Central nervous system regenerative failure: role of oligodendrocytes, astrocytes, and microglia. *Cold Spring Harb Perspect Biol,* 7**,** a020602.
SIMON, D. J., WEIMER, R. M., MCLAUGHLIN, T., KALLOP, D., STANGER, K., YANG, J., O'LEARY, D. D., HANNOUSH, R. N. & TESSIER-LAVIGNE, M. 2012. A caspase cascade regulating developmental axon degeneration. *J Neurosci,* 32**,** 17540-53.
SMITH, P. D., SUN, F., PARK, K. K., CAI, B., WANG, C., KUWAKO, K., MARTINEZ-CARRASCO, I., CONNOLLY, L. & HE, Z. 2009. SOCS3 deletion promotes optic nerve regeneration in vivo. *Neuron,* 64**,** 617-23.
SOARES, L., PARISI, M. & BONINI, N. M. 2014. Axon injury and regeneration in the adult Drosophila. *Sci Rep,* 4**,** 6199.
SONG, Y., ORI-MCKENNEY, K. M., ZHENG, Y., HAN, C., JAN, L. Y. & JAN, Y. N. 2012. Regeneration of Drosophila sensory neuron axons and dendrites is regulated by the Akt pathway involving Pten and microRNA bantam. *Genes Dev,* 26**,** 1612-25.
SONG, Y., SRETAVAN, D., SALEGIO, E. A., BERG, J., HUANG, X., CHENG, T., XIONG, X., MELTZER, S., HAN, C., NGUYEN, T. T., BRESNAHAN, J. C., BEATTIE, M. S., JAN, L. Y. & JAN, Y. N. 2015. Regulation of axon regeneration by the RNA repair and splicing pathway. *Nat Neurosci,* 18**,** 817-25.
SPIRA, M. E., OREN, R., DORMANN, A., ILOUZ, N. & LEV, S. 2001. Calcium, protease activation, and cytoskeleton remodeling underlie growth cone formation and neuronal regeneration. *Cell Mol Neurobiol,* 21**,** 591-604.
STARKEY, M. L., DAVIES, M., YIP, P. K., CARTER, L. M., WONG, D. J., MCMAHON, S. B. & BRADBURY, E. J. 2009. Expression of the regeneration-associated protein SPRR1A in primary sensory neurons and spinal cord of the adult mouse following peripheral and central injury. *J Comp Neurol,* 513**,** 51-68.
STONE, M. C., ALBERTSON, R. M., CHEN, L. & ROLLS, M. M. 2014. Dendrite injury triggers DLK-independent regeneration. *Cell Rep,* 6**,** 247-53.
STONE, M. C., NGUYEN, M. M., TAO, J., ALLENDER, D. L. & ROLLS, M. M. 2010. Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. *Mol Biol Cell,* 21**,** 767-77.
STONE, M. C., RAO, K., GHERES, K. W., KIM, S., TAO, J., LA ROCHELLE, C., FOLKER, C. T., SHERWOOD, N. T. & ROLLS, M. M. 2012. Normal spastin gene dosage is specifically required for axon regeneration. *Cell Rep,* 2**,** 1340-50.
STONE, M. C., ROEGIERS, F. & ROLLS, M. M. 2008. Microtubules have opposite orientation in axons and dendrites of Drosophila neurons. *Mol Biol Cell,* 19**,** 4122-9.
SUN, F. & HE, Z. 2010. Neuronal intrinsic barriers for axon regeneration in the adult CNS. *Curr Opin Neurobiol,* 20**,** 510-8.

SUN, F., PARK, K. K., BELIN, S., WANG, D., LU, T., CHEN, G., ZHANG, K., YEUNG, C., FENG, G., YANKNER, B. A. & HE, Z. 2011. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. *Nature,* 480**,** 372-5.

