Title

The roles of X11 and rheb in DLK/Wnd-dependent axonal growth and regeneration in Drosophila

Author

Leung, Lok Kwan

Publication Date

2016

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The roles of *X11* and *rheb* in DLK/Wnd-dependent axonal growth and regeneration in *Drosophila*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Lok Kwan Leung

2016

ABSTRACT OF THE DISSERTATION

The roles of *X11* and *rheb*
in DLK/Wnd-dependent axonal growth and
regeneration in *Drosophila*

by

Lok Kwan Leung
Doctor of Philosophy in
Molecular, Cellular, and Integrative Physiology
University of California, Los Angeles, 2016
Professor Ming Guo, Chair

In the mature mammalian central nervous system (CNS), axons do not regenerate after injury. To improve regeneration, past studies have focused on removing the cell extrinsic signals that block regeneration. However, recent studies suggest that the decline of intrinsic growth capacity of neurons might be the major reason why axons do not regenerate in the adult CNS. The Dual Leucine Zipper Kinase (DLK) pathway is a key intrinsic signal for regeneration after axonal injury. Overexpression of *dlk* leads to robust regeneration while loss of *dlk* completely abolishes regeneration after injury. In contrast to the extensive studies on DLK and its downstream signaling cascade, upstream signaling pathways that control DLK remain largely unknown. This dissertation aims to

identify the upstream regulators of *dlk*. In chapter two, we showed that the scaffolding proteins X11Lα and X11Lβ are negative regulators of the *Drosophila* homolog of DLK*,* Wallenda (Wnd). We found that loss of *X11* leads to enhanced axonal regeneration that is dependent on the DLK/Wnd pathway because inhibition of this pathway suppresses the regeneration phenotypes. Our data suggest that X11 normally prevents DLK/Wnd from promoting axonal growth. Upon injury, increase in intracellular Ca2+ triggers degradation of X11 by the Ca2+-dependent protease CalpB. The degradation of X11 in turn activates DLK/Wnd and initiates axonal regeneration. In Chapter three, we identified a small GTPase, *rheb*, that greatly enhances axonal regeneration and suppresses axonal degeneration in *Drosophila* when overexpressed. Enhanced axonal regeneration and reduced axonal degeneration caused by *rheb* overexpression are dependent on the DLK/Wnd pathway. Furthermore, we found that overexpression of *rheb* leads to an increase of DLK/Wnd protein level. Finally, we found that inhibition of the mTORC1 pathway suppresses *rheb*-mediated axonal overgrowth. Taken together, our results suggest that *rheb* regulates Wnd level by increasing protein translation of DLK/Wnd through the mTORC1 pathway. This thesis provides mechanistic insights into DLK/Wnd regulation by *X11* and *rheb* that will help develop therapeutic strategies for regeneration of the injured CNS.

The dissertation of Lok Kwan Leung is approved.

Mark Arthur Frye

Alvaro Sagasti

Yibin Wang

Ming Guo, Committee Chair

University of California, Los Angeles

2016

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

ACKNOWLEDGEMENTS

First, I would like to thank my thesis advisor Dr. Ming Guo for the endless amount of support and encouragement she has provided me the past several years. The road to becoming an independent scientist is long and difficult. I am thankful for her patience while she guided me through this path. She has supported me in every possible way, from giving me the opportunities to work on different projects to helping me apply for fellowships. I consider myself extremely lucky to have had her as a mentor and a role model early on in my career and will never forget the generous help she has provided.

For great advice and mentorship, I would like to thank my committee members Dr. Mark Fyre, Dr. Yibin Wang, and Dr. Alvaro Sagasti. I really appreciate their time, expertise, and guidance. I would like to thank the past and present Guo lab members for help and friendship. In particular, I would like to thank Dr. Garrett Gross and Dr. Mohiddin Lone for their mentorships early on in my graduate career. I would like to thank Michael Lizzio for designing DNA constructs and for generating some transgenic flies for this work. Special thanks go to Dr. Jina Yun for her generous help both inside and outside of the lab. I would also like to acknowledge the UCLA dissertation year fellowship and the Whitcome predoctoral fellowship through which this work was funded.

Chapter 2 of this thesis is a close collaboration between Dr. Mohiddin Lone and me. Dr. Lone first initiated the project and lay the groundwork for this chapter. Together with me, he characterized the phenotypes of *X11* mutants after injury. He also did the work on the

subcellular localizations of X11 and Wnd in the wildtype flies and *X11* mutants flies. His works were invaluable to this chapter.

I owe my deepest gratitude to my parents, Leung Koon On and Lau May Kwan. I am thankful for their unlimited love and support. They have made many sacrifices so that I can pursue my dream, and have always been there to support me. I can never thank them enough for what they have done for me. In addition, I would like to thank my brothers and sisters for their love and support. Without my family's support, this research would not have been possible.

BIOGRAPHICAL SKETCH

Lok Kwan Leung

Education

2011-present	University of California, Los Angeles, CA Ph.D. candidate, *Department of Molecular, Cellular, and Integrative Physiology*
2009-2011	University of California, Los Angeles, CA Graduate student, Department of *Molecular, Cellular, and Integrative Physiology*
2005-2007	University of California, Los Angeles, CA B.S., *Department of Molecular, Cell, and Developmental Biology*

Awards

2015-2016	UCLA Dissertation Year Fellowship
2013-2014	Whitcome Predoctoral Training Fellowship
2012-2013	UCLA University Fellowship
2007	UCLA Departmental highest honor Department of Molecular, Cell, and Developmental Biology

Research Experience

2009-present	Doctorial Research (Thesis Advisor: Ming Guo, M.D., Ph.D.) *Department of Molecular, Cellular, and Integrative Physiology, UCLA*
2007-2009	Research Associate (Advisor: Ming Guo Ph.D.) *Department of Neurology, UCLA*
2006 – 2007	Undergraduate research (Advisor: Alvaro Sagasti, Ph.D.) *Department Molecular, Cellular, and Developmental Biology, UCLA*

Publications

Dodson W. M., **Leung L.K.**, Lone G.M., Lizzio A. M., Guo M. Disease Models & Mechanisms (2014). Novel alleles of the Drosophila LRRK2 homolog reveal a crucial role in endolysosomal functions and autophagy in vivo.

Gross G.G.*, Lone G.M.*, **Leung L.K.**, Hartenstein V, Guo M. Journal of Neuroscience (2013). X11/Mint genes control polarized localization of axonal membrane proteins in vivo. (cover story and featured articles)

Presentations

Leung, **L.K.**, Lone, G. M., Gross, G.G., and Guo, M. (2014) The role of X11 proteins in axon injury and regeneration. Oral Presentation - Annual retreat of Molecular, Cellular, and Integrative Physiology program (MCIP).

Leung, **L.K.**, Lone, M., Gross, G.G., and Guo, M. (2014). X11/Mint genes control polarized localization of axonal membrane proteins in vivo. Poster presentation – Annual retreat of Molecular Biology Institute interdepartmental program.

Leung, **L.K.**, Lone, M., Gross, G.G., and Guo, M. (2013). X11/Mint genes control polarized localization of axonal membrane proteins in vivo. Poster presentation – Neurobiology of *Drosophila* conference at Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Leung, **L.K.**, Lone, M., Gross, G.G., and Guo, M. (2011) The X11L genes regulate synaptic growth during development. Poster presentation – Neurobiology of *Drosophila* conference at Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Teaching Experience

Spring 2011	Life Science 2 teaching assistant *Department of Life Science, UCLA*
Winter 2012	Life Science 23L teaching assistant *Department of Life Science, UCLA*

CHAPTER 1

INTRODUCTION

The central nervous system (CNS) consists of the brain and spinal cord while the peripheral nervous system (PNS) consists of the nerves and ganglia outside of the brain and spinal cord. A major difference between the nervous systems is their ability to regenerate following injuries. While the adult mammalian PNS has the ability to regenerate, the CNS has little or no capacity to do so (Yiu and He, 2006). As a result, injuries to the CNS, such as traumatic brain injury (TBI) and traumatic spinal cord injury (SCI), usually result in permanent disabilities. There are an estimated 1.4 million new cases of TBI, with over 50,000 deaths and 80,000 disabilities in the United States. Moreover, there are about 12,000 spinal cord injuries every year in the United States, with more than a quarter of a million Americans currently living with spinal cord injuries. The total cost of caring for TBI and SCI patients well exceeds $70 billion dollars a year. As a result of its lack of regenerative capability, there is no effective treatment for injuries to the CNS. Current treatment aims at stabilizing the patient and minimizing secondary damages by pharmacological agents, but improvement of clinical outcome is only modest (Clausen and Bullock, 2001; Faden, 1996; Maas et al., 2004; Marklund et al., 2006; Royo et al., 2003). Therefore, there is an urgent need to identify ways to enhance regeneration in the CNS.

Injury to the PNS is arguably a bigger healthcare problem in the United States as even more people are affected by PNS injuries than CNS injuries; It is estimated that twenty million Americans suffer from periphery nerve injuries (Lundborg, 2003), and

approximately $150 billion are spent on these patients annually in the United States (Taylor et al., 2008). The majority of PNS nerve injuries are of traumatic causes (Kouyoumdjian, 2006). Although the PNS can regenerate after injury, it does so imperfectly; PNS regeneration only leads to minimal functional recovery (Grinsell and Keating, 2014). One reason is that the regeneration of PNS is slow. It is estimated that damaged axons regenerate at a speed of about 1-2mm/day (Pfister et al., 2011), and muscle fibrosis and atrophy begin almost immediately after denervation of the axons (Lee and Wolfe, 2000). Thus, it will be beneficial for these patients if there are ways to speed up axonal regeneration in the PNS.

Together, injuries to the CNS and PNS affect tens of millions of people in the United States alone. In order to develop effective therapies for injuries to the CNS and PNS, a better understanding of why and how PNS regenerates while CNS does not at the molecular level is needed. Decades of research have demonstrated that whether a neuron can regenerate is determined by its intrinsic growth capacity and whether it is surrounded by an inhibitory or permissive extracellular environment.

Inhibitory extrinsic factors in the CNS prevent axon regeneration

Ramón y Cajal observed as early as 1928 that the CNS does not have the ability to regenerate (Horner and Gage, 2000). Several observations suggest that there are factors present in the CNS that prevent regeneration. For example, the mammalian Dorsal Root Ganglion (DRG) neurons, which have their cell bodies located outside of the CNS, have a bifurcated axon that projects one of its branch to the PNS (the peripheral branch) and

the other to the CNS (the central branch). Interestingly, only the peripheral branch that is projected to the PNS regenerates after injury(Richardson and Issa, 1984) . Since the branches originate from the same axon of the same neuron, the only difference is whether the branch is projected into the CNS or PNS. This strongly suggests that there are inhibitory molecules in the CNS that inhibit axon regeneration. In addition, a series of elegant works done by David and Aguayo demonstrated that at least some injured CNS neurons were able to regenerate if they are surrounded by PNS graft transplants, suggesting that inhibitory factors in the CNS environment might be primarily responsible for the failure of axons to regenerate in the CNS (David and Aguayo, 1981). Thus, extensive studies in the past decades have focused on finding and characterizing the molecular identities and functions of the inhibitors that are present in the CNS. Over the years, several inhibitors of axonal regeneration in the CNS have been identified, including the myelin-associated inhibitors that signal through the Nogo receptor and the chondroitin sulfate proteoglycans that are found in the glial scars (Liu et al., 2006). However, mutations of these inhibitors and their receptors only lead to modest regeneration in the CNS *in vivo* (Yiu and He, 2006), suggesting that removing the inhibitory molecules in the CNS alone is not sufficient for axon to regenerate, and the difference in intrinsic growth capability between neurons might also be crucial in determining whether neurons can regenerate.

Intrinsic growth capacity of neurons

In order for axons to regenerate after injury, new axonal structures need to be formed. Therefore, gene expression and new protein synthesis are thought to be required (Rishal

and Fainzilber, 2014). Indeed, studies with mouse DRG neurons suggest that transcription-dependent changes in gene expression after injury correlates with the neuron's ability to regenerate (Costigan et al., 2002; Hoffman and Cleveland, 1988). As previously described, the DRG neurons have two axon branches: one central branch and one peripheral branch. The cell body responds differently to injuries at these two branches. Injury to the peripheral branch triggers transcriptional changes that lead to expression of growth associated proteins (Hoffman and Cleveland, 1988), while injury to the central branch does not (Richardson and Issa, 1984). Interestingly, a prior lesion, known as the preconditioning lesion, to the peripheral branch dramatically increases regenerative responses to a second lesion at either the peripheral branch or the central branch (Richardson and Issa, 1984). This remarkable observation suggests that boosting up the intrinsic growth capacity of neurons could enable axonal regeneration even in the inhibitory CNS environment. Thus, understanding the intrinsic mechanisms that promote axonal regeneration in the PNS neurons might not only helps treating injures to the PNS but also helps developing new strategies to improve the regenerative ability of CNS neurons after injury. However, which genes/pathways regulate the intrinsic growth potential of neurons remained elusive until recently.

The Dual Leucine Zipper-bearing Kinase (DLK) pathway is required for axonal regeneration

One key intrinsic signaling pathway that is essential for axonal regeneration is the Dual Leucine Zipper-bearing Kinase (DLK) pathway. In a large-scale genetic screen, Hammarlund et al. identified the pathway as playing a key role in axonal regeneration

using a mutant strain of *Caenorhabditis elegans* which has fragile axons that breaks spontaneously when the worm moves (Hammarlund et al., 2009). By labeling the axonal membranes with GFP, they were able to observe the spontaneous breakage and regeneration of axons, and thus were able to screen for mutants that suppress axonal regeneration by using RNAi. From the screen, they identified *dlk* as a key gene that is required for axonal regeneration. Loss of *dlk* completely blocks axon regeneration while overexpression of *dlk* increases both the speed and the success rate of regeneration. Since *dlk* is a MAP Kinase Kinase Kinase (MAPKKK), they did a targeted screen to search for the downstream MAP Kinase Kinase (MAPKK) and MAP Kinase (MAPK) and identified *mkk-4* and *pmk-3* as the MAPKK and MAPK respectively (Hammarlund et al., 2009). Subsequent studies show that the role of the DLK pathway in axonal regeneration is conserved in both *Mus musculus* and in *Drosophila melanogaster* (Itoh et al., 2009; Xiong et al., 2010), suggesting that the DLK pathway is a key evolutionarily conserved pathway used by neurons to promote regeneration. Interestingly, DLK is required at the time of injury, as expressing DLK protein only at the time of injury, but not before or after injury, in the *dlk* mutant restores axonal regeneration (Hammarlund et al., 2009). In addition, preconditioning-mediated enhanced axonal regeneration requires DLK as there is no preconditioning effect in *dlk* mutant of both mouse and flies (Valakh et al., 2015; Xiong and Collins, 2012). How might injury activate the DLK pathway? In both *C. elegans* and *Drosophila,* DLK levels are controlled by the E3 ubiquitin ligase RPM-1 during synapse development (Collins et al., 2006; Nakata et al., 2005). One hypothesis is that DLK level is also regulated by RPM-1 after injury. Indeed, *rpm-1* mutant has increased axonal regeneration in comparison with wildtype (Hammarlund et al., 2009; Xiong et al.,

2010). In addition, it has been shown that after injury, the level of Highwire (Hiw), the homolog of Rpm-1 in *Drosophila,* is downregulated, concomitant with an increase of the level of Wallenda (Wnd), the homolog of DLK in *Drosophila* (Xiong et al., 2010). Another mechanism that regulates DLK activity is through the increase of intracellular calcium. In *C. elegans*, there is a long isoform of DLK known as DLKL and there is a short isoform of DLK known as DLKS (Yan and Jin, 2012). DLKL normally binds to DLKS and together they form an inactive heteromeric complex. An increase of intracellular concentration causes DLKL to switch its binding partner and form a homodimer with itself that is active. However, DLKS is not conserved across phyla, raising the possibility that calcium could regulate DLK via other mechanisms. A third mechanism regulating DLK activation has recently been described. In a genetic screen, Valakh et al. found that cytoskeleton disruption leads to increase synaptic growth and axonal regeneration through the DLK/Wnd pathway in *Drosophila* (Valakh et al., 2013). Disrupting cytoskeleton in mouse also leads to DLK activation and enhanced axonal regeneration (Valakh et al., 2015), suggesting this is an evolutionarily conserved mechanism. However, it remains unclear how cytoskeleton disruption could lead to the activation of DLK, and whether this is relevant to physical injuries to the axons although remodeling of cytoskeleton after axonal injury is a critical step for axonal regeneration (Spira et al., 2003).

The PTEN/PI3K/AKT pathway and intrinsic growth capacity of neurons

Another important signaling pathway that determines the intrinsic growth capacity of neuron is the PTEN/PI3K/AKT pathway. For most cells there are specific mechanisms that prevent overgrowth once development is completed. Park et al. reason that this might

be why mature CNS lose their ability to growth. To test their hypothesis, they generated conditional knockout mice that deletes candidate negative regulator of cell growth in the retinal ganglion cells (RGC) and tested whether there is axonal regeneration after optic nerve injury. From their screen, they found that knocking out the tumor suppressor gene Phosphatase and tensin homolog (PTEN) allows robust axonal regeneration of RGC after injury in the CNS (Park et al., 2008). PTEN is a major tumor suppressor that is frequently mutated or deleted somatically in various human cancers (Stiles et al., 2002). PTEN converts phosphatidylinositol (3,4,5)-triphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) while phosphatidylinositol 3-kinase (PI3K) phosphorylates PIP2 to produce PIP3. Thus, PTEN act as a negative regulator of the PI3K signaling. PIP3 produced by PI3K signaling recruits PDK1 to the membrane that in turn activates AKT. AKT then phosphorylates its many downstream targets and stimulates cell cycle progression and cell growth (Saucedo et al., 2003; Stiles et al., 2002). Multiple subsequent studies have confirmed that inhibition of PTEN or activation of PI3K enhances axonal regeneration in both the CNS and PNS (Abe et al., 2010; Saijilafu et al., 2013; Yang et al., 2014). PTEN inhibition of axonal regeneration also appears to be evolutionarily conserved in invertebrates as PTEN mutants show enhanced axonal regeneration in flies and worms (Byrne et al., 2014; Song et al., 2012). Interestingly, not all neurons show enhanced axonal regeneration in PTEN mutant. While PTEN inhibits axon regeneration in the GABA neurons of *C. elegans* (Byrne et al., 2014), it does not play a role in axonal regeneration in the mechanosensory neurons (Chen et al., 2011), suggesting that PTEN regulation of intrinsic growth potential might be dependent on the types of neurons. Although multiple studies confirmed that activating PI3K or inactivating

PTEN leads to enhanced axonal regeneration, it is less clear what is the downstream signaling pathway that mediates axonal regeneration. A major downstream signaling pathway of PTEN/PI3K/AKT in other contexts is the mammalian Target of Rapamycin (mTOR) pathway. Downstream of PI3K, AKT activation removes the inhibition of TSC. TSC1 and TSC2 negatively regulates a small GTP-binding protein, Ras homolog enriched in brain (Rheb). Rheb in turn controls the activity of TOR, a serine-threonine kinase. mTOR binds to Raptor and mLST8 to form the mTORC1 complex that regulates ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP). Phosphorylation of 4E-BP by mTORC1 initiates cap-dependent translation while phosphorylation of S6K by mTORC1 promotes mRNA biogenesis and translation initiation/elongation (Laplante and Sabatini, 2012). The mTORC1 pathway is a critical determinant of growth during development, regulating a number of cellular functions including translation, mRNA turnover, and protein stability. However, it is less clear if the mTORC1 pathway also mediates PI3K/AKT signaling to regulate axonal regeneration. In the adult mouse CNS, diminished ability of axons to regenerate correlates with decreasing activities of the mTORC1 pathway (Liu et al., 2010; Park et al., 2008). However, it has not been tested whether mTORC1 activity is required for axonal regeneration in the CNS of *pten* mutant. In the adult rodent PNS, involvement of mTOR in PTEN/PI3K/AKT-mediated axonal regeneration has been investigated, but yielding conflicting results. Two study suggested that mTOR signaling is dispensable for enhancing axonal regeneration induced by PTEN inhibition (Christie et al., 2010; Saijilafu et al., 2013), whereas one study showed that inhibition of mTOR suppresses axonal regeneration in the DRG neurons (Abe et al., 2010). As these studies were conducted in

neuronal culture, it is unclear whether mTOR is required for axonal regeneration *in vivo*. Interestingly, studies in both worms and mouse showed that PTEN/PI3K/AKT-mediated axonal regeneration cannot bypass the requirement of DLK (Byrne et al., 2014; Watkins et al., 2013), suggesting that DLK is a key downstream target of the PTEN/PI3K/AKT pathway. But how these two pathways interact to regulate axon regeneration remain unclear.

Role of calcium in axonal regeneration

One of the earliest signaling event of axonal regeneration is the influx of extracellular calcium from the injury site. Several evidences support that calcium plays a key role in axonal regeneration. First, after axotomy multiple organisms, intracellular calcium concentration rises rapidly at the site of transection (Cho et al., 2013; Ghosh-Roy et al., 2010; Ziv and Spira, 1995). Second, microinjecting calcium ionophore is sufficient to induce axonal growth in an intact axon (Ziv and Spira, 1997). Third, in *C. elegans*, the level of calcium in transected axons correlates with regenerative responses (Ghosh-Roy et al., 2010). Fourth, axotomy of DRG neurons in a calcium-free medium greatly impairs growth cone formation (Chierzi et al., 2005). These evidences suggest that calcium is an evolutionarily conserved upstream signal to trigger axonal regeneration. One recent study found that calcium influx after injury causes nuclear export of HDAC5 in a PKC-dependent manner and the injury-induced nuclear export of HDAC5 enhances histone acetylation, presumably this activates proregenerative genes (Cho et al., 2013). Interestingly, changes in calcium levels can also activate mouse DLK in cell culture (Mata et al., 1996). In addition, two studies in *C. elegans* have shown that DLK can be activated by calcium

signaling. The first study showed that DLK is activated by its direct binding to calcium, which mediates a switch of DLK from an inactive to an active state (Yan and Jin, 2012). Another study showed that calcium-mediated activation of core apoptotic executioner proteins CED-3 and CED-4 acts upstream of DLK in regulating axonal regeneration (Pinan-Lucarre et al., 2012). These studies suggest that calcium also plays a key role regulating the DLK pathway. It remains unclear whether increased intracellular calcium level also triggers activation of the PI3K/PTEN pathway after injury as it has not been tested.

Another important target of calcium signaling after axonal injury is the Calpain family of protease, which is activated by calcium after injury. Calpains have previously been shown to be activated by calcium influx to the axons after injury (Spira et al., 2001; Ziv and Spira, 1998). In rat models of spinal cord injury, the level of Calpain in spinal cord increases significantly compares with uninjured rats (Li et al., 1996; Ray et al., 2000). Interestingly, growth cone formation after axotomy can be inhibited by the application of calpeptin, an inhibitor of calpain in *Aplysia* neurons (Gitler and Spira, 2002). Moreover, when calpeptin is added to *Aplysia* neurons 15 minutes before axotomy, there is no growth cone formation, but if calpeptin is added 5 minutes after axotomy, growth cone formation is not affected (Gitler and Spira, 2002). This suggests there is only a short window of opportunity, similar to the temporal requirement of DLK in axonal regeneration in *C. elegans*, for Calpain-mediated growth cone formation after injury. However, whether Calpain activities are required for DLK activation remain unclear.