SUN, L., SHAY, J., MCLOED, M., ROODHOUSE, K., CHUNG, S. H., CLARK, C. M., PIRRI, J. K., ALKEMA, M. J. & GABEL, C. V. 2014. Neuronal regeneration in C. elegans requires subcellular calcium release by ryanodine receptor channels and can be enhanced by optogenetic stimulation. *J Neurosci,* 34**,** 15947-56.

TAKAGISHI, Y., KATANOSAKA, K., MIZOGUCHI, H. & MURATA, Y. 2016. Disrupted axon-glia interactions at the paranode in myelinated nerves cause axonal degeneration and neuronal cell death in the aged Caspr mutant mouse shambling. *Neurobiol Aging,* 43**,** 34-46.

TAKEI, K., SHIN, R. M., INOUE, T., KATO, K. & MIKOSHIBA, K. 1998. Regulation of nerve growth mediated by inositol 1,4,5-trisphosphate receptors in growth cones. *Science,* 282**,** 1705-8.

TAO, J., FENG, C. & ROLLS, M. M. 2016. The microtubule-severing protein fidgetin acts after dendrite injury to promote their degeneration. *J Cell Sci,* 129**,** 3274-81.

TAO, J. & ROLLS, M. M. 2011. Dendrites have a rapid program of injury-induced degeneration that is molecularly distinct from developmental pruning. *J Neurosci,* 31**,** 5398-405.

TASKEN, K. & AANDAHL, E. M. 2004. Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol Rev,* 84**,** 137-67.

TEDESCHI, A. & BRADKE, F. 2013. The DLK signalling pathway--a double-edged sword in neural development and regeneration. *EMBO Rep,* 14**,** 605-14.

TERMAN, J. R. & KOLODKIN, A. L. 2004. Nervy links protein kinase a to plexin-mediated semaphorin repulsion. *Science,* 303**,** 1204-7.

THOMPSON-PEER, K. L., DEVAULT, L., LI, T., JAN, L. Y. & JAN, Y. N. 2016. In vivo dendrite regeneration after injury is different from dendrite development. *Genes Dev,* 30**,** 1776-89.

TUVIKENE, J., PRUUNSILD, P., ORAV, E., ESVALD, E. E. & TIMMUSK, T. 2016. AP-1 Transcription Factors Mediate BDNF-Positive Feedback Loop in Cortical Neurons. *J Neurosci,* 36**,** 1290-305.

VALAKH, V., FREY, E., BABETTO, E., WALKER, L. J. & DIANTONIO, A. 2015. Cytoskeletal disruption activates the DLK/JNK pathway, which promotes axonal regeneration and mimics a preconditioning injury. *Neurobiol Dis,* 77**,** 13-25.

VALAKH, V., WALKER, L. J., SKEATH, J. B. & DIANTONIO, A. 2013. Loss of the spectraplakin short stop activates the DLK injury response pathway in Drosophila. *J Neurosci,* 33**,** 17863-73.

VOELZMANN, A., OKENVE-RAMOS, P., QU, Y., CHOJNOWSKA-MONGA, M., DEL CANO-ESPINEL, M., PROKOP, A. & SANCHEZ-SORIANO, N. 2016. Tau and spectraplakins promote synapse formation and maintenance through Jun kinase and neuronal trafficking. *Elife,* 5.

VONHOFF, F., KUEHN, C., BLUMENSTOCK, S., SANYAL, S. & DUCH, C. 2013. Temporal coherency between receptor expression, neural activity and AP-1-dependent transcription regulates Drosophila motoneuron dendrite development. *Development,* 140**,** 606-16.

WAN, H. I., DIANTONIO, A., FETTER, R. D., BERGSTROM, K., STRAUSS, R. & GOODMAN, C. S. 2000. Highwire regulates synaptic growth in Drosophila. *Neuron,* 26**,** 313-29.