Axonal degeneration after injury

Axons are particularly vulnerable to degeneration. Axonal degeneration has been observed in physically injured nerves (Coleman, 2005), by toxic insults (Silva et al., 2006; Spencer and Schaumburg, 1974), and in neurodegenerative diseases (Conforti et al., 2007; Raff et al., 2002). Axons that are detached from the cell bodies after physically injuries undergo a characteristic way of degeneration, in which after a lapse period, axons are simultaneously fragmented into beads and are then removed, a process commonly referred to as Wallerian degeneration (Coleman and Freeman, 2010). Originally thought to be a passive process due to the lack of trophic factors delivered to the axons, Wallerian degeneration is now accepted as an active process that is distinct from apoptosis (Burne et al., 1996; Finn et al., 2000). In 1989, Lunn et al. identified a strain of mutant mouse called *slow wallerian degeneration, Wld*^{S}. The axons of Wld^{S} mouse degenerate much slower after injury (Lunn et al., 1989). In these animals, the axons survive and maintain their functions for up to 3 weeks after injury, as opposed to a few days in wildtype mouse. This results clearly demonstrated that the distal axons are actively degraded, not by the lack of growth factors supplied from the cell body.

Axonal degeneration and Wlds/NMNAT

Extensive studies have been carried out to understand the nature of Wld^{S} mutation and how it protects axons from degeneration. The Wld^{S} mutation is caused by chromosome rearrangement that leads to the creation of a fusion protein that contains the full sequence of Nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) and the N-terminal 70 amino acid of gene Ube4b, linked by a unique 18 amino acid sequence (Conforti et al.,

2000; Mack et al., 2001). Further studies demonstrated that *nmnat1* is the core component of the Wld^S mutation, but not the *ube4b* gene (Coleman and Freeman, 2010). Nmnat1 is a key enzyme of the NAD+ salvage pathway. However, it is unclear whether NAD+ is the protective enzyme product. The total NAD+ levels in brain extracts from the Wld^S mouse are not significantly altered (Mack et al., 2001), and genetically manipulating the NAD+ synthesis pathway to increase NAD+ levels has no protective effect from degeneration (Sasaki et al., 2009). Further complicating matters, the enzymatic activity of Nmnat is dispensable for its neuroprotective function at least in flies (Zhai et al., 2008). Further studies are needed to understand how NMNAT protects axons from degeneration.

Axonal degeneration and the Ubiquitin-Proteasome system (UPS)

Multiple studies suggest that the ubiquitin-proteasome system (UPS) plays a critical role in axonal degeneration. In *Drosophila*, axonal degeneration requires the UPS (Hoopfer et al., 2006). Similarly, in rats, treating protease inhibitors delay axonal degeneration following injury (Zhai et al., 2003). Thus, these studies suggest that UPS action in facilitating axon fragmentation after injury is a conserved feature from flies to mammals.

Axonal degeneration and RPM-1/Hiw, NMNAT, and DLK/Wnd

What is the role UPS plays in axonal degeneration? One possible hypothesis is that the UPS is required for bulk degradation of axonal and axonal membrane proteins through many different ubiquitin ligases after injury or other neuronal insults. An alternative model is that one or several specific E3 ligases target the destruction of key inhibitors of the axonal degeneration process. Interestingly, mutations in *rpm-1*/*hiw* strongly inhibit the

initiation of Wallerian degeneration in multiple neuronal types and developmental stages in *Drosophila* (Xiong et al., 2012). Xiong et al. also showed that knockdown of *nmnat* strongly suppressed the protective phenotype of *rpm-1/hiw* mutant, both in the axons and in the neuromuscular junction (NMJ), suggesting that NMNAT function is an important component of Hiw's role in degeneration. As RPM-1/Hiw is an E3 ubiquitin ligase, one attractive hypothesis is that RPM-1/Hiw controls the level of NMNAT through ubiquitinating NMNAT and targets its degradation through the UPS. Consistent with this, overexpression of *rpm-1/hiw* but not a ligase-dead version of *rpm-1/hiw*, leads to reduction of NMNAT while *rpm-1hiw* mutant leads to increase level of NMNAT. These findings suggest that Rpm-1/Hiw plays a direct role in regulating the levels of NMNAT protein. In addition to NMNAT, DLK/Wnd is also an important component of Hiw's role in degeneration as knockdown of *dlk/wnd* abolishes *rpm-1*/*hiw's* protective phenotype in the motor neurons. However, *dlk/wnd* appears to play opposite roles in both fly sensory neurons and mouse DRG neurons. In these neurons, loss of function mutations of *dlk/wnd* modestly delay Wallerian degeneration (Miller et al., 2009), suggesting that *dlk/wnd* may play different role in axonal degeneration in different cell types and in different organisms. Further studies will be needed to understand the precise role of *dlk1/wnd* in axonal degeneration.

Drosophila as a model organism to study axonal regeneration and degeneration

Drosophila melanogaster has been used to study biological processes and diseases as essential genes are conserved but with less redundancy than vertebrates (Rubin, 2000). The life cycle of *Drosophila* is relatively short (~10 days), and maintenance is easy and

inexpensive. In addition, numerous genetic tools are available for gene expression, knockdown, deletion, as well as mutagenesis. There is a long history of using *Drosophila* to study neurobiology. In fact, many of the important neuronal proteins were first described in *Drosophila (Bellen et al., 2010)*. Moreover, *Drosophila* has been used to elucidate the mechanisms of axon guidance and synapse development. More recently, *Drosophila* has also been used to understand the basic biology of axon regeneration and degeneration (Brace and DiAntonio, 2016).

Larval models of axonal regeneration and degeneration in *Drosophila*

Several models of axon regeneration and degeneration have been developed in *Drosophila*. The Collins lab first described a nerve crush injury model that recapitulated many aspects of PNS regeneration observed in other models (Xiong et al., 2010). In the *Drosophila* larval brain, the motor neuron cell bodies are located in the ventral nerve cord (VNC), with their axons projecting to the periphery and innervating the muscles. Since the motor axons run just under the cuticle on the ventral side of the larva, they can be injured with a pair of forceps by grapping the ventral part of the larvae. Using this model, the Collins lab found that the Wnd/DLK pathway plays a critical role in axonal regeneration. This model was later adopted to study axonal degeneration by following the fate of the severed distal axons (Xiong and Collins, 2012). Another larval model of axonal regeneration using the Class IV ddaC sensory neurons was described by Song et al. (Song et al., 2012). Class IV ddaC neurons, which have their cell bodies in the periphery and project axons into the VNC, can be visualized and monitored by expression of ppk-GAL4. Using the model, they found that loss of function mutants in *pten* leads to an

increase in sprouting of injured axons in the CNS of *Drosophila* (Song et al., 2012), demonstrating that like their mammalian counterpart, the PTEN/AKT pathway plays an evolutionarily conserved role in regulating axon regeneration in flies.

Adult model of axon regeneration and degeneration in *Drosophila*

Although the *Drosophila* larval is a great model to study axonal regeneration and degeneration, it has several limitations. First, *Drosophila* larvae is still undergoing development at the time of injury. It is known that developing neurons have better regenerative capacity than adult neurons, thus insights generated from larval models might not be directly translatable to injured adult neurons. Second, after injuring the larvae, there are at most 2-3 days before the larvae turns into pupae. As a result, no larval models in *Drosophila* allow sufficient time for injured axons to regenerate to their targets. To circumvent these problems, the Bonini lab established the wing injury model to study axonal regeneration in the adult fly (Soares et al., 2014). There are approximately 255 mechanosensory and chemosensory neurons in the *Drosophila* wing (Fang et al., 2012). Their axons run along the L1 longitudinal wing vein and project into the CNS. By labeling these axons with GFP, they were able to visualize the axons and ablate them with a laser. Using this model, the Bonini lab showed that although the majority of axons regenerate, none of the them were able to grow pass the injury site as a noticeable scar forms at the injury site, suggesting that like the adult mammalian CNS, the adult *Drosophila* axons also have limited regenerative abilities. This model has also been used to study axonal degeneration. Using this model, Fang et al. were able to study axonal degeneration in the

adult neurons and revealed an essential role of NMNAT in maintaining axonal integrity (Fang et al., 2012).

Goal of the Dissertation

Decades of research have focused on characterizing the inhibitory factors in the CNS that limit regeneration, yet much less research has been done to understand the intrinsic growth capacity of neurons that determines their ability to regenerate. As removing the inhibitory factors in the CNS only lead to modest regeneration *in vivo*, research has now begun to focus on understanding the genes and pathways that determine the intrinsic growth potential of neurons. The goal of this dissertation is to use *Drosophila* as a model system to identify and characterize genes that regulate the intrinsic regenerative capacity of neurons. Numerous studies have been done to understand the two intrinsic growth pathway that leads to axonal regeneration after injury: the DLK1 pathway and the PTEN/PI3K pathway. Studies show that both the DLK1 pathway and PTEN/PI3K pathway are of ancient origin and are functionally conserved across highly divergent species. However, we still do not completely understand how they are normally kept inactivated, and how they are activated after injury. It also remains unclear how and if the two pathways interact to control the regeneration and degeneration process. In chapter 2 of this thesis, I will describe the roles of the adaptor protein X11 play in axonal growth during development and axonal regeneration after injury. In chapter 3, I will describe the small GTPase *rheb*, and its role in axonal growth, axonal regeneration and degeneration. In chapter 4, I will summarize my findings as well as discussing possible future directions on the research on axonal regeneration and degeneration.

REFERENCES

Abe, N., Borson, S.H., Gambello, M.J., Wang, F., and Cavalli, V. (2010). Mammalian target of rapamycin (mTOR) activation increases axonal growth capacity of injured peripheral nerves. J Biol Chem *285*, 28034-28043.

Bellen, H.J., Tong, C., and Tsuda, H. (2010). 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. Nat Rev Neurosci *11*, 514-522.

Brace, E.J., and DiAntonio, A. (2016). Models of axon regeneration in Drosophila. Exp Neurol.

Burne, J.F., Staple, J.K., and Raff, M.C. (1996). Glial cells are increased proportionally in transgenic optic nerves with increased numbers of axons. J Neurosci *16*, 2064-2073.

Byrne, A.B., Walradt, T., Gardner, K.E., Hubbert, A., Reinke, V., and Hammarlund, M. (2014). Insulin/IGF1 signaling inhibits age-dependent axon regeneration. Neuron *81*, 561-573.

Chen, L., Wang, Z., Ghosh-Roy, A., Hubert, T., Yan, D., O'Rourke, S., Bowerman, B., Wu, Z., Jin, Y., and Chisholm, A.D. (2011). Axon regeneration pathways identified by systematic genetic screening in C. elegans. Neuron *71*, 1043-1057.

Chierzi, S., Ratto, G.M., Verma, P., and Fawcett, J.W. (2005). The ability of axons to regenerate their growth cones depends on axonal type and age, and is regulated by calcium, cAMP and ERK. Eur J Neurosci *21*, 2051-2062.

Cho, Y., Sloutsky, R., Naegle, K.M., and Cavalli, V. (2013). Injury-induced HDAC5 nuclear export is essential for axon regeneration. Cell *155*, 894-908.

Christie, K.J., Webber, C.A., Martinez, J.A., Singh, B., and Zochodne, D.W. (2010). PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. J Neurosci *30*, 9306-9315.

Clausen, T., and Bullock, R. (2001). Medical treatment and neuroprotection in traumatic brain injury. Curr Pharm Des *7*, 1517-1532.

Coleman, M. (2005). Axon degeneration mechanisms: commonality amid diversity. Nat Rev Neurosci *6*, 889-898.

Coleman, M.P., and Freeman, M.R. (2010). Wallerian degeneration, wld(s), and nmnat. Annu Rev Neurosci *33*, 245-267.

Collins, C.A., Wairkar, Y.P., Johnson, S.L., and DiAntonio, A. (2006). Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron *51*, 57-69.

Conforti, L., Adalbert, R., and Coleman, M.P. (2007). Neuronal death: where does the end begin? Trends Neurosci *30*, 159-166.

Conforti, L., Tarlton, A., Mack, T.G., Mi, W., Buckmaster, E.A., Wagner, D., Perry, V.H., and Coleman, M.P. (2000). A Ufd2/D4Cole1e chimeric protein and overexpression of Rbp7 in the slow Wallerian degeneration (WldS) mouse. Proc Natl Acad Sci U S A *97*, 11377-11382.

Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E., and Woolf, C.J. (2002). Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. BMC Neurosci *3*, 16.

David, S., and Aguayo, A.J. (1981). Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science *214*, 931-933.

Faden, A.I. (1996). Pharmacological treatment of central nervous system trauma. Pharmacol Toxicol *78*, 12-17.

Fang, Y., Soares, L., Teng, X., Geary, M., and Bonini, N.M. (2012). A novel Drosophila model of nerve injury reveals an essential role of Nmnat in maintaining axonal integrity. Curr Biol *22*, 590-595.

Finn, J.T., Weil, M., Archer, F., Siman, R., Srinivasan, A., and Raff, M.C. (2000). Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. J Neurosci *20*, 1333-1341.

Ghosh-Roy, A., Wu, Z., Goncharov, A., Jin, Y., and Chisholm, A.D. (2010). Calcium and cyclic AMP promote axonal regeneration in Caenorhabditis elegans and require DLK kinase. J Neurosci *30*, 3175-3183.

Gitler, D., and Spira, M.E. (2002). Short window of opportunity for calpain induced growth cone formation after axotomy of Aplysia neurons. J Neurobiol *52*, 267-279.

Grinsell, D., and Keating, C.P. (2014). Peripheral nerve reconstruction after injury: a review of clinical and experimental therapies. Biomed Res Int *2014*, 698256.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.

Hoffman, P.N., and Cleveland, D.W. (1988). Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific beta-tubulin isotype. Proc Natl Acad Sci U S A *85*, 4530-4533.

Hoopfer, E.D., McLaughlin, T., Watts, R.J., Schuldiner, O., O'Leary, D.D., and Luo, L. (2006). Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron *50*, 883-895.

Horner, P.J., and Gage, F.H. (2000). Regenerating the damaged central nervous system. Nature *407*, 963-970.

Itoh, A., Horiuchi, M., Bannerman, P., Pleasure, D., and Itoh, T. (2009). Impaired regenerative response of primary sensory neurons in ZPK/DLK gene-trap mice. Biochem Biophys Res Commun *383*, 258-262.

Kouyoumdjian, J.A. (2006). Peripheral nerve injuries: a retrospective survey of 456 cases. Muscle Nerve *34*, 785-788.

Laplante, M., and Sabatini, D.M. (2012). mTOR Signaling. Cold Spring Harb Perspect Biol *4*.

Lee, S.K., and Wolfe, S.W. (2000). Peripheral nerve injury and repair. J Am Acad Orthop Surg *8*, 243-252.

Li, Z., Hogan, E.L., and Banik, N.L. (1996). Role of calpain in spinal cord injury: increased calpain immunoreactivity in rat spinal cord after impact trauma. Neurochem Res *21*, 441-448.

Liu, B.P., Cafferty, W.B., Budel, S.O., and Strittmatter, S.M. (2006). Extracellular regulators of axonal growth in the adult central nervous system. Philos Trans R Soc Lond B Biol Sci *361*, 1593-1610.

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B.*, et al.* (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat Neurosci *13*, 1075-1081.

Lundborg, G. (2003). Richard P. Bunge memorial lecture. Nerve injury and repair--a challenge to the plastic brain. J Peripher Nerv Syst *8*, 209-226.

Lunn, E.R., Perry, V.H., Brown, M.C., Rosen, H., and Gordon, S. (1989). Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve. Eur J Neurosci *1*, 27-33.

Maas, A.I., Marmarou, A., Murray, G.D., and Steyerberg, E.W. (2004). Clinical trials in traumatic brain injury: current problems and future solutions. Acta Neurochir Suppl *89*, 113-118.

Mack, T.G., Reiner, M., Beirowski, B., Mi, W., Emanuelli, M., Wagner, D., Thomson, D., Gillingwater, T., Court, F., Conforti, L.*, et al.* (2001). Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. Nat Neurosci *4*, 1199-1206.

Marklund, N., Bakshi, A., Castelbuono, D.J., Conte, V., and McIntosh, T.K. (2006). Evaluation of pharmacological treatment strategies in traumatic brain injury. Curr Pharm Des *12*, 1645-1680.

Mata, M., Merritt, S.E., Fan, G., Yu, G.G., and 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. J Biol Chem *271*, 16888-16896.

Miller, B.R., Press, C., Daniels, R.W., Sasaki, Y., Milbrandt, J., and DiAntonio, A. (2009). A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci *12*, 387-389.

Nakata, K., Abrams, B., Grill, B., Goncharov, A., Huang, X., Chisholm, A.D., and Jin, Y. (2005). Regulation of a DLK and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell *120*, 407-420.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M.*, et al.* (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science *322*, 963-966.

Pfister, B.J., Gordon, T., Loverde, J.R., Kochar, A.S., Mackinnon, S.E., and Cullen, D.K. (2011). Biomedical engineering strategies for peripheral nerve repair: surgical applications, state of the art, and future challenges. Crit Rev Biomed Eng *39*, 81-124.

Pinan-Lucarre, B., Gabel, C.V., Reina, C.P., Hulme, S.E., Shevkoplyas, S.S., Slone, R.D., Xue, J., Qiao, Y., Weisberg, S., Roodhouse, K.*, et al.* (2012). The core apoptotic executioner proteins CED-3 and CED-4 promote initiation of neuronal regeneration in Caenorhabditis elegans. PLoS Biol *10*, e1001331.

Raff, M.C., Whitmore, A.V., and Finn, J.T. (2002). Axonal self-destruction and neurodegeneration. Science *296*, 868-871.

Ray, S.K., Matzelle, D.D., Wilford, G.G., Hogan, E.L., and Banik, N.L. (2000). Increased calpain expression is associated with apoptosis in rat spinal cord injury: calpain inhibitor provides neuroprotection. Neurochem Res *25*, 1191-1198.

Richardson, P.M., and Issa, V.M. (1984). Peripheral injury enhances central regeneration of primary sensory neurones. Nature *309*, 791-793.

Rishal, I., and Fainzilber, M. (2014). Axon-soma communication in neuronal injury. Nat Rev Neurosci *15*, 32-42.

Royo, N.C., Shimizu, S., Schouten, J.W., Stover, J.F., and McIntosh, T.K. (2003). Pharmacology of traumatic brain injury. Curr Opin Pharmacol *3*, 27-32.

Saijilafu, Hur, E.M., Liu, C.M., Jiao, Z., Xu, W.L., and Zhou, F.Q. (2013). PI3K-GSK3 signalling regulates mammalian axon regeneration by inducing the expression of Smad1. Nat Commun *4*, 2690.

Sasaki, Y., Vohra, B.P., Lund, F.E., and Milbrandt, J. (2009). Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. J Neurosci *29*, 5525-5535.

Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nat Cell Biol *5*, 566-571.

Silva, A., Wang, Q., Wang, M., Ravula, S.K., and Glass, J.D. (2006). Evidence for direct axonal toxicity in vincristine neuropathy. J Peripher Nerv Syst *11*, 211-216.

Soares, L., Parisi, M., and 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., and 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*, 1612-1625.

Spencer, P.S., and Schaumburg, H.H. (1974). A review of acrylamide neurotoxicity. Part II. Experimental animal neurotoxicity and pathologic mechanisms. Can J Neurol Sci *1*, 152-169.

Spira, M.E., Oren, R., Dormann, A., and Gitler, D. (2003). Critical calpain-dependent ultrastructural alterations underlie the transformation of an axonal segment into a growth cone after axotomy of cultured Aplysia neurons. J Comp Neurol *457*, 293-312.

Spira, M.E., Oren, R., Dormann, A., Ilouz, N., and Lev, S. (2001). Calcium, protease activation, and cytoskeleton remodeling underlie growth cone formation and neuronal regeneration. Cell Mol Neurobiol *21*, 591-604.

Stiles, B., Gilman, V., Khanzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X., and Wu, H. (2002). Essential role of AKT-1/protein kinase B alpha in PTEN-controlled tumorigenesis. Mol Cell Biol *22*, 3842-3851.

Taylor, C.A., Braza, D., Rice, J.B., and Dillingham, T. (2008). The incidence of peripheral nerve injury in extremity trauma. Am J Phys Med Rehabil *87*, 381-385.

Valakh, V., Frey, E., Babetto, E., Walker, L.J., and 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., and DiAntonio, A. (2013). Loss of the spectraplakin short stop activates the DLK injury response pathway in Drosophila. J Neurosci *33*, 17863-17873.

Watkins, T.A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J.S., Tessier-Lavigne, M., and 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*, 4039-4044.

Xiong, X., and Collins, C.A. (2012). A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. J Neurosci *32*, 610-615.

Xiong, X., Hao, Y., Sun, K., Li, J., Li, X., Mishra, B., Soppina, P., Wu, C., Hume, R.I., and 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., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J Cell Biol *191*, 211-223.

Yan, D., and Jin, Y. (2012). Regulation of DLK kinase activity by calcium-mediated dissociation from an inhibitory isoform. Neuron *76*, 534-548.

Yang, L., Miao, L., Liang, F., Huang, H., Teng, X., Li, S., Nuriddinov, J., Selzer, M.E., and Hu, Y. (2014). The mTORC1 effectors S6K1 and 4E-BP play different roles in CNS axon regeneration. Nat Commun *5*, 5416.

Yiu, G., and He, Z. (2006). Glial inhibition of CNS axon regeneration. Nat Rev Neurosci *7*, 617-627.

Zhai, Q., Wang, J., Kim, A., Liu, Q., Watts, R., Hoopfer, E., Mitchison, T., Luo, L., and He, Z. (2003). Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron *39*, 217-225.

Zhai, R.G., Zhang, F., Hiesinger, P.R., Cao, Y., Haueter, C.M., and Bellen, H.J. (2008). NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration. Nature *452*, 887-891.

Ziv, N.E., and Spira, M.E. (1995). Axotomy induces a transient and localized elevation of the free intracellular calcium concentration to the millimolar range. J Neurophysiol *74*, 2625-2637.

Ziv, N.E., and Spira, M.E. (1997). Localized and transient elevations of intracellular Ca2+ induce the dedifferentiation of axonal segments into growth cones. J Neurosci *17*, 3568-3579.

Ziv, N.E., and Spira, M.E. (1998). Induction of growth cone formation by transient and localized increases of intracellular proteolytic activity. J Cell Biol *140*, 223-232.

CHAPTER 2

X11L/Mint as an Upstream Regulator of the DLK/Wnd Pathway in Axonal Regeneration

Abstract:

Dro*sophila* Wallenda (Wnd) is an evolutionarily conserved MAP Kinase Kinase Kinase that plays a central role in axonal regeneration after injury. However, the mechanisms underlying its activation are still not completely understood. Here, we report that in *Drosophila* the *X11* genes act as negative regulators of *wnd*. We demonstrated that Wnd dependent axonal regeneration after crush injuries is enhanced in *X11* mutant. Enhanced axonal regeneration in *X11* mutants is abrogated by inhibition of *wnd* and its downstream targets. Furthermore, we found that X11 and Wnd proteins physically interact and colocalize at the Golgi. In *X11* mutants, we observed that Wnd is redistributed from the Golgi to the cytosol, suggesting that X11 anchors Wnd to the Golgi. We also demonstrate that, in addition to retrograde transport, anterograde axonal transport of cargos is also required for Wnd dependent axonal regeneration. Our data suggest that X11 normally prevents Wnd from promoting axonal growth by restraining Wnd on the Golgi. Upon injury, increase in intracellular Ca2+ triggers X11 degradation by the Ca2+-dependent protease CalpB. The degradation of X11 in turn releases Wnd to the cytosol and initiates axonal regeneration. Taken together, this work provides mechanistic insights into X11 and Wnd regulation upon injury that will help develop therapeutic strategies for regeneration of the injured brain.

Introduction:

Axons in the central nervous system (CNS) do not regenerate. However, axons in the peripheral nervous system (PNS) can regenerate after injury (Bradke et al., 2012). How effective axons can regenerate is determined by a combination of extrinsic and intrinsic factors. An inhibitory CNS environment, together with a weak intrinsic regeneration program, contributes to a poor regenerative response in the CNS neurons (Park et al., 2008; Smith et al., 2009). Much focus has gone to the study of extrinsic inhibitors like the molecules associated with myelin and glial scar (Busch and Silver, 2007) and less is known about the intrinsic growth pathways affecting axonal regeneration after injury. Compared to the CNS, the PNS neurons activate an intrinsic regeneration program in response to injury that results in a strong and robust regenerative response. This regeneration program is widely believed to be a dormant developmental program that is reactivated in injured neurons (Cavalli et al., 2005; Hoffman, 2010). Our knowledge of how these programs are regulated both during development and in response to injury remains scarce.

The Dual leucine zipper-bearing kinase (DLK) pathway, an evolutionarily conserved MAP kinase cascade, is one of the intrinsic pathways activated upon injury. DLK, an upstream regulator of cJun N-terminal kinase (JNK) and p38 MAPK, plays a key role in synaptogenesis (Collins et al., 2006; Nakata et al., 2005) and in regenerative response after axotomy from *Drosophila* to *mammals* (Hammarlund et al., 2009; Shin et al., 2012; Xiong and Collins, 2012; Xiong et al., 2010; Yan et al., 2009). Axotomy results in initiation and extension of the growth cone at injury site and retrograde transport of injury signal to

the nucleus to elicit the activation of the transcription factors that turn on an intrinsic proregenerative program. DLK has been identified as a key intermediate required for axonal injury to activate this regenerative program. Although the mammalian genome has two genes that encode the closely related DLK kinases (DLK and LZK), *Drosophila* has only one gene encoding the DLK kinase, named *wallenda* (*wnd*) (Blouin et al., 1996; Collins et al., 2006; Sakuma et al., 1997). Genetic studies have revealed that DLK/Wallenda is negatively regulated by the E3 ubiquitin ligase known as Pam/Highwire/RPM-1 (PHR) proteins, mouse Phr1, human pam, *Drosophila* Highwire and *C .elegans* RPM-1 (Collins et al., 2006; Lewcock et al., 2007; Nakata et al., 2005). In *C. elegans* and *Drosophila,* DLK/Wnd mutants suppress the synapse defects caused by RPM mutants. Recent studies have shown similar regulatory role of PHR proteins on DLKs during regeneration after axotomy (Hammarlund et al., 2009; Xiong et al., 2010).

Regeneration is initiated by signals arising from the injury site. The involvement of calcium in axonal regeneration after axonal injury is supported by several experiments. First, after axotomy in multiple organisms, intracellular calcium concentration rises rapidly at the site of injuries (Cho et al., 2013; Ghosh-Roy et al., 2010; Ziv and Spira, 1995). Second, microinjecting calcium ionophore leads to the formation of growth cone and neurites in intact axons (Ziv and Spira, 1997). Third, the level of calcium in transected axons is correlated with regenerative responses in *C. elegans* (Ghosh-Roy et al., 2010) . Forth, axotomy of mammalian dorsal root ganglion (DRG) in a calcium-free medium greatly impairs growth cone formation (Chierzi et al., 2005). These evidences suggest that calcium is an evolutionarily conserved signal to trigger axon regeneration.

The influx of calcium at the injury sites trigger multiple signaling events. One recent study found that calcium influx causes nuclear export of HDAC5 that enhances histone acetylation to activate proregenerative genes (Cho et al., 2013). Interestingly, changes in Ca^{2+} levels can also activate mouse DLK in cell culture (Mata et al., 1996). In addition, one study in *C. elegans* has shown that DLK is activated by directly binding to Ca^{2+}, which mediates a switch of DLK from an inactive state to an active state (Yan and Jin, 2012). Another study showed that Ca^{2+}-mediated activation of core apoptotic executioner proteins CED-3 and CED-4 requires DLK to mediate axonal regeneration (Pinan-Lucarre et al., 2012), suggesting that calcium also plays a key role in regulating the DLK pathway.

Another important target of calcium signaling after axonal injury is the calcium-activated cystein protease Calpain (Spira et al., 2001; Ziv and Spira, 1998). In rat models of spinal cord injury, the level of Calpain increases significantly compared to those uninjured (Li et al., 1996; Ray et al., 2000). Growth cone formation after axotomy can also be inhibited by the application of calpeptin, an inhibitor of calpain in *Aplysia* neurons. Interestingly, when calpeptin is added to *Aplysia* before axotomy, there is no growth cone formation, but if calpeptin is added 5 minutes after axotomy, growth cone formation is present (Gitler and Spira, 2002). This suggests that similar to the temporal requirement of DLK in *C. elegans*, Calpain activities are required at the time of injury for axons to regenerate. However, whether Calpain activities are required for DLK activation remains unclear.

The X11/Mint family of multi-domain adaptor proteins, through their interactions with diverse neuronal molecules, have been shown to regulate various aspects of synaptic organization, neurotransmission and synaptic plasticity (Biederer and Sudhof, 2000; Borg et al., 1999; Borg et al., 1998; Butz et al., 1998; Verhage et al., 2000). Earlier studies have reported changes in the levels of X11α in the hippocampus of animal models of disease for epilepsy (Scorza et al., 2003) and ischemia (Nishimura et al., 2000).Thus the levels of X11α dramatically decrease immediately after an assault before they increase again, indicating the role of *X11* in hippocampal plasticity. However, the principal physiological function of *X11L* is not well understood.

Previous studies have established that the DLK/Wnd pathway mediates axonal regeneration after injury. Despite extensive studies on DLK/Wnd and its downstream targets, very little is known about the mechanism of activation of the DLK/Wnd pathway. Here we report the *Drosophila X11Lα* and *X11Lβ* genes as upstream regulators of the DLK/Wnd signaling pathway. We showed that *X11* regulates this pathway after injury. In addition, we demonstrated that *X11* regulates the localization of Wnd in *Drosophila* motor neurons. Our data suggest that after injury, influx of calcium leads to degradation of X11 which in turn activates the Wnd pathway.

Materials and Methods:

Molecular Biology:

To silence *X11Lβ*, two separate RNAi constructs were generated targeting two different regions (coding region and UTR) in the *X11β* transcript using a microRNA technology

previously described (Gross et al., 2013; Yun et al., 2014). To silence *X11Lα*, a construct was generated against coding region. PCR products of 3 these microRNA precursors were cloned into pUASt. To generate *UAS-X11Lα*, *UAS-X11Lα-myc*, *UAS-X11LαΔPDZ-myc*, the *X11α* cDNA (EST clone from *Drosophila* Genome Research Center, LD20981), was subcloned into pUASt. *UAS-Wnd* was cloned using gold clone (LD14856). All cloned PCR products were confirmed by DNA sequencing.

Fly Stocks:

X11Lα loss-of-function allele $X11\alpha^{80}$ was generated by imprecise excision of a P element, which was previously isolated in a genetic modifier screen for suppressors of the reporter amyloid precursor protein (Gross et al., 2008; Gross et al., 2013; Guo et al., 2003). Breakpoints were mapped by genomic polymerase chain reaction (PCR) followed by sequencing. UAS- *hX11β* was obtained from Kenji Matsuno and Toshiharu Suzuki (Hase et al., 2002), Canton S (wild-type), *wnd1*, *wnd2,* UAS–Wnd (Collins et al., 2006), and DVGLUT-Gal4 were provided by Cathy Collins and A. DiAntonio (University of Michigan and Washington University, St. Louis, MO). The following fly lines were obtained from the Bloomington Stock Center: $OK6^{Gal4}$ from Corey Goodman (Aberle et al., 2002), Pdf-Gal4 (Renn et al., 1999), UAS-mCD8-GFP (Lee and Luo, 1999), *UAS-Bsk*^{DN} (Weber et al., 2000), *UAS-Fos*^{DN} (Fbz) and *UAS-Fos*^{DN} (Jbz) (Eresh et al., 1997). UAS–GFP-*wnd*^{KD} (Xiong et al., 2010), UAS-p150 (*Glued*)^{DN} 96B (Allen et al., 1999), m12-Gal4 $(P(GAL4)^{5053A}$ (Ritzenthaler et al., 2000), puc-lacz stock, *puc*-$lacZ^{E69}$ (Martin-Blanco, 1997).

Immunocytochemistry:

Wandering third-instar larvae were dissected in PBS and fixed in a PBS solution containing 3.7% formaldehyde for 30 minutes. Blocking and staining was performed in PBS containing 0.1% Triton-X. For nc82 and GlutR11A larvae were fixed in Bouin's solution (a 1:5:15 ratio of acetic acid/formalin/picric acid) for 15 min. Antibodies were used at the following dilutions: FITC-conjugated goat α-HRP (Jackson Immunoresearch) at 1:250, rabbit α-Wnd (Collins et al., 2006) at 1:250, rabbit anti-X11 (1:100). Conjugated α-rabbit and α-mouse secondary antibodies were used at 1:250. Stained brains were mounted in Vectashield (Vector Laboratory; H-1000).

Imaging and quantitative analysis:

Images were acquired on a Zeiss confocal microscope. The NMJ on muscle 4 of the abdominal segment A2 was used for quantifications, α-CSP staining was used to count the number of individual boutons and α-HRP to count the number of branches in control and experimental samples that were processed and stained simultaneously, imaged using the same confocal settings. All quantifications were performed while blinded to genotype. Statistical analysis was performed and graphs were generated using Excel. Student’s t-test was used to determine statistical significance.

Lysate Preparation and Western Blotting:

Third instar larval brains were disrupted in lysis buffer with complete protease inhibitor cocktail (Roche) using a sonicator-3000 from MISONIX. Samples were boiled for 10 minutes, centrifuged, and total protein from 10 brains per genotype was analyzed by

Western blotting analysis. Antibodies used were rabbit anti-Wnd (Collins et al., 2006) and mouse anti-Tubulin (1: 10,000; Sigma). Secondary rabbit and mouse anti-HRP antibodies were purchased from Invitrogen and used at the manufacturer's recommended concentrations.

Co-immunoprecipitation:

Transfected S2 cells were collected and lysed in 500µl of cell lysis buffer containing protease inhibitor. Immuno-precipitation experiments were carried out using Peirce protein A/G magnetic Beads and following the recommended instructions. Anti-bodies used for the experiment were rabbit anti-GFP (Roche, 1: 5,000) mouse anti-myc (Millipore, 1: 10,000) and mouse anti-actin (Sigma, 1:1,000). Secondary antibodies used are HRP conjugated anti-rabbit (1:10,000), anti-mouse (1: 5,000).

Results:

mRNA expressions of both *X11Lα and X11Lβ* are restricted to the differentiated CNS neurons:

Drosophila genome has two paralogues of *X11* gene named *X11Lα* and *X11Lβ*. These multi-domain proteins comprise of a less-conserved N-terminal region followed by a highly conserved phosphotyrosine binding (PTB) domain, and two PSD95/Dlg/ZO-1 (PDZ) domains (Tomita et al., 1999) (Figure 1A). To generate *X11Lα* mutants, P element insertion in its 5' UTR was used to create a large deletion ($X11L\alpha^{80}$) and a miRNA-expressing line against *X11Lα* was also generated. Additionally, two independent miRNA-expressing lines against two distinct regions of *X11Lβ* were created (Figure 1A). We used

the Gal4/UAS system (Brand and Perrimon, 1993) to drive the expression of miRNA targeting *X11L*β in combination with either *X11Lα*^{80} or mi-RNA against *X11Lα* (both referred as *X11* mutants) to generate *X11* mutants. The expressions of both *Drosophila* X11L proteins are restricted to differentiated neurons in the CNS (Figure 1B, D). *X11Lα*^{80} mutant embryos contain no detectable *X11Lα* transcript (Figure 1B, C), suggesting *X11Lα*^{80}is a null allele. Since no loss-of-function mutants of *X11Lα* are available, we generated two synthetic miRNA-expressing transgenic fly lines targeting two distinct regions of *X11Lα*^{80} (Figure 1A). Embryos from flies expressing either construct using the UAS-GAL4 system (Brand and Perrimon, 1993) show robust silencing of the *X11Lα*^{80} transcript (Figure 1D,E), and they produced highly similar if not identical phenotypes when coupled with *X11Lα*^{80} or miRNAs targeting *X11Lα* we generated (Figure 1F-G''). The two paralogues of *X11L* gene are redundant in function as loss of *X11Lα* alone causes only mild NMJ phenotype (Ashley et al., 2005), and loss of both *X11Lα and X11L*β shows a strong phenotype in the mushroom body (Gross et al., 2013).

X11L regulates the Wnd dependent regeneration after axonal injury:

Nerve crush injury induces a regenerative response in larval motor neuron axons in the form of quantifiable sproutings at the proximal end of the injured neurons within hours after injury (Xiong et al., 2010). Given that this regenerative response is mediated by the *wnd* pathway, we tested whether *X11* mutants play a role in axonal regeneration after injury in the motor neuron axons. We used a previously described nerve crush injury paradigm (Figure 2A,B) and quantification techniques to look at the sprouting at the proximal stumps (Ritzenthaler et al., 2000; Xiong et al., 2010). *m12-Gal4* is expressed in

a single motor neuron that forms a single axon within each segmental nerve. Expression of UAS-mCD8-GFP was used to label these neurons. We measured the response at different time intervals and observed that in *X11* mutants, injured neurons take less time to initiate a response and wild type axons take at least 4 hours more to display equal extant of branching (Figure 2C). These results indicate that loss of *X11s* lead to enhanced regenerative response after injury.

We hypothesized that the accelerated regeneration phenotype of *X11* mutants would be dependent on the DLK/Wnd pathway. To test this hypothesis, we looked at the time point of 12 hours after injury for comparison and quantification of phenotypes. At 12 hours after injury, compared to wildtype control axons, the sprouting is significantly increased and becomes more extensive in *X11* mutant axons (Figure 2D,E). Interestingly, overexpression of wnd^{DN} or bsk^{DN} in these mutant neurons significantly suppressed the regeneration phenotypes (Figure 2F-I), indicating that *X11* acts through the *wnd* pathway to regulate regeneration response after injury.

Nerve crush injury leads to upregulation of the JNK pathway as indicated by increased *puc-lacZ* expression in the nucleus of the motor neurons (Xiong et al., 2010). We observed a striking similarity in the *puc-lacZ* pattern between *X11* mutants and injured neurons (Figure 2L-M). Parallel to the enhancement in the regeneration response, the injury causes enhancement of the phenotype in *X11* mutants as indicated by the increase in the number of nuclei stains with β-gal and the enhancement of β-gal intensity in the nuclei of VNC motor neurons (Figure 2J). Interestingly, the strong phenotype due to injury

in the *X11* mutants background was strongly suppressed by overexpressing wnd^{DN} or bsk^{DN} in these neurons (Figure 2P,Q). These results further confirm that *X11* regulates injury response through the Wnd pathway.

X11L physically binds to Wnd and regulates its localization to the TGN Golgi:

To gain insights into how *X11Lα* regulates the Wnd pathway we examined the subcellular localization of the X11L*α* protein. In order to find out which cellular compartment X11L is localized to, we expressed various organelle and cell compartment specific GFP-tagged markers in the motor neurons. Among the different markers tested, we found that X11L does not co-localize with either an early endosomal compartment molecule (Clathrin-GFP), another endosomal marker (Rab5-GFP) or Endoplasmic reticulum marker (KDEL-GFP) (figure S1 A-I), but significantly co-localizes with Golgi reassembly stacking protein of 65kD (GRASP65-GFP), a trans-Golgi protein, indicating that X11L*α* is localized to the Golgi (Figure 3A-C). We then examined the subcellular localization of Wnd. Because the available antibody is unable to detect endogenous Wnd, we used UAS-GFP-wnd^{KD} expression (Xiong et al., 2010) to test the localization of Wnd protein in motor neurons. In the motor neuron cell bodies, we found that Wnd protein is predominantly localized to the Golgi (Figure 3D). This was revealed by its co-localization with the Golgi marker *Drosophila* GM130 (cis-Golgi) (Sinka et al., 2008) (Figure 3J-L). Interestingly, mammalian DLK (Wnd homologue) also predominantly localizes to the Golgi (Mata et al., 1996). Given that Wnd and X11L*α* work in the same pathway, we tested whether the two proteins co-localize in the neuronal cell bodies. Larval brains expressing *UAS-GFP-*wnd^{KD} in motor neurons were stained with *anti-X11Lα* antibody and we observed that the two proteins

significantly co-localize in the Golgi (Figure 3D-F). Therefore, we tested whether they physically bind to each other using co-immunoprecipitation/Pull-down assay in S2 cells. We observed that *UAS-X11Lmyc* pulled down *UAS-wndGFP,* but not the control GFP-tagged construct. We further validated the binding by performing the reverse binding assay and found that *UAS-wnd-GFP* pulled down myc-tagged *X11Lα* but not a control myc-tagged construct (Figure 3P). All these results indicate that Wnd and X11L physically bind to each other.

As an independent test of this physical binding, we examined the localization of Wnd-GFP in *X11* mutants background. We observed that discrete punctate localization of the *wnd-GFP* in wild type was abolished in *X11* mutants. Instead Wnd appeared more diffuse in the cell bodies (Figure 3D-I). The mis-localization of Wnd-GFP could be secondary to any change in the structure or localization of Golgi caused by *X11* mutation. To rule out this possibility, we stained *X11* mutant brains with a Golgi marker (GM130), and found no change in either the structure or localization of Golgi (Figure 3J-O). Another reason for the mis-localization of Wnd-GFP could be that the levels of Wnd-GFP is increased in *X11* mutant background. To rule out this possibility, we expressed Wnd-GFP and compared it with the Wnd-GFP levels in *X11* mutant brains. Our Western Blot analysis showed that the levels of Wnd-GFP do not change in mutants and are comparable to Wnd-GFP expression in the wildtype background (Figure 3Q). Taken together, these results indicate that X11 physically binds to Wnd and tethers Wnd to the Golgi.

The PDZ domains of X11 are important for the regulation of Wnd and axonal growth

Because X11 proteins contain one PTB domain and two PDZ domains, we tested which of these domains might play a role in the regulation of the Wnd pathway. We found that overexpression of *X11* without the PTB domain (X11ΔPTB) could suppress the overgrowth phenotype in the larval neuromuscular junction of *X11* mutants (Fig. 4A, C, D), but overexpression of *X11* without the PDZ domains (X11ΔPDZ) could not (Fig. 4A, E, F), suggesting that the PDZ domains are important for the regulation of Wnd and axonal growth. To rule out the possibility that X11ΔPDZ fails to suppress the axonal overgrowth phenotype of *X11* mutants due to lower expression level, we examined the expression levels of *UAS-X11α, UAS-X11ΔPTB*, and *UAS-X11ΔPDZ* by both immunostaining (Fig. 4H-K) and by Western Blot analysis (Figure 4L). We found that both *UAS-X11ΔPTB* and *UAS-X11ΔPDZ* are expressed at similar levels. Finally, we tested if overexpression of *UAS-X11α*, *UAS-X11ΔPTB*, or *UAS-X11ΔPDZ* would affect the localization of Wnd by co-expressing Wnd-GFP. We found that *UAS-X11α* and *UAS-X11ΔPTB* colocalize perfectly with Wnd-GFP while X11ΔPDZ does not colocalize. These data further suggest that the PDZ domains of X11 are important for the regulation of Wnd.

The N terminal of Wnd is both necessary and sufficient for the Golgi localization of Wnd:

Because X11 physically binds to Wnd and tethers it to the Golgi, we generated Wnd constructs with different regions deleted in order to identify which region of Wnd is important for its binding to X11 and its Golgi localization (Figure 5A). We overexpressed the FLAG-tagged Wnd deletion constructs in S2 cellls and co-stained the cells with FLAG and GM130, a Golgi protein, and found that overexpressing the N terminal of Wnd is

sufficient for its Golgi localization. Conversely, Wnd with only the N-terminal deleted does not localize to the Golgi, but instead localize to the cytosol. These data suggest that the N terminal of Wnd is both necessary and sufficient for the proper localization of Wnd to the Golgi.

Antereograde transport, but not retrograde transrport, is critical for *X11* induced phenotypes:

Axonal injury signal travels to the nucleus to elicit the activation of intrinsic regenerative program observed in the form of increased levels of transcriptional factors in cell body nuclei. The enhanced levels of transcription factors in nuclei thus amplify the expression of axonal growth promoting proteins to enhance regrowth after injury (Hoffman, 2010). Earlier studies have shown that axonal transport is vital for axonal regeneration after injury (Abe and Cavalli, 2008; Hanz and Fainzilber, 2006; Xiong et al., 2010). More recently, mouse DLK was identified as necessary for the retrograde transport of injury signal to the nucleus (Shin et al., 2012). We therefore tested whether *X11*-dependent enhanced regeneration phenotype is dependent on axonal transport. We blocked axonal transport by disrupting Dynactin complex critically required for dynein cargo binding (Schroer, 2004). This was achieved by expressing a DN-truncated subunit, p150/Glued Glued^{DN} (Allen et al., 1999) in motor neurons of *X11* mutants. This resulted in strong suppression of regeneration response in *X11* mutants after injury (Figure 6A-D). We infer that axonal transport plays critical role in accelerated regeneration response after injury in *X11* mutants although Glued^{DN} expression inhibit both anterograde and retrograde

transports (Allen et al., 1999; Barkus et al., 2008; Haghnia et al., 2007), so it is difficult to conclude whether it is anterograde, retrograde, or both are playing a role here.

We then examined the effects of blocking axonal transport on the NMJ growth. Interestingly, expression of Glued^{DN} in motor neurons significantly suppressed the overgrowth NMJ phenotype of *X11* mutants (Figure 6F-I). However, Glued^{DN} expression had no impact on *puc-lacZ* activation (Figire 6L-O). This indicates that activation of the Wnd pathway alone is not sufficient to cause axonal growth. We infer that anterograde transport of growth promoting factors to the injury site is also necessary for axonal growth. Taken together, these results indicate that anterograde axonal transport of cargo is required downstream of the Wnd pathway to mediate axonal regeneration.

X11 proteins are degraded by CalpB after axonal injury:

We have shown that *X11* regulates the Wnd pathway during development by restraining Wnd to the Golgi. Loss of *X11* causes mislocalization of Wnd to the cytosol that leads to the overactivation of the Wnd pathway. We hypothesize that in injury situation, loss or mislocalization of *X11* in the axon may be the reason the Wnd pathway is activated. Earlier studies have reported changes in the levels of X11α in the hippocampus of animal models of disease for epilepsy (Scorza et al., 2003) and ischemia (Nishimura et al., 2000). They showed that the levels of X11α dramatically decrease immediately after an assault before it increases again. Therefore, we examined the levels of X11 in injured axons. Although our immunostaining analysis did not reveal any change in the levels or localization of X11 protein after injury in the axon or cell body, Western Blot analysis of

the whole brains after the nerve crush injury (crushing all the axons just outside the VNC in segment A1) showed a decrease in X11 levels (Figure 7A). One of the following three reasons could explain why we do not observe a decrease in levels of X11 protein after injury. First, that the decrease in the levels of X11 is temporary and very short lived to capture. Second, the immune-staining may be a less sensitive assay to appreciate a decrease in X11 levels. Third, the antibody may still be able to detect the fragmented products of the protein.