WANG, J. T., MEDRESS, Z. A. & BARRES, B. A. 2012a. Axon degeneration: molecular mechanisms of a self-destruction pathway. *J Cell Biol,* 196**,** 7-18.
WANG, X., KIM, J. H., BAZZI, M., ROBINSON, S., COLLINS, C. A. & YE, B. 2013. Bimodal control of dendritic and axonal growth by the dual leucine zipper kinase pathway. *PLoS Biol,* 11**,** e1001572.
WANG, Z., JIANG, H., CHEN, S., DU, F. & WANG, X. 2012b. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell,* 148**,** 228-43.
WATERHAM, H. R., KOSTER, J., VAN ROERMUND, C. W., MOOYER, P. A., WANDERS, R. J. & LEONARD, J. V. 2007. A lethal defect of mitochondrial and peroxisomal fission. *N Engl J Med,* 356**,** 1736-41.
WATKINS, T. A., WANG, B., HUNTWORK-RODRIGUEZ, S., YANG, J., JIANG, Z., EASTHAM-ANDERSON, J., MODRUSAN, Z., KAMINKER, J. S., TESSIER-LAVIGNE, M. & LEWCOCK, J. W. 2013. DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. *Proc Natl Acad Sci U S A,* 110**,** 4039-44.
WEBER, U., PARICIO, N. & MLODZIK, M. 2000. Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. *Development,* 127**,** 3619-29.
WELSBIE, D. S., YANG, Z., GE, Y., MITCHELL, K. L., ZHOU, X., MARTIN, S. E., BERLINICKE, C. A., HACKLER, L., JR., FULLER, J., FU, J., CAO, L. H., HAN, B., AULD, D., XUE, T., HIRAI, S., GERMAIN, L., SIMARD-BISSON, C., BLOUIN, R., NGUYEN, J. V., DAVIS, C. H., ENKE, R. A., BOYE, S. L., MERBS, S. L., MARSH-ARMSTRONG, N., HAUSWIRTH, W. W., DIANTONIO, A., NICKELLS, R. W., INGLESE, J., HANES, J., YAU, K. W., QUIGLEY, H. A. & ZACK, D. J. 2013. Functional genomic screening identifies dual leucine zipper kinase as a key mediator of retinal ganglion cell death. *Proc Natl Acad Sci U S A,* 110**,** 4045-50.
WILLS, Z. P., MANDEL-BREHM, C., MARDINLY, A. R., MCCORD, A. E., GIGER, R. J. & GREENBERG, M. E. 2012. The nogo receptor family restricts synapse number in the developing hippocampus. *Neuron,* 73**,** 466-81.
WINDING, M., KELLIHER, M. T., LU, W., WILDONGER, J. & GELFAND, V. I. 2016. Role of kinesin-1-based microtubule sliding in Drosophila nervous system development. *Proc Natl Acad Sci U S A,* 113**,** E4985-94.
WOLF, J. A., STYS, P. K., LUSARDI, T., MEANEY, D. & SMITH, D. H. 2001. Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. *J Neurosci,* 21**,** 1923-30.
WONG, C. O., PALMIERI, M., LI, J., AKHMEDOV, D., CHAO, Y., BROADHEAD, G. T., ZHU, M. X., BERDEAUX, R., COLLINS, C. A., SARDIELLO, M. & VENKATACHALAM, K. 2015. Diminished MTORC1-Dependent JNK Activation Underlies the Neurodevelopmental Defects Associated with Lysosomal Dysfunction. *Cell Rep,* 12**,** 2009-20.
WONG, W. & SCOTT, J. D. 2004. AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol,* 5**,** 959-70.
WU, C. C., WU, H. J., WANG, C. H., LIN, C. H., HSU, S. C., CHEN, Y. R., HSIAO, M., SCHUYLER, S. C., LU, F. L., MA, N. & LU, J. 2015. Akt suppresses DLK for maintaining self-renewal of mouse embryonic stem cells. *Cell Cycle,* 14**,** 1207-17.

XIAO, Y., FAUCHERRE, A., POLA-MORELL, L., HEDDLESTON, J. M., LIU, T. L., CHEW, T. L., SATO, F., SEHARA-FUJISAWA, A., KAWAKAMI, K. & LOPEZ-SCHIER, H. 2015a. High-resolution live imaging reveals axon-glia interactions during peripheral nerve injury and repair in zebrafish. *Dis Model Mech,* 8**,** 553-64.