Axonal injury triggers rapid Ca^{2+} influx into the cell cytoplasm one of the earliest events that ultimately leads to axonal regeneration in *C.elegans* (Ghosh-Roy et al., 2010). We hypothesized that elevating Ca^{2+} levels in the cell could degrade X11. To test this possibility, we used the S2 cell cultures. Treatment of S2 cells with 5uM ionomycin (Ca^{2+} ionophore) for 30 minutes caused partial degradation whereas longer duration caused complete degradation of X11, suggesting that Ca^{2+} influx may lead the X11 degradation (Figure 7B). Earlier studies have reported that X11 proteins are degraded by the Calpains in a neurodegeneration model (Yoon et al., 2007). Therefore, to probe further into the mechanisms of X11 degradation, we treated S2 cells with ionomycin together with the Calpain inhibitor calpeptin and found that calpeptin strongly blocked the degradation of X11 (Figure 7C). This indicates that X11 is degraded by Calpains. There are four *calpain* genes in *Drosophila* (*calpa, calpb, calpc, and calpd*) (Friedrich et al., 2004). To identify whether there is a specific *calp* gene responsible for the degradation of X11 in *Drosophila*, we knockdown each of the 4 *calp* gene independently. Interestingly, only knockdown of *calpb* completely blocks the degradation of X11 upon ionomycin treatment but not the

others (Figure 6D). Taken together, these results indicate that X11 is degraded specifically by CalpB after Ca^{2+} influx. We interpret that X11 is degraded by CalpB after axonal injury and this degradation of X11 cause activation of the Wnd pathway that leads to regenerative responses.

Discussions:

The DLK kinases are key regulators of axonal regeneration after injury across animal kingdom from worms to mammals (Hammarlund et al., 2009; Shin et al., 2012; Xiong and Collins, 2012; Xiong et al., 2010; Yan et al., 2009). Here, we report the discovery of an upstream regulator of DLK/Wnd pathway and showed that it regulates axonal regeneration. We demonstrated that axonal regeneration is enhanced in *X11* mutants, and enhanced regeneration of axons in *X11* mutants is abrogated by inhibition of *wnd* and its downstream targets. We presented data showing that in the motor neurons, X11 and Wnd proteins coalize at Golgi. In *X11* mutants, we observed that Wnd no longer localizes to the Golgi but instead localize to the cytosol. In addition, in *X11* mutants, the Wnd pathway is activated as indicated by the increased expression of the *puc-lacz* reporter. We showed *in vitro* evidence that X11 and Wnd proteins physically bind to each other. We also showed that the PDZ domains of are important for its regulation of Wnd. In addition, the N terminal region of Wnd is both necessary and sufficient for the Golgi localization of Wnd. We demonstrated that anterograde axonal transport of cargo is required downstream of the *wnd* pathway to regulate regenerative growth response after axotomy. Finally, we showed that the levels of X11 proteins decrease on injury and provide *in vitro* evidence that X11 is degraded by *calpb* upon an increase of intracellular

calcium. Our findings indicate that X11/Mint proteins negatively regulate synaptic growth and axonal regeneration after axotomy via the *wnd/Jnk* pathway.

How does *X11* regulate Wnd?

The key finding of our study is that X11 localizes Wnd to the Golgi network. We observed that discrete X11 punctate inside the motor neuronal cell body show colocalization with the cis-Golgi marker GRASP-65-GFP indicating that X11 localizes to the Golgi. We also confirmed that X11 and Wnd proteins colocalize on the Golgi network and physically binds to each other. Finally, we demonstrated that in absence of *X11* function Wnd is no longer localized to the Golgi and is instead mislocalized to the cytosol. This mislocalization of Wnd to cytosol leads to activation of the *wnd/Jnk* pathway that causes axonal regeneration. So the question raised is what could be the mechanism of regulation of *wnd/Jnk* pathway by *X11*? Mammalian DLK has been shown to occur in 2 different pools, membrane associated non-phosphorylated form (inactive) and the cytosolic phosphorylated (active) pool (Mata et al., 1996). Based on the observation that Wnd protein localize to the cytosol in X11 mutant background, we speculate that Wnd though mainly localized to Golgi may have a small active part of it diffuse in the cytosol like its mammalian homologue (DLK) that cannot be detected by available antibody. A better Wnd antibody would give a more reliable readout as the available antibody is unable to detect endogenous in neurons. X11/Mint proteins through their protein binding domains interact with the multitude of proteins and thus mediate assembly of functional multiple protein complexes (Rogelj et al., 2006). Based on these arguments, we believe that *X11* plays a role in constraining Wnd on the Golgi (probably in a mutliprotein complex) and

releases it to the cytosol where it gets activated and in turn transduces signals to downstream molecules in the pathway. In the absence of *X11*, the restrain on Wnd is gone and as our data indicated, Wnd is no longer localized to the Golgi but instead mislocalized to the cytosol. The cytosolic Wnd then activates its downstream targets.

_X11_ proteins act as intermediates between the injury and Wnd activation.

Several observations suggest a link between neuronal injury and *X11* mutation. First, there is a remarkable similarity in the pattern of the *Jnk* pathway activation as indicated by *puc-lacZ* reporter assay between *X11* mutant and injured neurons. Second, both injury and *X11* mutation lead to Wnd activation, as the expression of dominant negative wallenda (*UAS-wnd*^{DN}) blocks the activation of the Wnd pathway in individual as well as injury in the *X11* mutants background (Figure 2M-R, Figure 5L-Q). Third, the axonal regeneration phenotype is significantly enhanced in injured *X11* mutants. Forth, X11 protein is degraded by CalpB as a result of increase in Ca^{2+} levels immediately after injury. Previous reports indicate that mammalian homologues of X11 (Mints) are targeted by Calpains *in vitro* during neuronal assault (Yoon et al., 2007). Moreover the levels of X11 protein have been shown to change following different neuronal insults i.e, Epilepsy and Ischemia models (Nishimura et al., 2000; Scorza et al., 2003). Furthermore, previous studies have shown that a rapid increase in Ca^{2+} on injury promotes axon regeneration in a DLK dependent manner (Ghosh-Roy et al., 2010). Based on these findings and our own data, we envision the following mechanism for the action of X11 during injury: Injury induces increase of intracellular Ca^{2+} levels that results in the activation of CalpB. Subsequently, X11 is degraded by CalpB. The loss of *X11* in turn leads to activation of

the Wnd pathway. Importantly, in *C. elegans,* DLK has recently been shown to be regulated by Ca^{2+}-mediated switch from an inactive to an active state (Yan and Jin, 2012) and mouse DLK activity changes with the change in Ca^{2+} levels inside the cell in cell culture (Mata et al., 1996). Recent studies in *C. elegans* also show that DLK could be activated by calcium directly (Yan and Jin, 2012) or indirectly (Pinan-Lucarre et al., 2012). Our data indicates a third mechanism that calcium can regulate Wnd is through the degradation of X11s.

Our genetic data clearly demonstrates that *X11/Mint* acts upstream of the Wnd pathway. Earlier studies have reported that *Hiw* and its *C.elegans* homologue RPM-1 also negatively regulate DLK/Wnd to mediate the synaptic growth and regeneration after injury (Collins et al., 2006; Hammarlund et al., 2009; Nakata et al., 2005; Xiong et al., 2010; Yan et al., 2009). Moreover, mouse homologue of *Hiw*, Phr1 and human Pam (Lewcock et al., 2007) also regulate DLK during synapse development. *Hiw* E3 ubiquitin ligase regulates the DLK/Wnd pathway by negatively controlling the levels of Wnd protein (Collins et al., 2006). Similarly, in *C. elegans* RPM-1 can stimulate ubiquitination of DLK and regulate its levels in the motor neurons (Nakata et al., 2005; Park et al., 2009). Our data clearly indicates that like *Hiw*, *X11/Mint* also acts upstream of wnd to downregulate the *wnd/Jnk* pathway. It will be of interest to determine whether the two proteins act in same or different pathways and how do the two regulators of the pathway play out in absence of one another if they regulate the pathway independently.

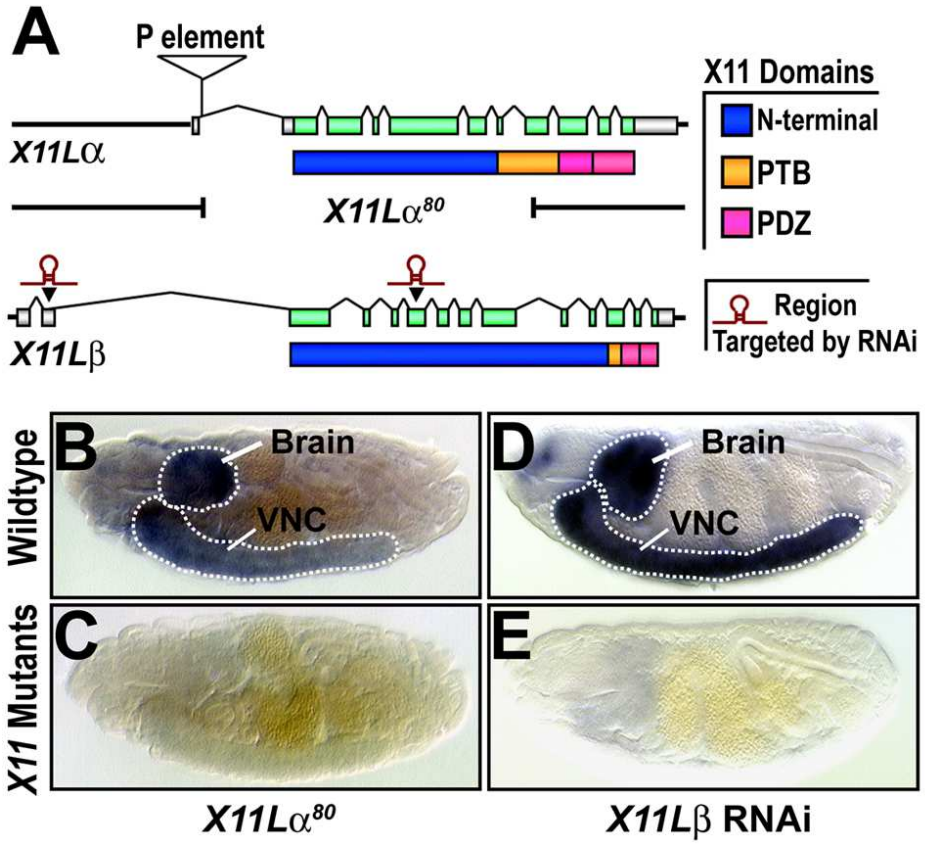


Figure 2-1. mRNA expressions of X11Lα and X11Lβ are restricted to the differentiated CNS. (A) Schematic depicting *Drosophila X11* paralogs *X11L*α and *X11L*β. X11 proteins consist of a N-terminal domain, PTB domain, and two PDZ domains. Loss-of-function mutant *X11L* α*80* was generated by imprecise excision of a P element inserted in the 5' UTR, which also excised a translational start codon, the N-terminal domain, and part of the PTB domain. *X11L*β was targeted by RNAi technology at two independent regions.

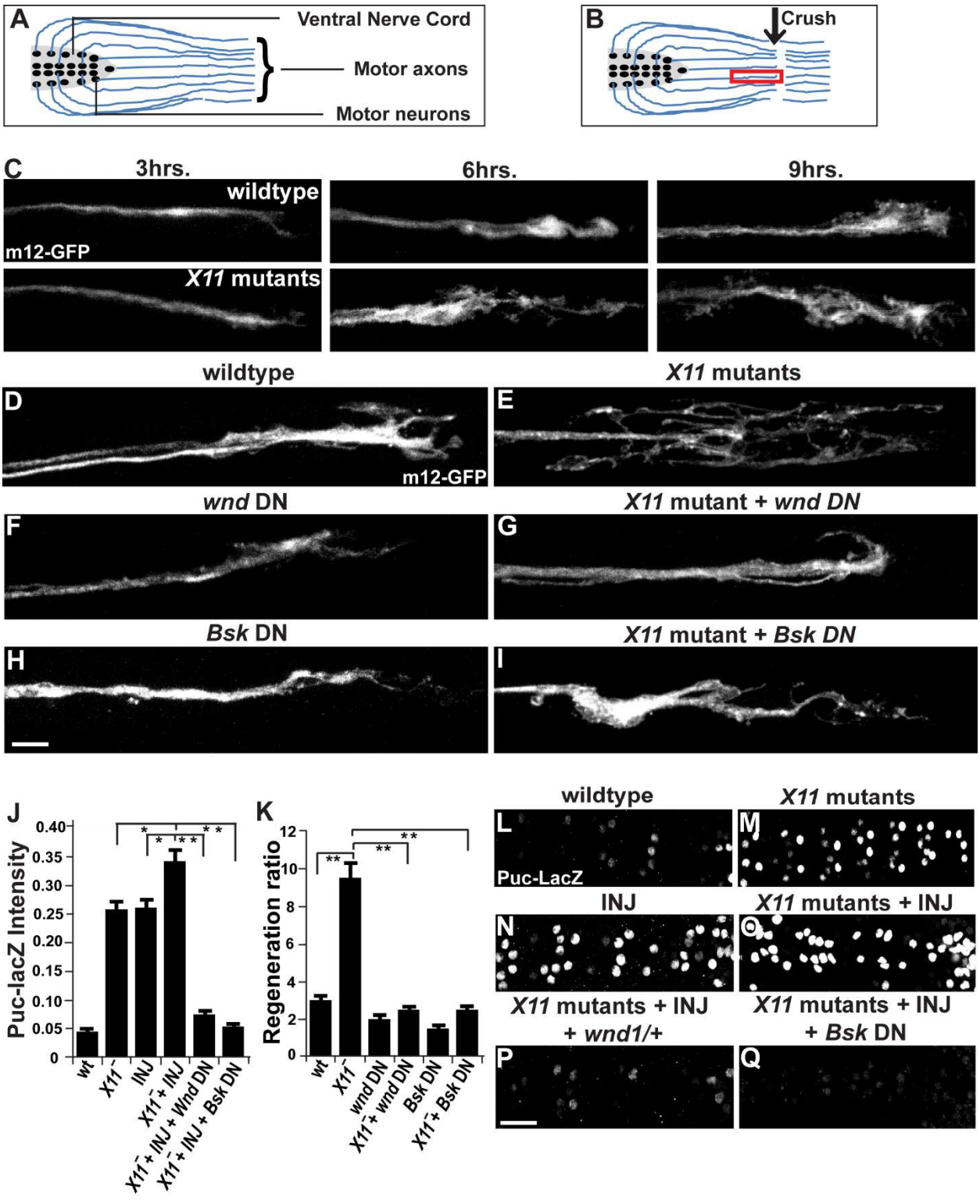

A
Ventral Nerve Cord
Motor axons
Motor neurons
B
Crush
C
3hrs.
6hrs.
9hrs.
wildtype
m12-GFP
X11 mutants
wildtype
X11 mutants
D
m12-GFP
E
wnd DN
X11 mutant + wnd DN
F
G
Bsk DN
X11 mutant + Bsk DN
H
I
J
Puc-lacZ Intensity
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
wt
X11-
INJ
X11- + INJ
X11- + INJ + Wnd DN
X11- + INJ + Bsk DN
K
Regeneration ratio
12
10
8
6
4
2
0
wt
X11-
wnd DN
X11- + wnd DN
Bsk DN
X11- + Bsk DN
wildtype
X11 mutants
L
Puc-LacZ
M
INJ
X11 mutants + INJ
N
O
X11 mutants + INJ + wnd1/+
X11 mutants + INJ + Bsk DN
P
Q

Figure 2-2. X11 mutants have enhanced axonal regeneration that is dependent on the Wnd pathway. (A) Schematics depicting third instar larval ventral nerve cord, motor neurons, and motor axons. (B) Schematics depicting third instar larval ventral nerve cord, motor neurons, and motor axons that is injured by a crush. (C-I) Larval motor axons labeled with UAS-mCD8-GFP driven with m12-gal4. (C) Regeneration of axons in wildtype and in X11 mutants at different time points (3hrs, 6hrs, and 9 hrs). (D) m12-gal4/+; UAS-mCD8-GFP/+ (Wiltype). (E) *X11Lα80, RNAi-X11Lβ/+; m12-gal4/+; UAS-mCD8-GFP/+ (X11 mutants),* (F) m12-gal4/UAS-Wnd^{DN}; UAS-mCD8-GFP/+ (UAS-Wnd^{DN}), (G) *X11Lα80, RNAi-X11Lβ/+; m12-gal4/*UAS-Wnd^{DN}*; UAS-mCD8-GFP/+ (X11 mutants* + UAS-Wnd^{DN}*).* (H) UAS-bsk^{DN}; m12-gal4/+; UAS-mCD8-GFP/+ (bsk^{DN}), (I) UAS-bsk^{DN}; m12-gal4/+; UAS-mCD8-GFP/RNAi-*X11Lα, RNAi-X11Lβ* (bsk^{DN}+*X11* mutant). (J) Quantification of the puc-lacz intensities of L-Q. (K) Quantification of the regeneration ratio of D-I. (L-Q) puc-lacz staining of various gentypes. (L) ok6-gal4/+; puc-lacz/+ (wildtype). (M) *X11Lα80, UAS-RNAi-X11Lβ/+;* ok6-gal4/+; puc-lacz/+. (N) ok6-gal4/+;pucl-lacz/+ (Injured wildtype). (O) *X11Lα80, UAS-RNAi-X11Lβ/+;* ok6-gal4/+; puc-lacz/+ (Injured *X11* mutants). (P) *X11Lα80, UAS-RNAi-X11Lβ/+;* ok6-gal4/+; puc-lacz/wnd^{-1} (Injured *X11* mutants + loss-of-wnd). (Q) UAS-bsk^{DN}; ok6-gal4/+; puc-lacz/UAS-RNAi-*X11Lα, RNAi-X11Lβ* (Injured *X11* mutants + bsk^{DN}).

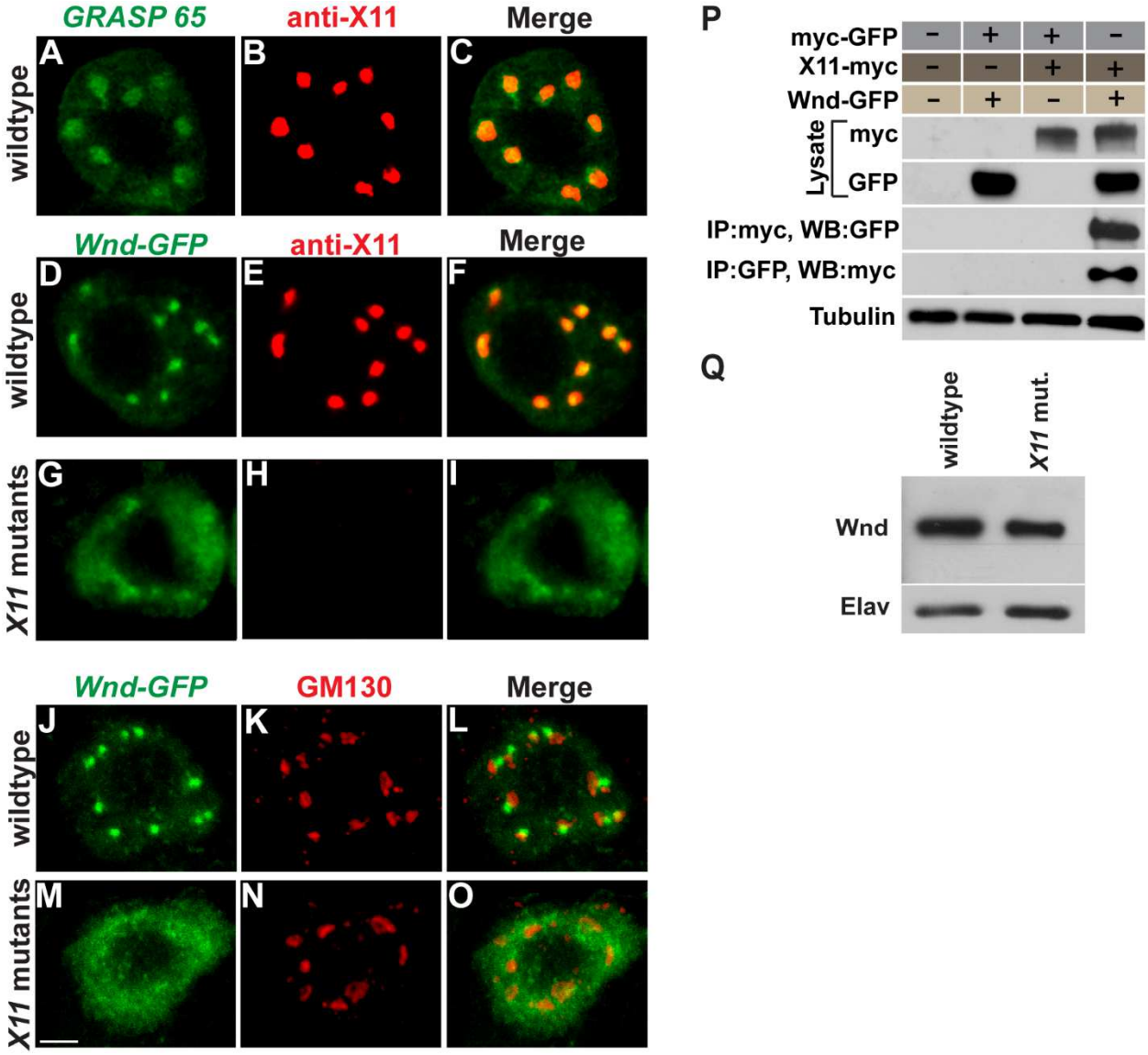


Figure 2-3. X11 physically binds to Wnd and tethers it to the Golgi. (A-O) Cell bodies of the third instar larval motor neurons. (A-F, J-K) wildtype. (A-C) UAS-GRASP65-GAP/ok6-gal4. (A) UAS-GRASP65-GFP (Golgi). (B) anti-X11. (C) Merged view of A and B. (D-F) ok6-gal4/+; UAS-GFP-Wnd-KD. (D) UAS-GFP-Wnd-KD. (E, H) anti-X11. (F) Merged view of D and E. (G-I, M-O) *X11Lα80, RNAi-X11Lβ/y; ok6-gal4/+; UAS-GFP-*

Wnd-KD/+ (X11 mutants). (K-N) anti-GM130 (Golgi marker). (L-O) merged view of J,K and M,N. (P) Co-immunoprecipitation of X11-Myc and Wnd-GFP. (Q) Wnd level in wildtype and *X11* mutants.

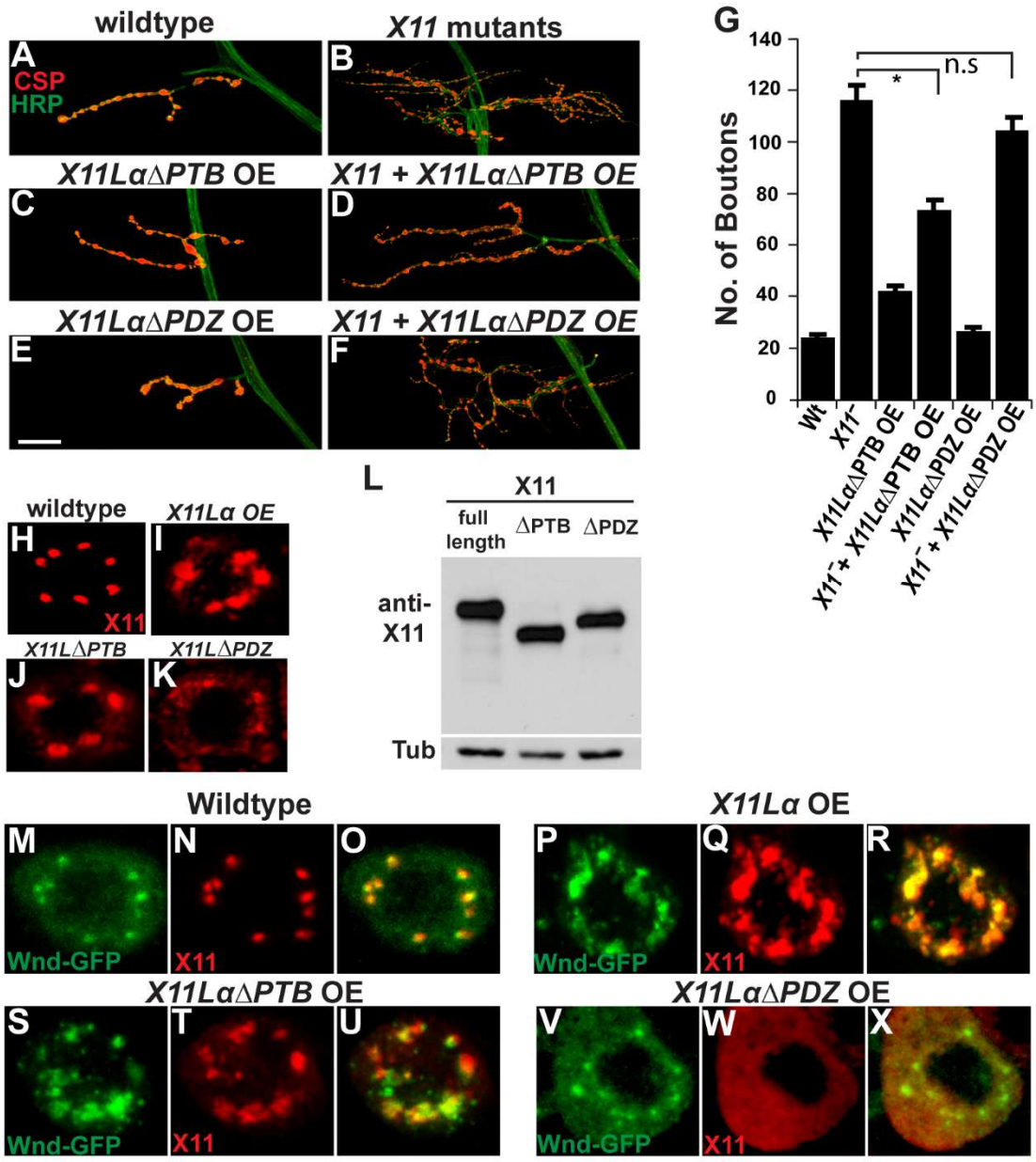


Figure 2-4. PDZ domain of X11 is important for the regulation of Wnd and axonal growth. (A-F) Third-instar larval NMJ synapses at muscle 4 are stained with the neuronal membrane marker HRP (green) and synaptic vesicle marker CSP (red). (A) wildtype, (B) *X11Lα80,* RNAi *X11L*β/+; ok6-gal4/+ (X11 mutants), (C) UAS- *X11L*αΔPTB/ok6-gal4 (*X11L*αΔPTB overexpression), (D) *X11Lα80,* RNAi *X11L*β/y; ok6-gal4/UAS-*X11L*αΔPTB)

(X11 mutants+ *X11L*αΔPTB overexpression), (E) ok6-gal4 /+; UAS-*X11L*αΔPDZ/+ (*X11L*αΔPDZ overexpression), (F) *X11Lα80,* RNAi *X11L*β/y; ok6-gal4/+; UAS-*X11L*αΔPDZ) (X11 mutants + *X11L*αΔPDZ overexpression). (G) Quantification of the average number of boutons of A-F. (H-K) Third-instar larval motor neurons stained with anti-X11. (H) wildtype, (I) UAS-*X11L*α/ok6-gal4, (J) UAS- *X11L*αΔPTB/ok6-gal4, (K) ok6-gal4 /+; UAS-*X11L*αΔPDZ/+. (L) Western blot analysis of adult head of UAS-*X11L*α, UAS-*X11L*αΔPTB, and UAS-*X11L*αΔPDZ expressed with GMR-gal4. (M-X) Overexpression of GFP-Wnd-KD with ok6-gal4 in the third instar larval motor neuron stained with anti-X11. (M, P, S, V) GFP-Wnd-KD. (N, Q, T, W) anti-X11. (O, R, U, X) merged view. (M-O) wildtype. (P-R) UAS-*X11L*α/ok6-gal4. (S-U) UAS-*X11L*αΔPTB. (V-X) UAS-*X11L*αΔPDZ.

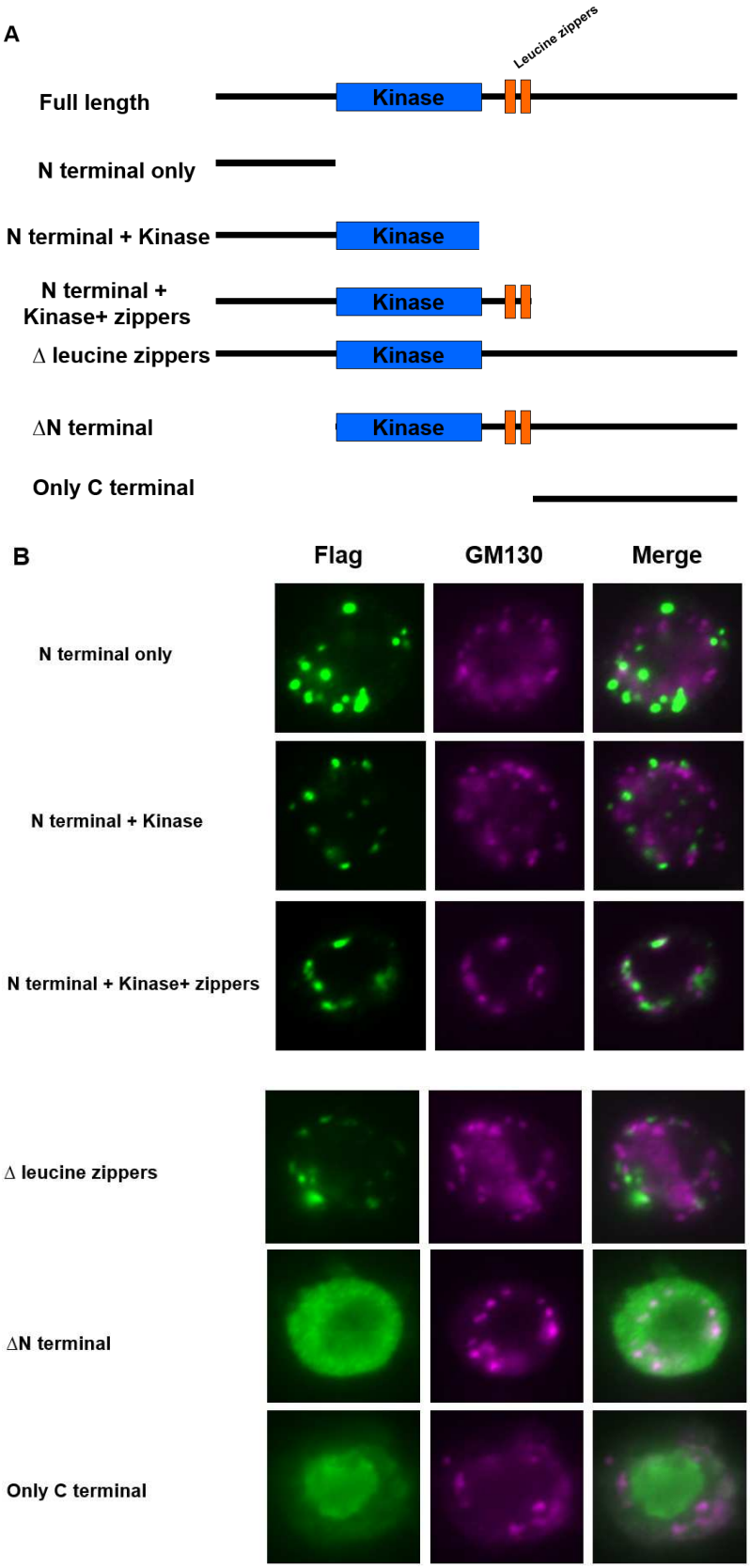

A
Leucine zippers
Full length
Kinase
N terminal only
N terminal + Kinase
Kinase
N terminal +
Kinase+ zippers
Kinase
Δ leucine zippers
Kinase
ΔN terminal
Kinase
Only C terminal
B
Flag
GM130
Merge
N terminal only
N terminal + Kinase
N terminal + Kinase+ zippers
Δ leucine zippers
ΔN terminal
Only C terminal

Figure 2-5. The N-terminal of Wnd is necessary and sufficient of Golgi localization of Wnd.

(A) Deletion constructs of Wnd with different regions or domains deleted. The following constructs were generated: UAS-FLAG-Wnd, UAS-FLAG-N-terminal-Wnd, UAS-FLAG-N-terminal-Kinase-Wnd, UAS-FLAG-N-terminal-Kinase-Zippers-Wnd, UAS-FLAG-Wnd-Δleucinezippers, UAS-FLAG-Wnd-ΔN-terminal, and UAS-FLAG-Wnd-C-terminal. (B) immunostaining of S2 cells overexpressing FLAG-tagged Wnd constructs. Magenta is GM130, a Golgi marker. Green is FLAG.

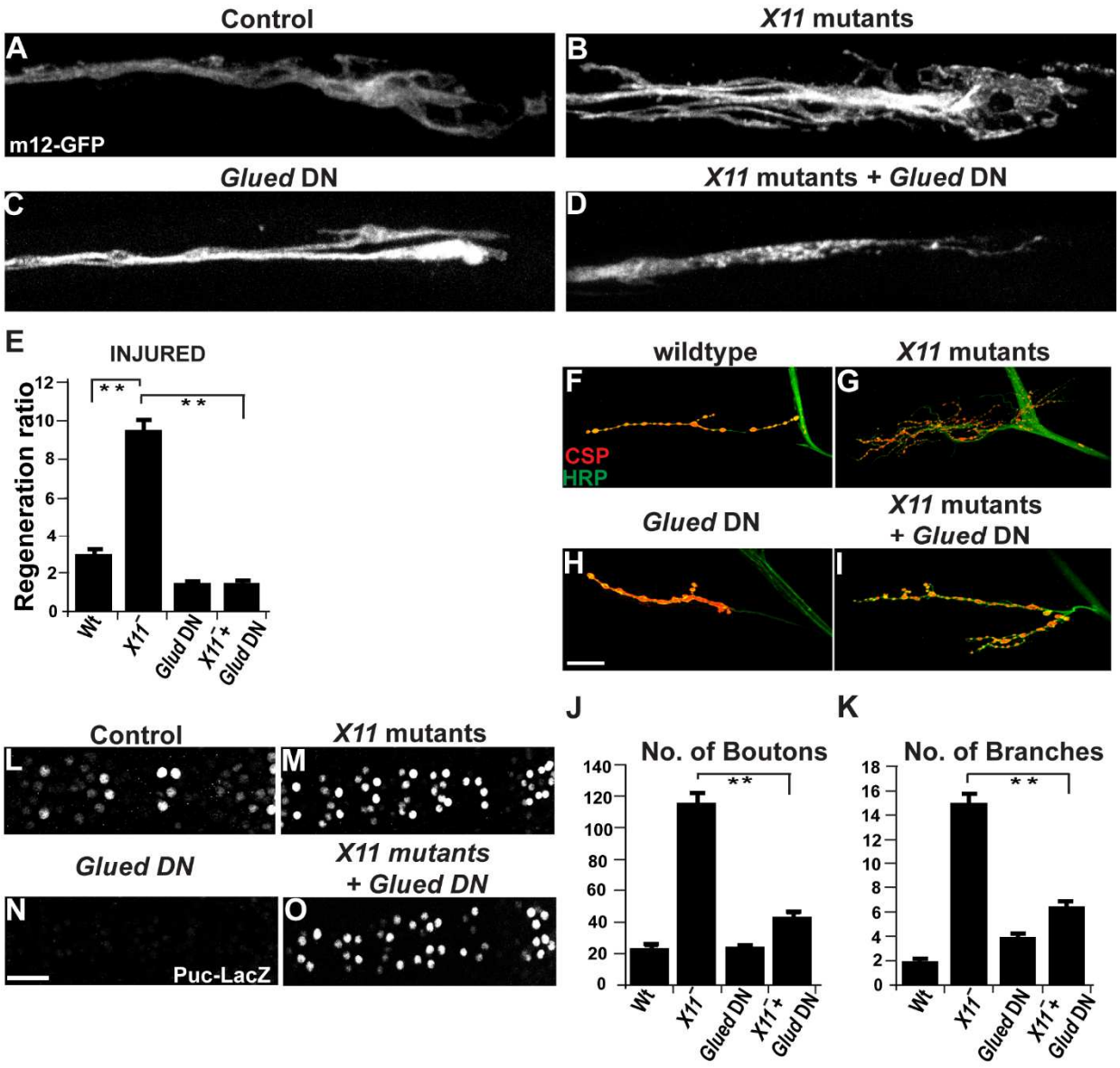


Figure 2-6 Glued^{DN} is required for axonal regeneration and NMJ overgrowth of *X11* mutant, but not puc-lacz activation. (A-D) Axons are labeled by driving expression of *UAS-mCD8-GFP* by *m12-Gal4*. Axons from (A) *UAS-mCD8-GFP/+; m12-gal4/+* (wildtype), (B) *X11Lα80,* RNAi *X11L*β/y; *UAS-mCD8-GFP/+; m12-gal4/+* (*X11* mutants), (C) *UAS-mCD8-GFP/UAS-Glued DN; m12-gal4/+* (Glued DN overexpression), (D) *X11Lα80,* RNAi *X11L*β/y; *UAS-mCD8-GFP/UAS-Glued DN; m12-gal4/+* (*X11* mutants +

Glued DN overexpression). (E) Quantification of the regeneration ratio of A-D. (F-I) Third-instar larval NMJ synapses at muscle 4 are stained with the neuronal membrane marker HRP (green) and synaptic vesicle marker CSP (red). (F) wildtype, (G) *X11Lα80,* RNAi *X11L*β/y; *ok6-gal4/+* (*X11* mutants), (H) *UAS-Glued DN/ok6-gal4* (*Glued DN* overexpression), (I) *X11Lα80,* RNAi *X11L*β/y; *ok6-gal4/UAS-Glued DN* (*X11* mutants + GlueDN overexpression). (J) Quantification of the average number of boutons of F-I. (K) Quantification of the number of branches of F-I. (L-O) Larval ventral nerve cords stained for puc-lacz expression with anti-βgal. (L) *ok6-gal4/+ ; puc-lacz/+* (control), (M) *X11Lα80,* RNAi *X11L*β/y; *ok6-gal4/+* (*X11* mutants), (N) *ok6-gal4/UAS-Glued DN; puc-lacz/+* (*Glued DN* overexpression), (O) *X11Lα80,* RNAi *X11L*β/y; *ok6-gal4/UAS-Glued DN; puc-lacz/+ (X11* mutant + *Glued DN*).

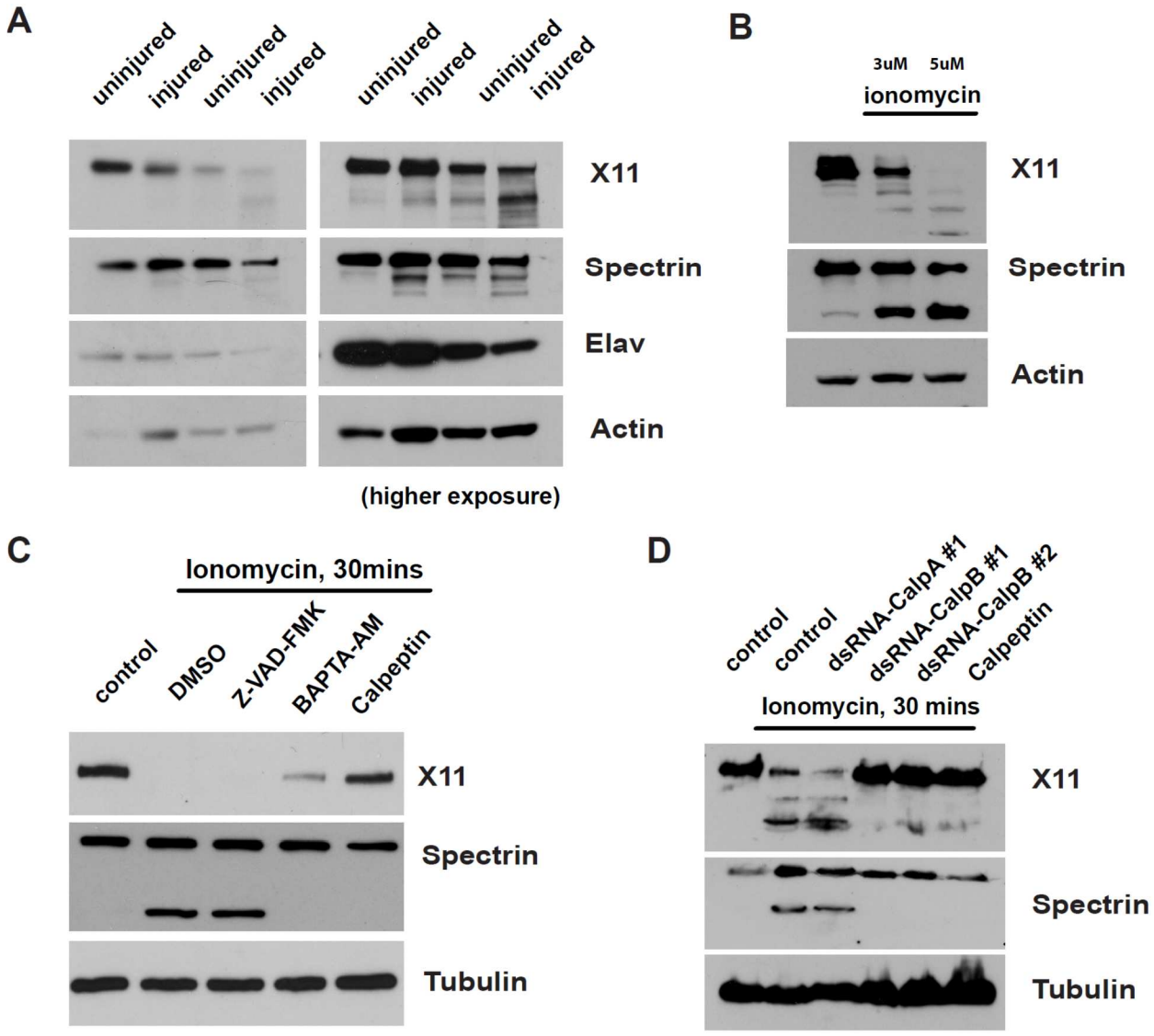


Figure 2-7. CalpB degrades X11 after injury. (A) Western blot analysis of the brain lysates of uninjured larvae and injured larvae. (B) Western blot analysis of S2 cells lysates that are treated with 3uM and 5uM of ionomycin and 1uM of calcium for 10 mins. (C) Western blot analysis of S2 cells lysates treated with ionomycin for 30mins with various inhibitors (Z-VAD-FMK: apoptosis inhibitor, BAPTA-AM: calcium chelator, calpeptin:

Calpain inhibitor). (D) Western blot analysis of S2 cells lysates treated with dsRNA to CalpA and CalpB.

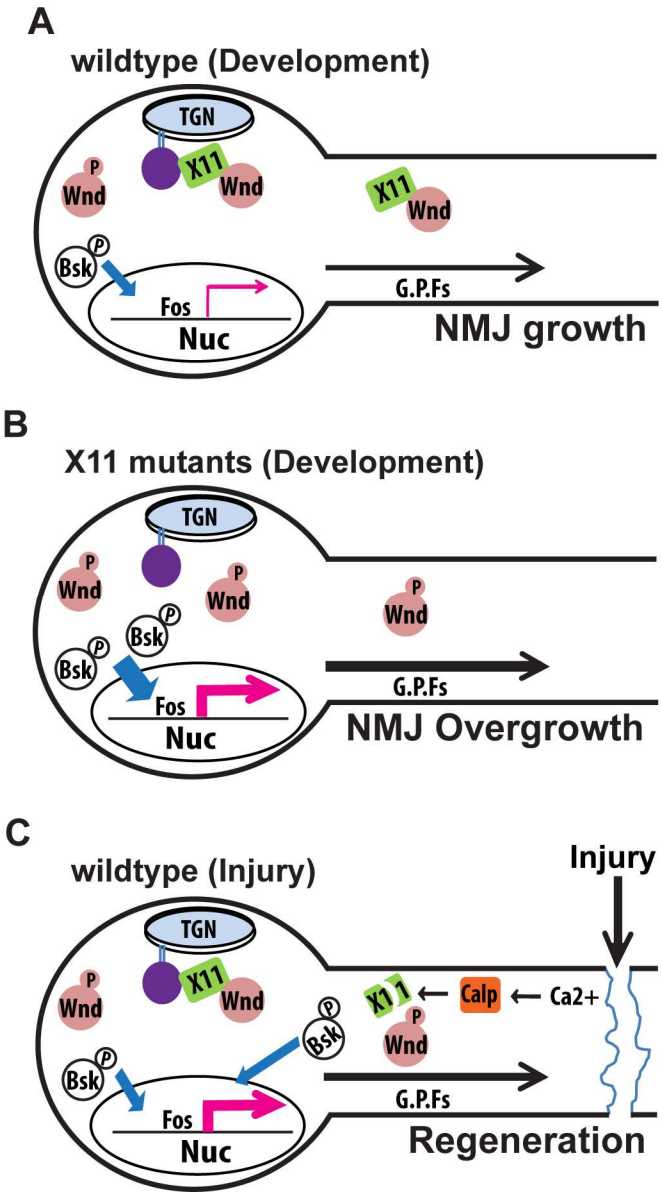
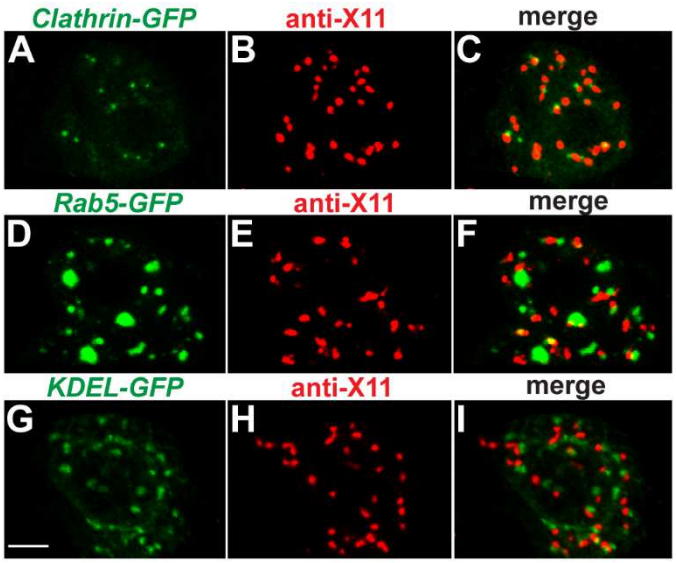


Figure 2-8. Summary diagram of X11 function during development and after injury. (A) During normal development, X11 proteins anchor Wnd to the Golgi apparatus and keep it inactive. (B) In *X11* mutant, Wnd localizes to the cytosol instead of the Golgi apparatus and activates downstream MAP kinase signaling to cause NMJ overgrowth. (C) After injury, influx of calcium activates CalpB, which degrades X11. Degradation of

X11 in turns lead to the release of Wnd from the Golgi to the cytosol, where it activates downstream signaling pathway and promotes axonal regeneration.