XIAO, Y., TIAN, W. & LOPEZ-SCHIER, H. 2015b. Optogenetic stimulation of neuronal repair. *Curr Biol,* 25**,** R1068-9.

XIONG, X. & COLLINS, C. A. 2012. A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. *J Neurosci,* 32**,** 610-5.

XIONG, X., HAO, Y., SUN, K., LI, J., LI, X., MISHRA, B., SOPPINA, P., WU, C., HUME, R. I. & COLLINS, C. A. 2012. The Highwire ubiquitin ligase promotes axonal degeneration by tuning levels of Nmnat protein. *PLoS Biol,* 10**,** e1001440.

XIONG, X., WANG, X., EWANEK, R., BHAT, P., DIANTONIO, A. & COLLINS, C. A. 2010. Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. *J Cell Biol,* 191**,** 211-23.

XUE, Y., REN, J., GAO, X., JIN, C., WEN, L. & YAO, X. 2008. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol Cell Proteomics,* 7**,** 1598-608.

XUE, Y., ZHOU, F., ZHU, M., AHMED, K., CHEN, G. & YAO, X. 2005. GPS: a comprehensive www server for phosphorylation sites prediction. *Nucleic Acids Res,* 33**,** W184-7.

YAN, D. & JIN, Y. 2012. Regulation of DLK-1 kinase activity by calcium-mediated dissociation from an inhibitory isoform. *Neuron,* 76**,** 534-48.

YAN, D., WU, Z., CHISHOLM, A. D. & JIN, Y. 2009. The DLK-1 kinase promotes mRNA stability and local translation in C. elegans synapses and axon regeneration. *Cell,* 138**,** 1005-18.

YANG, J., WU, Z., RENIER, N., SIMON, D. J., URYU, K., PARK, D. S., GREER, P. A., TOURNIER, C., DAVIS, R. J. & TESSIER-LAVIGNE, M. 2015. Pathological axonal death through a MAPK cascade that triggers a local energy deficit. *Cell,* 160**,** 161-76.

YING, Z., ZHAI, R., MCLEAN, N. A., JOHNSTON, J. M., MISRA, V. & VERGE, V. M. 2015. The Unfolded Protein Response and Cholesterol Biosynthesis Link Luman/CREB3 to Regenerative Axon Growth in Sensory Neurons. *J Neurosci,* 35**,** 14557-70.

YLERA, B., ERTURK, A., HELLAL, F., NADRIGNY, F., HURTADO, A., TAHIROVIC, S., OUDEGA, M., KIRCHHOFF, F. & BRADKE, F. 2009. Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon. *Curr Biol,* 19**,** 930-6.

YOSHIDA, H., MATSUI, T., YAMAMOTO, A., OKADA, T. & MORI, K. 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell,* 107**,** 881-91.

YOSHIHARA, M., ADOLFSEN, B., GALLE, K. T. & LITTLETON, J. T. 2005. Retrograde signaling by Syt 4 induces presynaptic release and synapse-specific growth. *Science,* 310**,** 858-63.

ZHEN, M., HUANG, X., BAMBER, B. & JIN, Y. 2000. Regulation of presynaptic terminal organization by C. elegans RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron,* 26**,** 331-43.

ZHONG, J., DIETZEL, I. D., WAHLE, P., KOPF, M. & HEUMANN, R. 1999. Sensory impairments and delayed regeneration of sensory axons in interleukin-6-deficient mice. *J Neurosci,* 19**,** 4305-13.

ZOU, H., HO, C., WONG, K. & TESSIER-LAVIGNE, M. 2009. Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci,* 29**,** 7116-23.
ZROURI, H., LE GOASCOGNE, C., LI, W. W., PIERRE, M. & COURTIN, F. 2004. The role of MAP kinases in rapid gene induction after lesioning of the rat sciatic nerve. *Eur J Neurosci,* 20**,** 1811-8.