Supplementary Figure S2-1. X11 localization with different subcellular markers. (A-I) Third-instar larval motor neuron overexpressing ok6-gal4. (A) Clathrin-GFP, (B,E,H) Stained with anti-X11. (D) Rab5-GFP, (E) KDEL-GFP, (C-I) Merged views of motor neurons.

References

Abe, N., and Cavalli, V. (2008). Nerve injury signaling. Curr Opin Neurobiol *18*, 276-283.

Aberle, H., Haghighi, A.P., Fetter, R.D., McCabe, B.D., Magalhaes, T.R., and Goodman, C.S. (2002). wishful thinking encodes a BMP type II receptor that regulates synaptic growth in Drosophila. Neuron *33*, 545-558.

Allen, M.J., Shan, X., Caruccio, P., Froggett, S.J., Moffat, K.G., and Murphey, R.K. (1999). Targeted expression of truncated glued disrupts giant fiber synapse formation in Drosophila. J Neurosci *19*, 9374-9384.

Ashley, J., Packard, M., Ataman, B., and Budnik, V. (2005). Fasciclin II signals new synapse formation through amyloid precursor protein and the scaffolding protein dX11/Mint. J Neurosci *25*, 5943-5955.

Barkus, R.V., Klyachko, O., Horiuchi, D., Dickson, B.J., and Saxton, W.M. (2008). Identification of an axonal kinesin-3 motor for fast anterograde vesicle transport that facilitates retrograde transport of neuropeptides. Mol Biol Cell *19*, 274-283.

Biederer, T., and Sudhof, T.C. (2000). Mints as adaptors. Direct binding to neurexins and recruitment of munc18. J Biol Chem *275*, 39803-39806.

Blouin, R., Beaudoin, J., Bergeron, P., Nadeau, A., and Grondin, G. (1996). Cell-specific expression of the ZPK gene in adult mouse tissues. DNA Cell Biol *15*, 631-642.

Borg, J.P., Lopez-Figueroa, M.O., de Taddeo-Borg, M., Kroon, D.E., Turner, R.S., Watson, S.J., and Margolis, B. (1999). Molecular analysis of the X11-mLin-2/CASK complex in brain. J Neurosci *19*, 1307-1316.

Borg, J.P., Straight, S.W., Kaech, S.M., de Taddeo-Borg, M., Kroon, D.E., Karnak, D., Turner, R.S., Kim, S.K., and Margolis, B. (1998). Identification of an evolutionarily

conserved heterotrimeric protein complex involved in protein targeting. J Biol Chem *273*, 31633-31636.

Bradke, F., Fawcett, J.W., and Spira, M.E. (2012). Assembly of a new growth cone after axotomy: the precursor to axon regeneration. Nat Rev Neurosci *13*, 183-193.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Busch, S.A., and Silver, J. (2007). The role of extracellular matrix in CNS regeneration. Curr Opin Neurobiol *17*, 120-127.

Butz, S., Okamoto, M., and Sudhof, T.C. (1998). A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. Cell *94*, 773-782.

Cavalli, V., Kujala, P., Klumperman, J., and Goldstein, L.S. (2005). Sunday Driver links axonal transport to damage signaling. J Cell Biol *168*, 775-787.

Chierzi, S., Ratto, G.M., Verma, P., and Fawcett, J.W. (2005). The ability of axons to regenerate their growth cones depends on axonal type and age, and is regulated by calcium, cAMP and ERK. Eur J Neurosci *21*, 2051-2062.

Cho, Y., Sloutsky, R., Naegle, K.M., and Cavalli, V. (2013). Injury-induced HDAC5 nuclear export is essential for axon regeneration. Cell *155*, 894-908.

Collins, C.A., and DiAntonio, A. (2007). Synaptic development: insights from Drosophila. Curr Opin Neurobiol *17*, 35-42.

Collins, C.A., Wairkar, Y.P., Johnson, S.L., and DiAntonio, A. (2006). Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron *51*, 57-69.

DiAntonio, A., Petersen, S.A., Heckmann, M., and Goodman, C.S. (1999). Glutamate receptor expression regulates quantal size and quantal content at the Drosophila neuromuscular junction. J Neurosci *19*, 3023-3032.

Eresh, S., Riese, J., Jackson, D.B., Bohmann, D., and Bienz, M. (1997). A CREB-binding site as a target for decapentaplegic signalling during Drosophila endoderm induction. EMBO J *16*, 2014-2022.

Friedrich, P., Tompa, P., and Farkas, A. (2004). The calpain-system of Drosophila melanogaster: coming of age. Bioessays *26*, 1088-1096.

Ghosh-Roy, A., Wu, Z., Goncharov, A., Jin, Y., and Chisholm, A.D. (2010). Calcium and cyclic AMP promote axonal regeneration in Caenorhabditis elegans and require DLK kinase. J Neurosci *30*, 3175-3183.

Gitler, D., and Spira, M.E. (2002). Short window of opportunity for calpain induced growth cone formation after axotomy of Aplysia neurons. J Neurobiol *52*, 267-279.

Grenningloh, G., Rehm, E.J., and Goodman, C.S. (1991). Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. Cell *67*, 45-57.

Gross, G.G., Feldman, R.M., Ganguly, A., Wang, J., Yu, H., and Guo, M. (2008). Role of X11 and ubiquilin as in vivo regulators of the amyloid precursor protein in Drosophila. PLoS One *3*, e2495.

Gross, G.G., Lone, G.M., Leung, L.K., Hartenstein, V., and Guo, M. (2013). X11/Mint genes control polarized localization of axonal membrane proteins in vivo. J Neurosci *33*, 8575-8586.

Guo, M., Hong, E.J., Fernandes, J., Zipursky, S.L., and Hay, B.A. (2003). A reporter for amyloid precursor protein gamma-secretase activity in Drosophila. Hum Mol Genet *12*, 2669-2678.

Haghnia, M., Cavalli, V., Shah, S.B., Schimmelpfeng, K., Brusch, R., Yang, G., Herrera, C., Pilling, A., and Goldstein, L.S. (2007). Dynactin is required for coordinated bidirectional motility, but not for dynein membrane attachment. Mol Biol Cell *18*, 2081-2089.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.

Hanz, S., and Fainzilber, M. (2006). Retrograde signaling in injured nerve--the axon reaction revisited. J Neurochem *99*, 13-19.

Hase, M., Yagi, Y., Taru, H., Tomita, S., Sumioka, A., Hori, K., Miyamoto, K., Sasamura, T., Nakamura, M., Matsuno, K.*, et al.* (2002). Expression and characterization of the Drosophila X11-like/Mint protein during neural development. J Neurochem *81*, 1223-1232.

Hoffman, P.N. (2010). A conditioning lesion induces changes in gene expression and axonal transport that enhance regeneration by increasing the intrinsic growth state of axons. Exp Neurol *223*, 11-18.

Hortsch, M., Bieber, A.J., Patel, N.H., and Goodman, C.S. (1990). Differential splicing generates a nervous system-specific form of Drosophila neuroglian. Neuron *4*, 697-709.

Johansen, J., Halpern, M.E., Johansen, K.M., and Keshishian, H. (1989). Stereotypic morphology of glutamatergic synapses on identified muscle cells of Drosophila larvae. J Neurosci *9*, 710-725.

Keshishian, H., Broadie, K., Chiba, A., and Bate, M. (1996). The drosophila neuromuscular junction: a model system for studying synaptic development and function. Annu Rev Neurosci *19*, 545-575.

Kittel, R.J., Wichmann, C., Rasse, T.M., Fouquet, W., Schmidt, M., Schmid, A., Wagh, D.A., Pawlu, C., Kellner, R.R., Willig, K.I.*, et al.* (2006). Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. Science *312*, 1051-1054.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451-461.

Lewcock, J.W., Genoud, N., Lettieri, K., and Pfaff, S.L. (2007). The ubiquitin ligase Phr1 regulates axon outgrowth through modulation of microtubule dynamics. Neuron *56*, 604-620.

Leyssen, M., Ayaz, D., Hebert, S.S., Reeve, S., De Strooper, B., and Hassan, B.A. (2005). Amyloid precursor protein promotes post-developmental neurite arborization in the Drosophila brain. EMBO J *24*, 2944-2955.

Li, Z., Hogan, E.L., and Banik, N.L. (1996). Role of calpain in spinal cord injury: increased calpain immunoreactivity in rat spinal cord after impact trauma. Neurochem Res *21*, 441-448.

Mackler, J.M., Drummond, J.A., Loewen, C.A., Robinson, I.M., and Reist, N.E. (2002). The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo. Nature *418*, 340-344.

Martin-Blanco, E. (1997). Regulation of cell differentiation by the Drosophila Jun kinase cascade. Curr Opin Genet Dev *7*, 666-671.

Mata, M., Merritt, S.E., Fan, G., Yu, G.G., and 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. J Biol Chem *271*, 16888-16896.

Nakata, K., Abrams, B., Grill, B., Goncharov, A., Huang, X., Chisholm, A.D., and Jin, Y. (2005). Regulation of a DLK and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell *120*, 407-420.

Nishimura, H., Matsuyama, T., Obata, K., Nakajima, Y., Kitano, H., Sugita, M., and Okamoto, M. (2000). Changes in mint1, a novel synaptic protein, after transient global ischemia in mouse hippocampus. J Cereb Blood Flow Metab *20*, 1437-1445.

Park, E.C., Glodowski, D.R., and Rongo, C. (2009). The ubiquitin ligase RPM-1 and the p38 MAPK PMK-3 regulate AMPA receptor trafficking. PLoS One *4*, e4284.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M.*, et al.* (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science *322*, 963-966.

Parnas, D., Haghighi, A.P., Fetter, R.D., Kim, S.W., and Goodman, C.S. (2001). Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Neuron *32*, 415-424.

Petersen, S.A., Fetter, R.D., Noordermeer, J.N., Goodman, C.S., and DiAntonio, A. (1997). Genetic analysis of glutamate receptors in Drosophila reveals a retrograde signal regulating presynaptic transmitter release. Neuron *19*, 1237-1248.

Pinan-Lucarre, B., Gabel, C.V., Reina, C.P., Hulme, S.E., Shevkoplyas, S.S., Slone, R.D., Xue, J., Qiao, Y., Weisberg, S., Roodhouse, K.*, et al.* (2012). The core apoptotic

executioner proteins CED-3 and CED-4 promote initiation of neuronal regeneration in Caenorhabditis elegans. PLoS Biol *10*, e1001331.

Ray, S.K., Matzelle, D.D., Wilford, G.G., Hogan, E.L., and Banik, N.L. (2000). Increased calpain expression is associated with apoptosis in rat spinal cord injury: calpain inhibitor provides neuroprotection. Neurochem Res *25*, 1191-1198.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell *99*, 791-802.

Ritzenthaler, S., Suzuki, E., and Chiba, A. (2000). Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. Nat Neurosci *3*, 1012-1017.

Rogelj, B., Mitchell, J.C., Miller, C.C., and McLoughlin, D.M. (2006). The X11/Mint family of adaptor proteins. Brain Res Rev *52*, 305-315.

Sakuma, H., Ikeda, A., Oka, S., Kozutsumi, Y., Zanetta, J.P., and Kawasaki, T. (1997). Molecular cloning and functional expression of a cDNA encoding a new member of mixed lineage protein kinase from human brain. J Biol Chem *272*, 28622-28629.

Schroer, T.A. (2004). Dynactin. Annu Rev Cell Dev Biol *20*, 759-779.

Scorza, C.A., Garrido Ydel, C., Arida, R.M., Amado, D., Cavalheiro, E.A., and Naffah-Mazzacoratti Mda, G. (2003). Levels of the synaptic protein X11 alpha/mint1 are increased in hippocampus of rats with epilepsy. Epilepsy Res *57*, 49-57.

Shin, J.E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., and DiAntonio, A. (2012). Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. Neuron *74*, 1015-1022.

Sinka, R., Gillingham, A.K., Kondylis, V., and Munro, S. (2008). Golgi coiled-coil proteins contain multiple binding sites for Rab family G proteins. J Cell Biol *183*, 607-615.

Smith, P.D., Sun, F., Park, K.K., Cai, B., Wang, C., Kuwako, K., Martinez-Carrasco, I., Connolly, L., and He, Z. (2009). SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron *64*, 617-623.

Spira, M.E., Oren, R., Dormann, A., Ilouz, N., and Lev, S. (2001). Calcium, protease activation, and cytoskeleton remodeling underlie growth cone formation and neuronal regeneration. Cell Mol Neurobiol *21*, 591-604.

Tomita, S., Ozaki, T., Taru, H., Oguchi, S., Takeda, S., Yagi, Y., Sakiyama, S., Kirino, Y., and Suzuki, T. (1999). Interaction of a neuron-specific protein containing PDZ domains with Alzheimer's amyloid precursor protein. J Biol Chem *274*, 2243-2254.

Torroja, L., Luo, L., and White, K. (1996). APPL, the Drosophila member of the APP-family, exhibits differential trafficking and processing in CNS neurons. J Neurosci *16*, 4638-4650.

Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M.*, et al.* (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science *287*, 864-869.

Wagh, D.A., Rasse, T.M., Asan, E., Hofbauer, A., Schwenkert, I., Durrbeck, H., Buchner, S., Dabauvalle, M.C., Schmidt, M., Qin, G.*, et al.* (2006). Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron *49*, 833-844.

Weber, U., Paricio, N., and Mlodzik, M. (2000). Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. Development *127*, 3619-3629.

Xiong, X., and Collins, C.A. (2012). A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. J Neurosci *32*, 610-615.

Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J Cell Biol *191*, 211-223.

Yan, D., and Jin, Y. (2012). Regulation of DLK kinase activity by calcium-mediated dissociation from an inhibitory isoform. Neuron *76*, 534-548.

Yan, D., Wu, Z., Chisholm, A.D., and Jin, Y. (2009). The DLK kinase promotes mRNA stability and local translation in C. elegans synapses and axon regeneration. Cell *138*, 1005-1018.

Yoon, S., Choi, J., Haam, J., Choe, H., and Kim, D. (2007). Reduction of mint-1, mint-2, and APP overexpression in okadaic acid-treated neurons. Neuroreport *18*, 1879-1883.

Yun, J., Puri, R., Yang, H., Lizzio, M.A., Wu, C., Sheng, Z.H., and Guo, M. (2014). MUL1 acts in parallel to the PINK1/parkin pathway in regulating mitofusin and compensates for loss of PINK1/parkin. Elife *3*, e01958.

Zeitlinger, J., and Bohmann, D. (1999). Thorax closure in Drosophila: involvement of Fos and the JNK pathway. Development *126*, 3947-3956.

Zinsmaier, K.E., Eberle, K.K., Buchner, E., Walter, N., and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of Drosophila. Science *263*, 977-980.

Ziv, N.E., and Spira, M.E. (1995). Axotomy induces a transient and localized elevation of the free intracellular calcium concentration to the millimolar range. J Neurophysiol *74*, 2625-2637.

Ziv, N.E., and Spira, M.E. (1997). Localized and transient elevations of intracellular Ca2+ induce the dedifferentiation of axonal segments into growth cones. J Neurosci *17*, 3568-3579.

Ziv, N.E., and Spira, M.E. (1998). Induction of growth cone formation by transient and localized increases of intracellular proteolytic activity. J Cell Biol *140*, 223-232.

Chapter 3

rheb overexpression enhances axonal regeneration and suppresses axon degeneration by regulating the level of Wallenda through the mTORC1 pathway

Abstract

Wallenda (Wnd) is an evolutionarily conserved Map Kinase Kinase Kinase (MAPKKK) that plays a key role in regulating synaptic growth during development and in regulating axonal regeneration and degeneration after injury. Highwire (Hiw), an E3 ubiquitin ligase, has been shown to regulate Wnd both during development and upon axonal injury. However, it remains unclear what other mechanisms and pathways might regulate Wnd. Here we report that overexpression of *rheb* greatly enhances axonal regeneration and prevents axonal degeneration in the *Drosophila* motor neuron. Enhanced axonal regeneration and reduced axonal degeneration caused by *rheb* overexpression is dependent on the Wnd pathway because loss-of-function mutations of this pathway strongly suppress these phenotypes. In addition to axonal regeneration after injury, *rheb* overexpression leads to overgrowth of the neuromuscular junctions (NMJs) during development. Interestingly, loss-of-function mutations of the Wnd pathway suppress the number of branches and the number of boutons of *rheb* overexpression, but not the size of the boutons or cell bodies, suggesting that rheb regulates cell size via a different pathway. Furthermore, we found that loss-of-function mutations of the mTORC1 pathway suppresses *rheb*-mediated synaptic overgrowth and axonal regeneration, but loss-of-function mutations of the mTORC2 pathway do not. Finally, we found that overexpression of *rheb* leads to an increase of Wnd protein level in the cell body while *hiw* mutant leads

to an increase of Wnd level in the neuropil, where synapses are found. Our results suggest that *rheb* acts in parallel to *hiw* in regulating Wnd level by increasing protein translation of Wnd through the mTORC1 pathway.

Introduction

Unlike axons in the peripheral nervous system (PNS), axons in the central nervous system (CNS) fail to undergo functional regeneration (Sun and He, 2010). The lack of functional regeneration in the CNS has enormous ramifications for patients who have suffered brain or spinal cord injuries. Past studies have primarily focused on the extrinsic inhibitory signals such as the myelin associated inhibitors and the chondroitin sulfate proteoglycans in the glia scar that limit axon regeneration. However, removal of these inhibitory signals has led to only minor improvements in axonal regeneration *in vivo* (O'Brien and Sagasti, 2009), suggesting additional regulators may exist. More recent studies have pointed to the importance of intrinsic growth potential of neurons (Lu et al., 2014). The evolutionarily conserved Dual Leucine Zipper-breaing Kinase (DLK) pathway is a key intrinsic signal for regeneration after axonal injuries. Regeneration is completely absent in animals with loss-of function mutations on DLK or any of its downstream components. Importantly, activating this pathway leads to robust axonal regeneration (Hammarlund et al., 2009; Xiong et al., 2010). Reduced expression of growth-promoting genes in the mature neurons has also been implicated to be the reason axons fail to regenerate in the adult neurons (Blackmore et al., 2010); thus, increasing protein synthesis might help regeneration of the mature neurons. One of the key pathway that regulates protein synthesis and cellular growth is the PI3K/AKT pathway. Activation of

PI3K or inhibiting PTEN, a negative regulator of the PI3K pathway, has recently been shown to promote axonal regeneration in the CNS of multiple organisms (Liu et al., 2010; Park et al., 2008; Song et al., 2012), suggesting that this function of the PI3K/AKT pathway is evolutionarily conserved. Acting downstream of the PI3K/AKT pathway, the Mammalian target of rapamycin (mTOR), a key regulator of protein synthesis, is thought to mediate the signals from PI3K/AKT for axonal regeneration. In the CNS, axotomy leads to reduced phosphorylation of S6K, a downstream target of mTOR (Park et al., 2008). However, it remains unclear if mTOR is required for axonal regeneration in CNS. In the PNS, there are conflicting results regarding the role of mTOR. While two studies showing that mTOR is required for axonal regeneration in *pten* mutants (Christie et al., 2010; Saijilafu et al., 2013), another study showed that mTOR is dispensable for axonal regeneration (Abe et al., 2010) . All three studies regarding the roles of mTOR in the PNS were conducted in neuronal cell cultures. Whether mTOR plays a role in axonal regeneration *in vivo* needs to be further addressed.

Two recent studies show that enhanced axonal regeneration of *pten* mutant cannot bypass the requirement of DLK (Byrne et al., 2014; Watkins et al., 2013), suggesting DLK is a key target of the PI3K pathway. However, it remains unclear how DLK is regulated by the PI3K pathway. Understanding how these pathways that control intrinsic growth capacity of neurons are regulated will provide useful insights for developing therapeutic strategies for axonal regeneration.

Rheb (Ras homologue enriched in brain) belongs the Ras superfamily of GTP-binding protein that is conserved from yeast to human (Aspuria and Tamanoi, 2004). Studies in *Drosophila* and mammalian cells have shown that Rheb increases growth and promotes cell cycle progression. Genetic and biochemical studies indicate that Rheb functions in the insulin signaling pathway acting downstream of Tsc1/Tsc2 and upstream of TOR. Thus, *rheb* plays a key role in the insulin/TOR/S6K signaling pathway. A recent paper shows that a constitutively active form of *rheb* enhances axon regeneration in adult neurons after a complete spinal cord injury. It is suggested that Rheb-mediated enhanced axonal regeneration occurs through the mTOR pathway because *rheb* is an activator of the mTOR pathway; nevertheless, this has not been specifically tested; it remains unclear whether Rheb-mediated axonal regeneration is dependent on mTOR signaling. In *Drosophila*, overexpression of *rheb* has been shown to increase synaptic growth in the neuromuscular junction and during developmental regrowth (Dimitroff et al., 2012; Knox et al., 2007; Yaniv et al., 2012). However, it is unknown whether Rheb may also plays a role in axonal regeneration in *Drosophila*.

Interestingly, the Wnd pathway has also been shown to regulate axonal degeneration. In *Drosophila*, *hiw* mutant has been shown to increase the level of both Wnd and NMNAT to prevent axon degeneration. Overexpression of *rheb* has also recently been shown to prevent axonal degeneration in a mouse model of degeneration of dopaminergic neurons (Cheng et al., 2011), suggesting that overexpression of *rheb* might also prevent axonal degeneration after injury.

Because Wnd regulates both synaptic growth during development and axonal regeneration after injury, we hypothesized that other pathways that controls synaptic growth during development might also regulate axonal regeneration after injury. Here we found that *rheb*, a protein that regulates synaptic growth during development, regulates both axonal regeneration and degeneration after injury. We further found that *rheb* regulates the Wnd pathway through the mTORC1 pathway. Our data suggest that rheb activate the Wnd pathway by by increasing protein translation of Wnd.

Materials and Methods

Molecular Biology:

To generate UAS-FLAG-rheb, the *rheb* cDNA (EST clone from *Drosophila* Genome Research Center, LD20981) was subcloned into pUASt. pUAST-GFP-Wnd is previously described (Collins et al., 2006). All cloned PCR products were confirmed by DNA sequencing.

Fly Stocks:

wnd1 and *wnd3* were provided by Cathy Collins and A. DiAntonio (University of Michigan and Washington University, St. Louis, MO). The following fly lines were obtained from the Bloomington Stock Center: UAS-rheb[av4], UAS-rheb, UAS-PI3KDN, UAS-TOR-TED, $OK6^{Gal4}$ from Corey Goodman, UAS-mCD8-GFP, *UAS-Bsk*^{DN}, *UAS-Fos*^{DN}, UAS–GFP-*wnd*^{KD,} m12-Gal4 ($P(GAL4)^{5053A}$, puc-lacz stock, *puc*-$lacZ^{E69}$.

Immunocytochemistry:

Wandering third-instar larvae were dissected in PBS and fixed in a PBS solution containing 3.7% formaldehyde for 30 minutes. Blocking and staining was performed in PBS containing 0.1% Triton-X. Antibodies were used at the following dilutions: mouse α-Dlg at 1:10, rabbit anti-rheb(abcam) at 1:200, mouse α-NC82 at 1:10, mouse α-GluR11A at 1:10, mouse α-CSP at 1:10 (Developmental Studies Hybridoma bank) at 1:10, mouse α- β-gal (Sigma) at 1:10, FITC-conjugated goat α-HRP (Jackson Immunoresearch) at 1:250, rabbit α-Wnd at 1:250. Conjugated α-rabbit and α-mouse secondary antibodies were used at 1:250. Stained brains were mounted in Vectashield (Vector Laboratory; H-1000).

Imaging and quantitative analysis:

Images were acquired on a Zeiss confocal microscope. The NMJ on muscle 4 of the abdominal segment A2 was used for quantifications, α-CSP staining was used to count the number of individual boutons and α-HRP to count the number of branches in control and experimental samples that were processed and stained simultaneously, imaged using the same confocal settings. All quantifications were performed while blinded to genotype. Statistical analysis was performed and graphs were generated using Excel. Student's t-test was used to determine statistical significance.

Lysate Preparation and Western Blotting:

Third instar larval brains were disrupted in lysis buffer with complete protease inhibitor cocktail (Roche) using a sonicator-3000 from MISONIX. Samples were boiled for 10 minutes, centrifuged, and total protein from 10 brains per genotype was analyzed by

Western blotting analysis. Antibodies used were rabbit anti-Wnd, anti-rheb (1:1000, ABCAM), and mouse anti-actin (1:5,000; Sigma). Secondary rabbit and mouse anti-HRP antibodies were purchased from Thermo Fisher and used at the manufacturer's recommended concentrations.

RNA isolation, cDNA synthesis, and quantitative PCR (qPCR)

RNA was isolated from whole flies using the Macherry-Nagel Nucleospin RNA II kit. cDNA synthesis was performed using the Clontech RNA to cDNA EcoDry Premix Kit, using a combination of Oligo-dT and random hexamer priming. Quantitative PCR was performed using the BioRadiTaq Fast Sybr Green enzyme mix, 10 µl reactions in triplicate, on a Roche Light Cycler 480.

Reverse transcription PCR (RT-PCR)

Total RNA was prepared as described above. RT-PCR was performed using Titanium One-Step RT-PCR Kit according to the manufacturer's instructions (Promega, Madison, WI).

Results

Overexpression of *rheb* leads to robust axon regeneration in injured motor axons that is dependent on the Wnd pathway

The *Drosophila* motor neuron is a great model system for studying axonal regeneration and degeneration. Their cell bodies and axons can clearly be identified (Fig.1A), and a motor axon injury paradigm in *Drosophila* has previously been established (Xiong et al.,

2010). To study motor axon regeneration, axons were labeled with GFP and were injured by pinching the ventral side of the larvae with a pair of forceps (Fig1A, 1B). Regeneration of axons were monitored by dissecting flies at various time points after injury. To test whether *rheb* affects the axon regeneration process, we overexpressed *rheb* in the motor neurons by expressing UAS-rheb with m12-gal4. We found that, compared with wildtype, injured axons of flies overexpressing *rheb* were able to regenerate faster (Fig.1C-1E). By 8hrs after injury more regenerating axons were observed in flies overexpressing *rheb*. Since the Wnd pathway, consisting of Wnd, BSK, and FOS, plays a key role in axon regeneration, we tested if *rheb*-mediated axonal regeneration might be dependent on this pathway. At 12 hours after injury, injured wildtype axons showed modest regeneration (Fig.1F). Injured axons from flies overexpressing *rheb*, on the other hand, showed a dramatic regeneration phenotype (Fig.1G). Knockdown of *wnd* completely suppresses axonal regeneration as previously reported by others (Fig. 1H). Interestingly, flies simultaneously overexpressing *rheb* with *wnd* RNAi showed a dramatic reduction of regeneration (Fig.1I), so as flies overexpressing *rheb* and bsk^{DN} (Fig. 1J), suggesting that *rheb*-mediated axon regeneration is dependent on the Wnd pathway.

Synaptic growth phenotype of *rheb* overexpression is mediated through the Wnd pathway

Next, we asked if the synaptic growth phenotype of *rheb* overexpression is also dependent on the Wnd pathway. As previously reported by others, overexpression of *rheb* led to synaptic growth in the larval neuromuscular junction (NMJ) compared with wildtype (Fig 2A and Fig 2B). Interestingly, the Wnd pathway is also required for the synaptic

growth phenotype of *rheb* ovexpression because *wnd* mutant, and mutations of the downstream components of the Wnd pathway, *bsk*, and *fos* suppress the overgrowth phenotype, suggesting the effect of both axon regeneration and synaptic development of rheb overepxression is mediated through the Wnd pathway. *rheb* overexpression also leads to an increase of cell bodies, axons and boutons sizes, but mutants of the Wnd pathway cannot suppress these phenotypes (data not shown), suggesting that *rheb* regulates cell sizes via different mechanisms.

rheb overexpression activates the Wnd pathway by increasing the protein level of Wnd

Because both axon regeneration and synaptic growth phenotype of *rheb* overexpression is suppressed by mutations of the Wnd pathway, we hypothesize that the overgrowth phenotype of *rheb* overexpression is due to the activation of the Wnd pathway. In *Drosophila*, the activities of the JNK pathway can be monitored by the Puc-lacz reporter flies. When the JNK pathway is activated, more *puc* will be transcribed (Fig.3A). Compared with wildtype flies (Fig.3B), flies overexpressing *rheb* showed a much higher level of *puc-lacz* expression (Fig.3C), suggesting that *rheb* activates the JNK pathway. Because loss of *wnd* can suppress the phenotype of *rheb* overexpression, we tested whether Rheb directly binds to Wnd. We found that Rheb and Wnd colocalize in both the motor neurons and in the S2 cells. To test whether the two proteins might interact, co-immunoprecipitation was performed, but the two proteins did not physically interact, suggesting that Wnd might not be a direct substrate of Rheb (data not shown). Next, we tested whether overexpression of *rheb* might affect the protein level or the subcellular

localization of Wnd. We found that overexpression of *rheb* leads to an increase of Wnd in the motor neurons and axons. To further confirm the results, we performed Western Blot and found that the Wnd level is indeed increased in flies overexpressing *rheb*. Taken together, *rheb* activates the Wnd pathway by increasing the protein level of Wnd.

Rheb, acting in parallel to Hiw, regulates Wnd

As both *hiw* and *rheb* regulate the level of Wnd, we tested whether *hiw* and *rheb* function in the same genetic pathway. Both *rheb* overexpression and *hiw* mutant show NMJ overgrowth phenotypes when compared with wildtype (Fig.4A, 4B, and 4F). Furthermore, overexpression of *rheb* in *hiw* mutant leads to further increase of synaptic growth (Fig. 4G), suggesting that *rheb* does not regulate synaptic growth through *hiw*. Instead they are likely to regulate through a parallel pathway. Interestingly, we found that *rheb* overexpression leads to an increase of Wnd in the motor neuron cell bodies and in the axon (Fig. 4H-4J) while *hiw* mutant leads to the increase of Wnd primarily in the neuropil (Fig. 4K-4M), where synapses reside. Consistent with what we have found, it has previously been reported that another substrate of hiw, NMNAT, is primarily increased in the neuropil, suggesting that *hiw* regulates the level of its substrate in the neuropil, but not in the cell body or axons. Taken together, these data suggest that *rheb* regulates the protein level of Wnd through a *hiw*-independent mechanism.

***rheb* overexpression prevents axonal degeneration**

After axons are injured, the proximal stumps that are still attached to the cell body undergo regeneration while the detached distal stumps undergo degeneration (Fig. 5A). *hiw*

mutant has been shown to prevent axonal degeneration by increasing the level of Wnd and NMNAT. Because overexpression of *rheb* increases the level of Wnd, we then asked if overexpression of *rheb* is sufficient to prevent axonal degeneration. 16 hours after injury, most damaged wildtype axons labeled with GFP are completely fragmented (Fig. 5B). In contrast, damaged axons in flies overexpressing *rheb* appear to be intact (Fig.5C), suggesting that *rheb* overexpression also protects damaged axons from undergoing degeneration. Next, we tested if overexpression of rheb would regulate the protein level of NMNAT, another key target of Hiw that regulates the axon degeneration process. We found that unlike hiw, overexpression of rheb does not regulate the level of NMNAT (Fig. 5D-5F).

***rheb*-mediated overgrowth is dependent on the mTORC1 pathway**

As *rheb* has been shown to regulate the mTOR pathway, we investigated whether knockdown this pathway might suppress *rheb*-mediated synaptic growth and axon regeneration. We found that the overgrowth phenotype of *rheb* overexpression can be suppressed when a mutant form of mTOR, TOR-TED, is expressed (Fig 6A, B), suggesting that the TOR pathway is important for the function of *rheb*. Because mTOR forms two different complexes, mTORC1 and mTORC2, we tested whether the mutants of mTORC1 pathway and mTOR2 pathway can suppressed the phenotypes of *rheb* overexpression. We found that loss-of-function mutant of the key downstream target of the mTORC1 pathway, S6K, can partially suppress *rheb* overpexression phenotypes (Fig. 6E and 6F), but not the subunit of mTORC2 comlex, *rictor* (Fig.6G), suggesting that *rheb*-mediated overgrowth is likely signaled through the mTORC1 pathway. In addition to

protein synthesis, mTORC1 signaling has also been shown to increase transcription of many genes (Laplante and Sabatini, 2013; Peng et al., 2002). To test if *rheb* overexpression increases the transcript level of *wnd*, qPCR was performed. We found that the transcript level of *wnd* slightly decreased (Fig.S1), possibly due to a compensatory mechanism. This suggest that the increase of Wnd level is not due to an increase of Wnd mRNA level. Rather, the increase of Wnd protein level likely through increase of protein synthesis.

Starvation leads to reduction of Wnd level

mTOR activity is controlled by multiple signals including nutrients and amino acid availability (Aspuria and Tamanoi, 2004). We tested if these signals might affect the level of Wnd. In S2 cells, starving the cells by growing them in HBSS or serum free media led to the reduction of Wnd level (data not shown), consistent with our data that Rheb regulates the level of Wnd. This suggest that nutrient availability can regulate the Wnd pathway.

Discussion

In this study, we found that *rheb* overexpression enhances axonal regeneration and prevents axonal degeneration in *Drosophila*. Our results demonstrated that *rheb* enhances axonal regeneration and prevents axonal degeneration through the mTORC1 pathway by activating the Wnd pathway, a key pathway that regulates axon regeneration. When *rheb* is overexpressed, we found that the protein level of Wnd is increased, both by immunostaining and by western blotting. Furthermore, we found that NMJ overgrowth

phenotype of *rheb* overexpression is also due to the activation of the Wnd pathway as mutants of this pathway can suppress the phenotype.

Although both Hiw and Rheb regulate the level of Wnd, we found that they regulate Wnd through distinct mechanisms. First, overexpression of *rheb* in *hiw* mutant leads to further increase of synaptic growth suggesting that they regulate *wnd* in parallel. Second, *rheb* overexpression in the motor neuron leads to an increase of Wnd primarily in the cell bodies and axons, but not the neuropil, while *hiw* mutant leads to an increase of Wnd level in the neuropil, but not in the cell bodies. This is consistent with a previous study showing that another substrate of *hiw*, NMNAT, is regulated mainly in the neuropil, suggesting that the main site of action of *hiw* might be in the neuropil.

Next, we tested whether overexpression of r*heb* suppresses axon degeneration. *hiw* mutant has previously been shown to prevent axon degeneration through increasing the level of Wnd and NMNAT, and *wnd* overexpression is sufficient to prevent axon degeneration. The distal end of injured axon normally undergoes degeneration, but we found that *rheb* overexpression could suppress this phenotype. Interestingly, knockdown of *wnd* could suppress the ability of *rheb* to suppress axonal degeneration, consistent with our data that *rheb* overexpression leads to an increase of Wnd protein level.

As *rheb* has been shown to regulate the mTOR pathway, we tested if inhibiting this pathway could abolish *rheb*-mediated axonal growth. We found that mutant of a downstream target of mTORC1, S6K, could suppress *rheb*-mediated overgrowth, but not

mutants of the mTORC2 pathway, suggesting that *rheb* regulates Wnd through the mTORC1 pathway. Furthermore, we found that in S2 cells, starvation leads to reduction of Wnd level, suggesting that nutrient availability directly regulates Wnd level through the mTORC1 pathway.

As the mTORC1 pathway can regulate both protein synthesis and gene transcription, we tested the mRNA level of *wnd* in flies overexpressing *rheb*. We found that the mRNA level of *wnd* is slightly decreased when *rheb* is overexpressed, suggesting that *rheb* is likely regulating the Wnd not at the level of transcription, but the at the level of translation or post translation. The mTOR pathway has previously been shown to controls axon formation and growth by selective translational upregulation of proteins (Choi et al., 2008). Further studies will be needed to understand how mTOR activation leads to the upregulation of these proteins. Taken together, our results indicate that *rheb* overexpression leads to enhanced axonal regeneration and delayed axonal degeneration through increasing the level of Wnd.

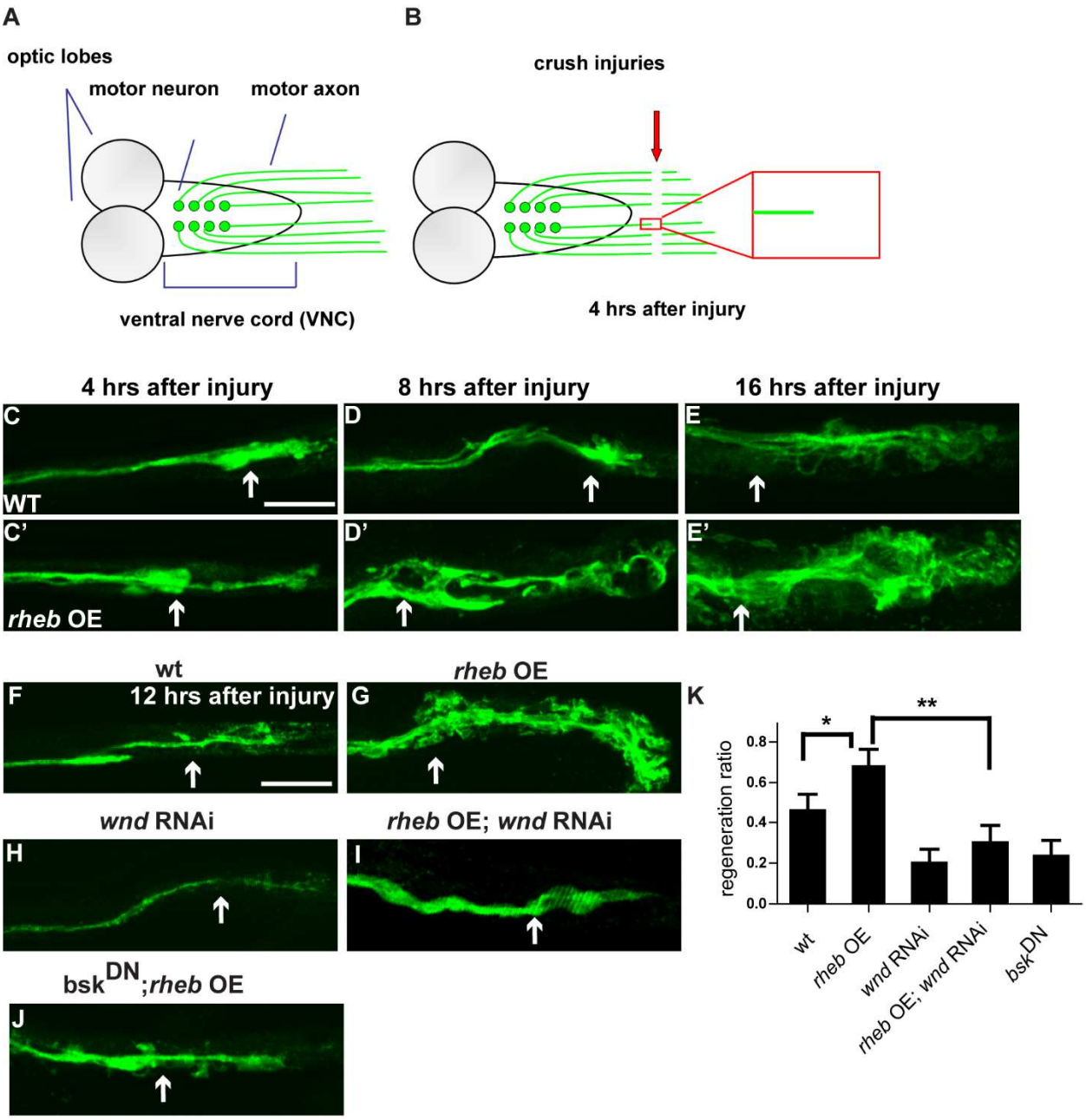


Figure 3-1. *rheb* overexpression enhances axon regeneration and the enhanced regeneration is dependent on the Wnd pathway. **(A)** Schematic diagram of the *Drosophila* third instar larval brain. **(B)** Schematic diagram of crushed motor axons of the *Drosophila* third instar larval brain. Red arrow indicates crush site. At 4 hours

after injury, no new axon sprouts are formed yet. **(C-E)** Time course of motor axon regeneration after injury in wildtype. (**C'-E')** Time course of motor axon regeneration after injury in flies overexpressing *rheb*. **(F-J)** Axons are labeled by expressing UAS-mCD8GFP with the motor axon driver m12-gal4. White arrow indicates injury site. Larvae were injured and were dissected after 12 hours. Scale bar: 10um. **(F)** At 12 hours after injury, new axons sprout from the injured site of the motor axon of wildtype. **(G)** *Rheb*-overexpressing larvae show enhanced axon regeneration compared with wildtype. **(H)** No new axons are formed in *wnd* rnai expressing larvae. **(I)** *wnd* rnai completely suppresses new axon sproutings in rheb overexpressing larvae. **(J)** BSK DN completely suppresses new axon sproutings in *rheb*-overexpressing larvae. **(K)** Quantification of the regeneration ratio of the various genotypes at 12 hours after injury. The statistical analysis was done using One-way ANOVA with Tukey's multiple comparisons test. *, $P < 0.05$. **, $P < 0.001$. Error bars indicate mean ± SEM. Scale bars, 10μm.

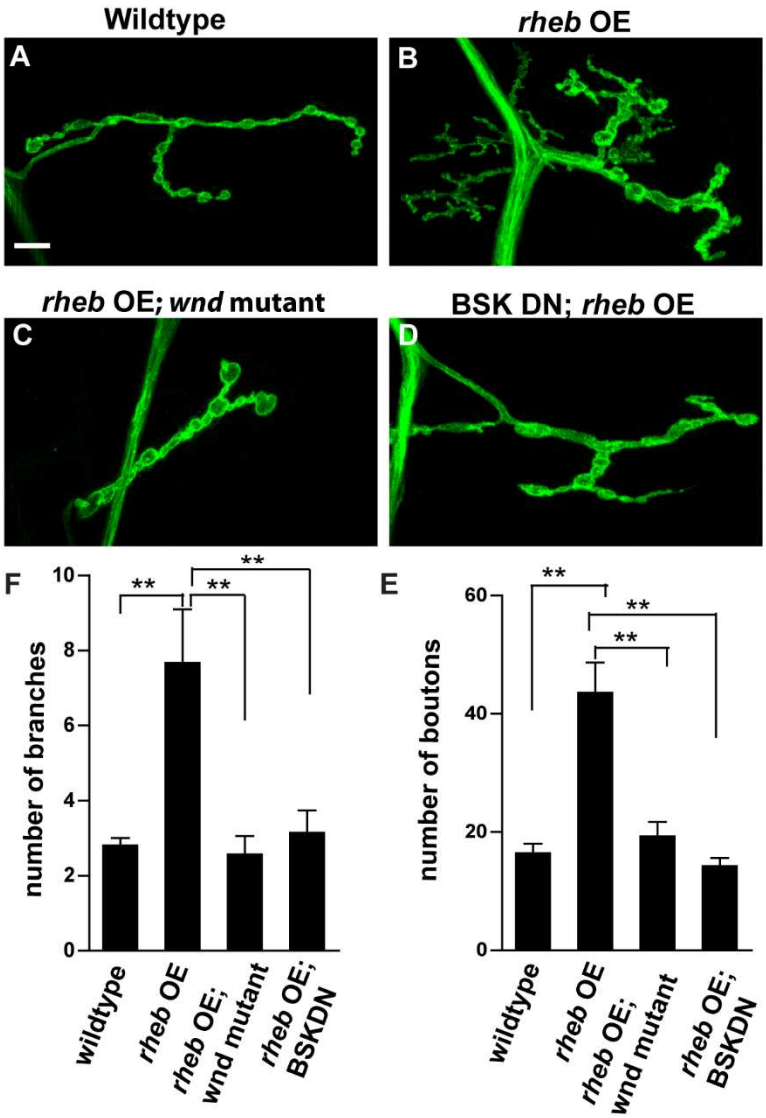


Figure 3-2. *rheb* overexpression leads to NMJ overgrowth and the growth phenotype is dependent on the Wnd pathway. **(A-D)** NMJs of muscle 4 were stained with the synaptic vesicle marker CSP (red) and the neuronal membrane marker HRP (green). **(A)** wildtype NMJ. **(B)** NMJ overgrowth phenotypes are seen in *rheb* overexpressing larvae. **(C)** Loss of *wnd* suppresses the overgrowth phenotype of

rheb overexpressing larvae. **(D)** BSK DN suppresses the overgrowth phenotype of *rheb* overexpressing larvae. **(E)** Quantification of the number of boutons of each genotype. **(F)** Quantification of the number of branches of each genotype. $n > 15$ per genotype. The statistical analysis was done using One-way ANOVA with Tukey's multiple comparisons test. **, $P < 0.001$. Error bars indicate mean ± SEM. Scale bars, 10μm.

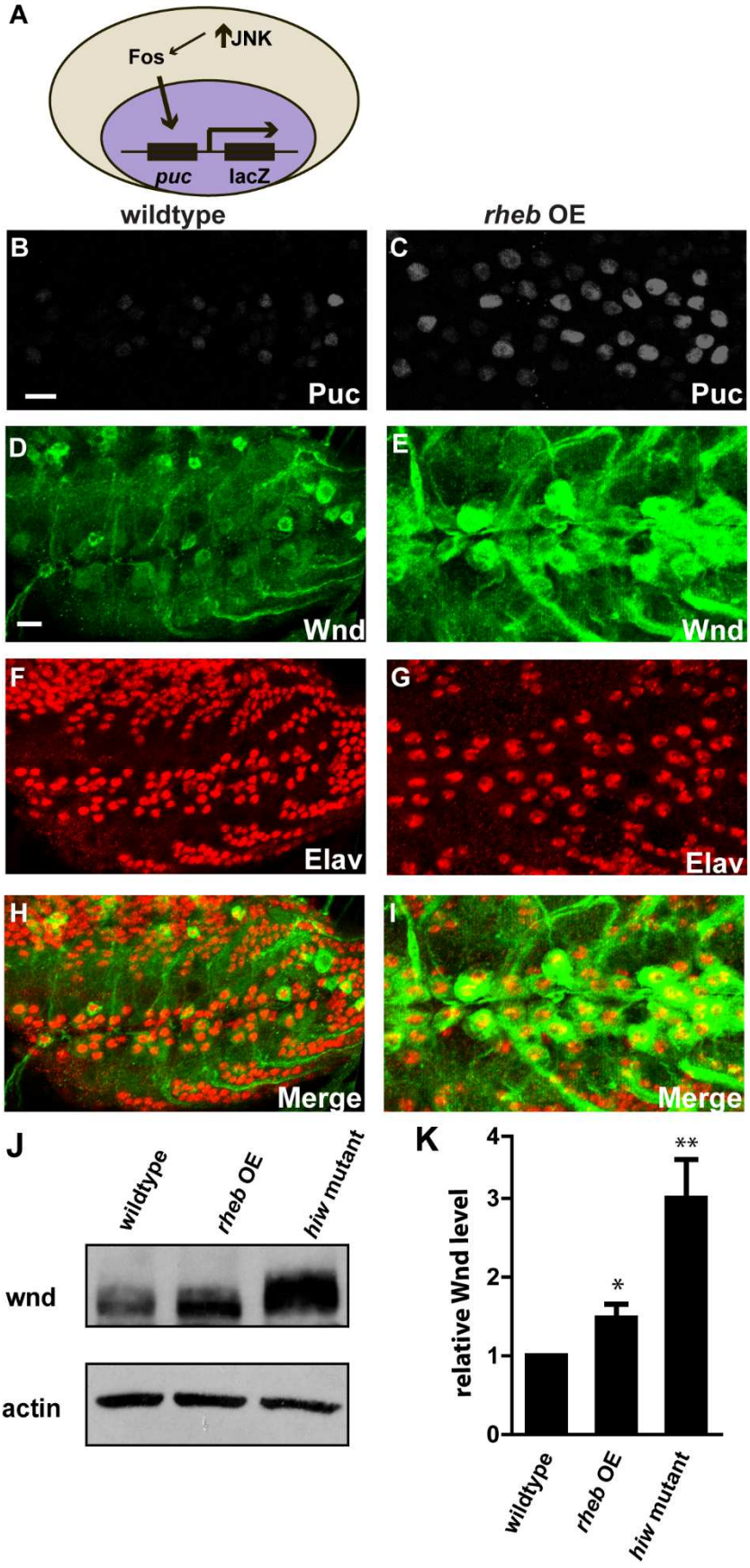

A
JNK
Fos
puc
lacZ
wildtype
rheb OE
B
Puc
C
Puc
D
Wnd
E
Wnd
F
Elav
G
Elav
H
Merge
I
Merge
J
wildtype
rheb OE
hiw mutant
wnd
actin
K
relative Wnd level
0
1
2
3
4
*
**
wildtype
rheb OE
hiw mutant

Figure 3-3. *rheb* overexpression activates the Wnd pathway by increasing the protein level of Wnd.

(A) Schematic diagram of the Puc-lacz reporter for JNK activities. When the JNK pathway is activated, more lacZ is transcribed. **(B)** In injured wildtype animal, basal level of Puc is detected in the neurons. **(C)** Elevated level of Puc is detected in the neruons of *rheb* overexpressing animals. *rheb* is overexpressed by the motor neuron driver ok6-gal4. **(D-E)** GFP-Wnd-KD is expressed in the motor neurons by ok6-gal4. **(D)** Wildtype larvae overexpressing GFP-Wnd-KD **(E)** Larvae co-expressing rheb and GFP-Wnd-KD. **(F-G)** Neuronal nuclei are stained with the neuronal marker Elav (red). Scale bars, 10μm. **(H-I)** Merged view of GFP-Wnd-KD-GFP and Elav. **(J)** Western blot of brain lysates of wildtype, *rheb* overexpression, and hiw mutant blot with anti-wnd. (K) Quantification of the western blot shown in J. The statistical analysis was done using One-way ANOVA with Tukey's multiple comparisons test. n=3. *, $P < 0.05$. **, $P < 0.001$.

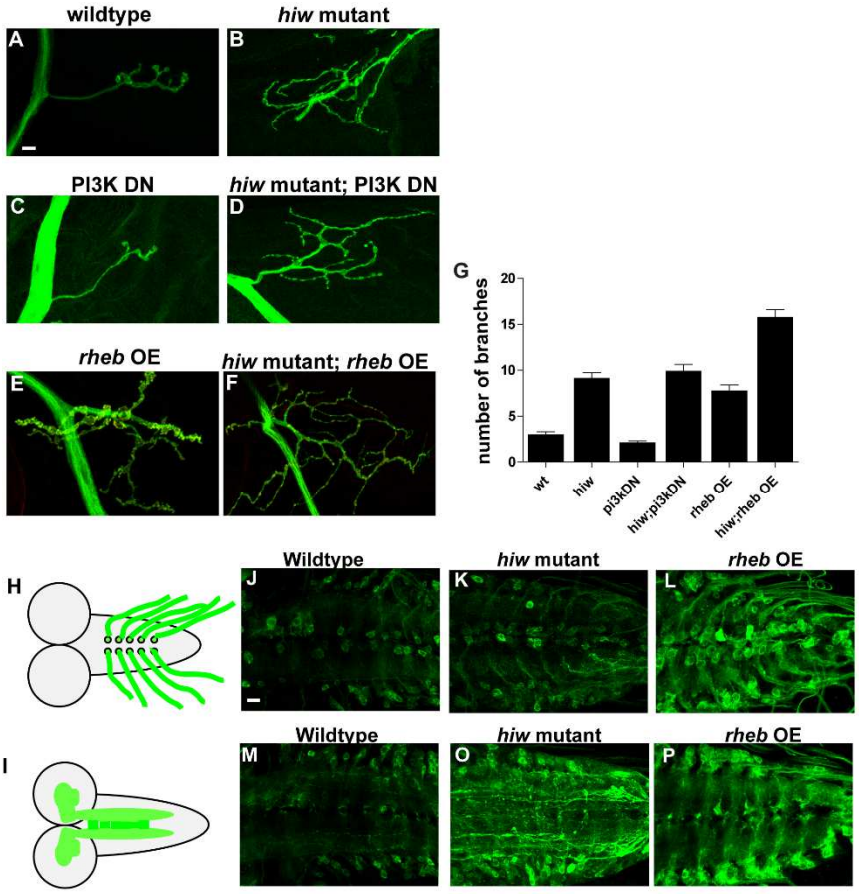


Figure 3-4. Rheb acts in parallel to Hiw to regulate Wnd. **(A-E)** NMJs of muscle 4 were stained with the neuronal membrane marker HRP (green). **(A)** NMJ of muscle 4 of *hiw* mutant. **(B)** NMJ of muscle 4 of larvae overexpressing $pi3k^{DN}$. **(C)** NMJ of muscle 4 of *hiw* mutant larvae overexpressing $pi3k^{DN}$. **(D)** NMJ of *rheb* overexpressing larvae. (E) NMJ of larvae overexpressing rheb. **(F)** NMJ of *hiw* mutant overexpressing *rheb*. Scale bars, 10μm.

(G) Quantification of the number of branches of each genotype.

(H) Schematic diagram of the *Drosophila* third instar larval brain, focusing on the neuronal cortex layer, where the neuronal cell bodies reside. **(I)** Schematic diagram of the *Drosophila* third instar larval brain, focusing on the neuropil layer, where axons and synapses are found. **(J-O)** GFP-Wnd-KD is expressed in the motor neurons by ok6-gal4. **(J)** GFP-Wnd-KD in the neuronal cell bodies of wildtype larvae. **(K)** GFP-Wnd-KD in the neuronal cell bodies of *hiw* mutant. **(L)** GFP-Wnd-KD in the neuronal cell bodies of *rheb* overexpressing larvae.

(M) GFP-Wnd-KD in the neuropil of wildtype larvae. **(N)** GFP-Wnd-KD in the neuropil of *hiw* mutant. **(O)** GFP-Wnd-KD in the neuropil of *rheb* overexpressing larvae. Scale bars, 10μm.

Fig.5 ***rheb*** overexpression suppresses degeneration.

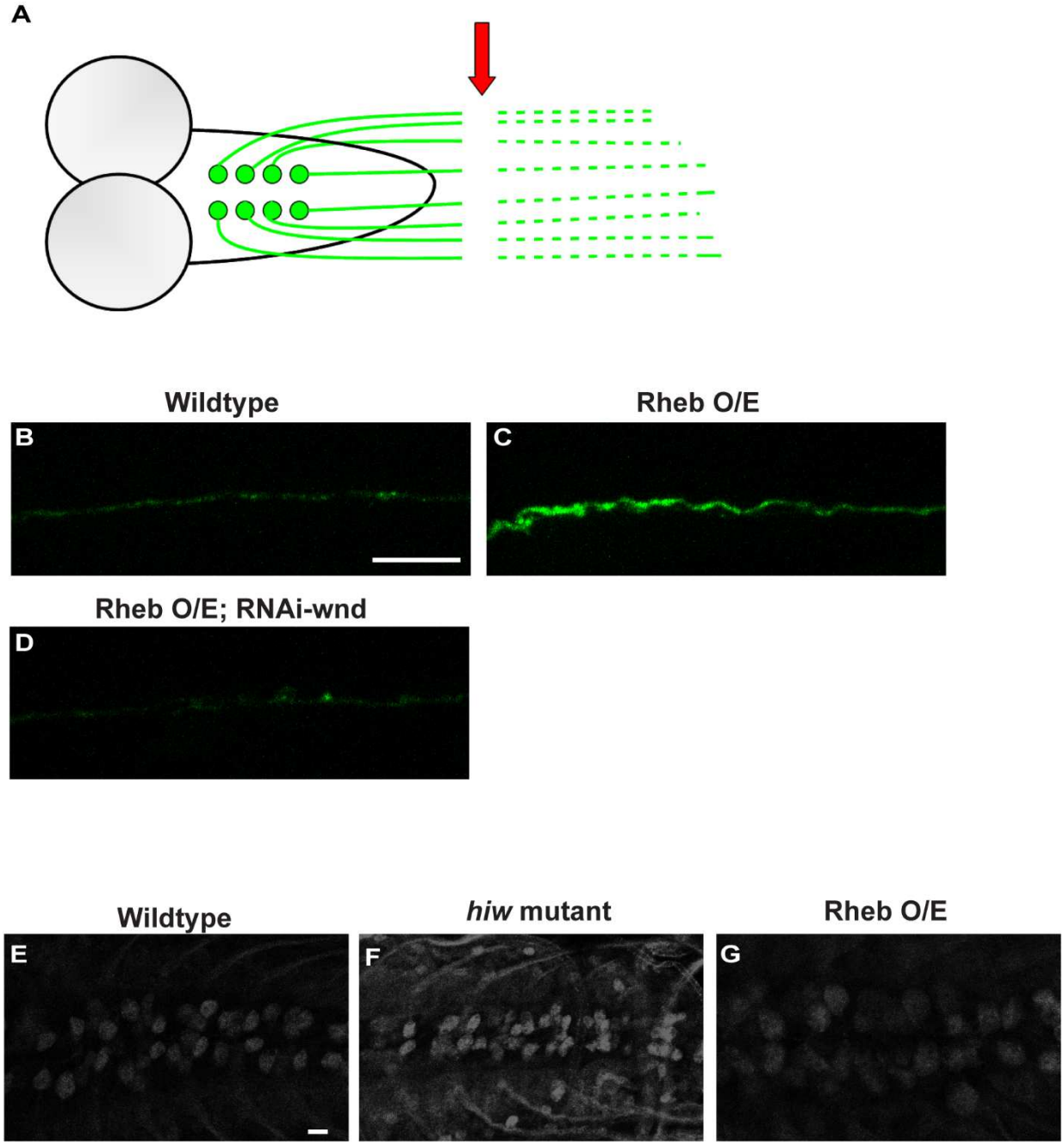


Figure 3-5. *rheb* overexpression suppresses degeneration. (A) Schematic diagram depicting the degeneration of severed axons distal to the injury sites. **(B-C)** motor

axons labeled with mCD8GFP by the m12-gal4 driver are injured and assayed after 12 hours. **(B)** Axons of wildtype larvae degenerate and show a fragmentation phenotype. **(C)** Axons of *rheb* overexpressing larvae do not degenerate 12 hours after injury. **(D-F)** NMNAT-HA is expressed in the motor neurons by ok6-gal4. **(D)** NMNAT-HA is expressed in wildtype larvae. **(E)** The level of NMNAT-HA in *hiw* mutant. **(F)** The level of NMNAT-HA in *rheb*-overexpressing larvae. Scale bars, 10μm.

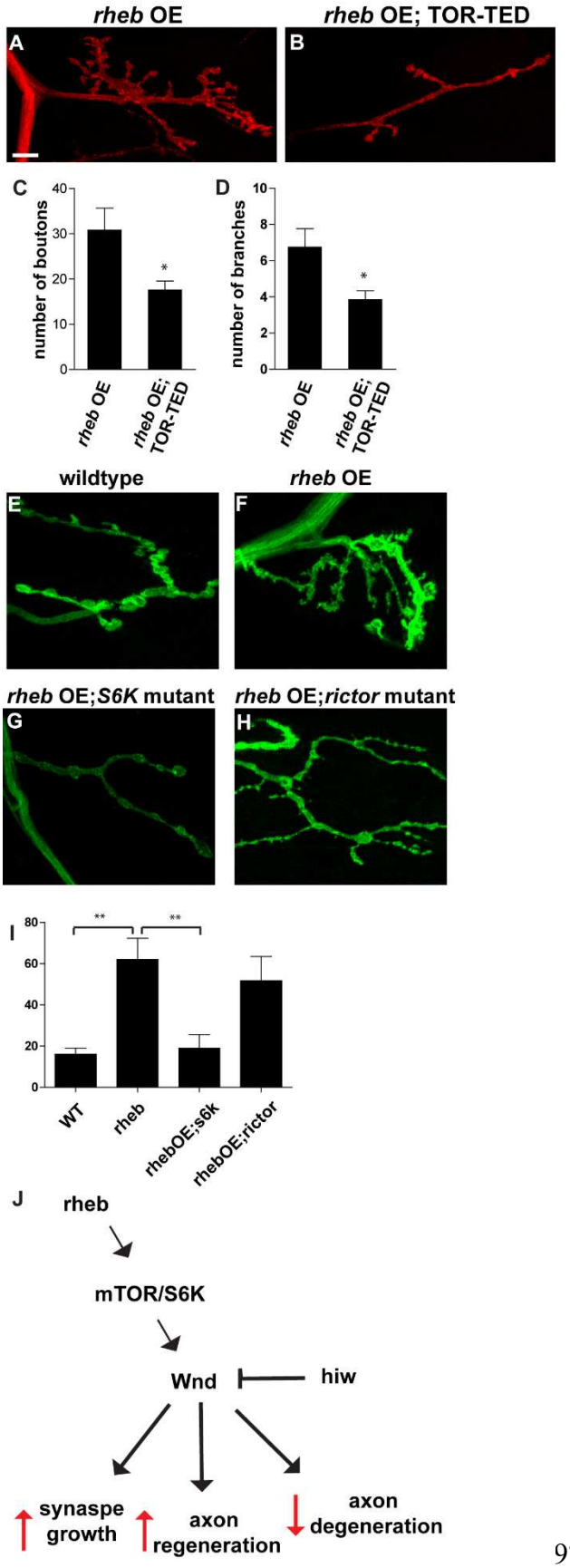

rheb OE
rheb OE; TOR-TED
A
B
C
number of boutons
40
30
20
10
0
*
rheb OE
rheb OE; TOR-TED
D
number of branches
10
8
6
4
2
0
*
rheb OE
rheb OE; TOR-TED
wildtype
rheb OE
E
F
rheb OE;S6K mutant
rheb OE;rictor mutant
G
H
I
80
60
40
20
0
**
**
WT
rheb
rhebOE;s6k
rhebOE;rictor
J
rheb
mTOR/S6K
Wnd
hiw
synaspe growth
axon regeneration
axon degeneration

Figure 3-6. NMJ overgrowth of rheb overexpression is dependent on the mTORC1 pathway.

(A) NMJ overgrowth phenotypes in *rheb* overexpressing larvae. **(B)** Expression of TOR-TED suppresses the NMJ overgrowth phenotype of *rheb* overexpression. **(C)** Quantification of the number of boutons of each genotype. **(D)** Quantification of the number of branches of each genotype. Scale bars, 10μm.

(E) NMJ of the muscle 4 of wildtype. **(F)** NMJ of the muscle 4 of larvae overexpressing *rheb*. **(G)** NMJ of the muscle 4 of S6K mutant overexpressing *rheb*. **(H)** NMJ of the muscle 4 of *rictor* mutant overexpressing *rheb*. **(I)** Quantification of the number of boutons in each genotype. **(J)** Rheb and Hiw regulate Wnd via independent mechanism to control synapse growth, axon regeneration, and axon degeneration. The statistical analysis was done using One-way ANOVA with Tukey's multiple comparisons test. n >15. *, $P < 0.05$. **, $P < 0.001$.

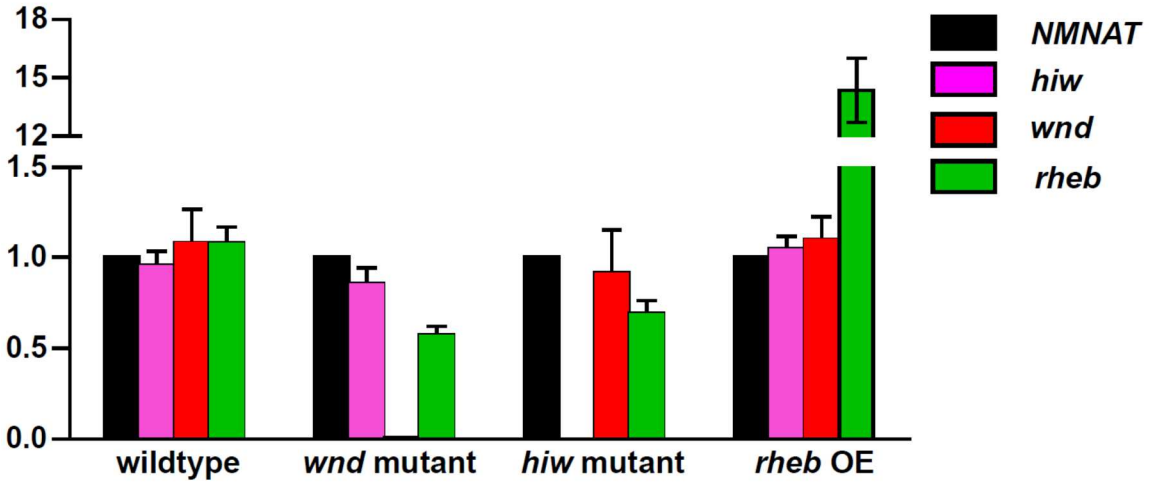


Figure S3-1. *rheb* overexpression does not increase the mRNA level of *NMNAT* and *wnd.* qPCR is performed to test the mRNA expression of the genes *NMNAT, hiw, wnd,* and *rheb* in each of the following genotypes: wildtype, *wnd* mutant, *hiw* mutant, and *rheb* overexpression.

References:

Abe, N., Borson, S.H., Gambello, M.J., Wang, F., and Cavalli, V. (2010). Mammalian target of rapamycin (mTOR) activation increases axonal growth capacity of injured peripheral nerves. J Biol Chem *285*, 28034-28043.

Aspuria, P.J., and Tamanoi, F. (2004). The Rheb family of GTP-binding proteins. Cell Signal *16*, 1105-1112.

Blackmore, M.G., Moore, D.L., Smith, R.P., Goldberg, J.L., Bixby, J.L., and Lemmon, V.P. (2010). High content screening of cortical neurons identifies novel regulators of axon growth. Mol Cell Neurosci *44*, 43-54.

Byrne, A.B., Walradt, T., Gardner, K.E., Hubbert, A., Reinke, V., and Hammarlund, M. (2014). Insulin/IGF1 signaling inhibits age-dependent axon regeneration. Neuron *81*, 561-573.

Cheng, H.C., Kim, S.R., Oo, T.F., Kareva, T., Yarygina, O., Rzhetskaya, M., Wang, C., During, M., Talloczy, Z., Tanaka, K.*, et al.* (2011). Akt suppresses retrograde degeneration of dopaminergic axons by inhibition of macroautophagy. J Neurosci *31*, 2125-2135.

Choi, Y.J., Di Nardo, A., Kramvis, I., Meikle, L., Kwiatkowski, D.J., Sahin, M., and He, X. (2008). Tuberous sclerosis complex proteins control axon formation. Genes Dev *22*, 2485-2495.

Christie, K.J., Webber, C.A., Martinez, J.A., Singh, B., and Zochodne, D.W. (2010). PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. J Neurosci *30*, 9306-9315.

Dimitroff, B., Howe, K., Watson, A., Campion, B., Lee, H.G., Zhao, N., O'Connor, M.B., Neufeld, T.P., and Selleck, S.B. (2012). Diet and energy-sensing inputs affect TorC1-mediated axon misrouting but not TorC2-directed synapse growth in a Drosophila model of tuberous sclerosis. PLoS One *7*, e30722.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.

Knox, S., Ge, H., Dimitroff, B.D., Ren, Y., Howe, K.A., Arsham, A.M., Easterday, M.C., Neufeld, T.P., O'Connor, M.B., and Selleck, S.B. (2007). Mechanisms of TSC-mediated control of synapse assembly and axon guidance. PLoS One *2*, e375.

Laplante, M., and Sabatini, D.M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. J Cell Sci *126*, 1713-1719.

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B.*, et al.* (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat Neurosci *13*, 1075-1081.

Lu, Y., Belin, S., and He, Z. (2014). Signaling regulations of neuronal regenerative ability. Curr Opin Neurobiol *27*, 135-142.

O'Brien, G.S., and Sagasti, A. (2009). Fragile axons forge the path to gene discovery: a MAP kinase pathway regulates axon regeneration. Sci Signal *2*, pe30.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M.*, et al.* (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science *322*, 963-966.

Peng, T., Golub, T.R., and Sabatini, D.M. (2002). The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. Mol Cell Biol *22*, 5575-5584.

Saijilafu, Hur, E.M., Liu, C.M., Jiao, Z., Xu, W.L., and Zhou, F.Q. (2013). PI3K-GSK3 signalling regulates mammalian axon regeneration by inducing the expression of Smad1. Nat Commun *4*, 2690.

Song, Y., Ori-McKenney, K.M., Zheng, Y., Han, C., Jan, L.Y., and 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*, 1612-1625.

Sun, F., and He, Z. (2010). Neuronal intrinsic barriers for axon regeneration in the adult CNS. Curr Opin Neurobiol *20*, 510-518.

Watkins, T.A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J.S., Tessier-Lavigne, M., and 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*, 4039-4044.

Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J Cell Biol *191*, 211-223.

Yaniv, S.P., Issman-Zecharya, N., Oren-Suissa, M., Podbilewicz, B., and Schuldiner, O. (2012). Axon regrowth during development and regeneration following injury share molecular mechanisms. Curr Biol *22*, 1774-1782.

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis explores the molecular mechanisms of axonal regeneration and degeneration using *Drosophila melanogaster* as a model organism. While the mammalian PNS can regenerate, the mammalian CNS has limited capacity to regenerate (Yiu and He, 2006). Therefore, there is no effective treatment for injuries to the CNS known as of now. Whether a neuron can regenerate is determined by the extrinsic factors in the CNS environment and the intrinsic regenerate potential of the neurons. Past studies have mainly focused on removing the inhibitory extrinsic factors in the extracellular environment. Much less has been done to understand what determines the intrinsic growth potential of neurons until recently. Thus, in this study, we focused on understanding the molecular pathways that regulate intrinsic growth capacity of the neurons. One of the major pathways that controls the intrinsic growth capacity of neurons after injury is the Dual Leucine-Zipper bearing Kinase (DLK) pathway. Activation of this pathway leads to enhanced axonal regeneration while mutations of this pathway completely block any axonal regeneration. We still do not completely understand how this pathway is normally kept inactive and how it is activated after injury. Another pathway that determines the intrinsic growth capacity of neurons after injury is the PI3K/AKT pathway. Downstream of PI3K/AKT, Mammalian Target of Rapamycin (mTOR) is thought to mediate the signals from PI3K/AKT for axonal regeneration although there are conflicting results regarding whether mTOR is required for regeneration (Abe et al., 2010; Saijilafu et al., 2013). As these studies were done in cell cultures, it remains unclear what

roles mTOR plays in axonal regeneration *in vivo*. Interestingly, enhanced regeneration requires the DLK pathway, suggesting that the DLK pathway might be a key downstream target of the PI3K/AKT pathway. However, it remains unclear how the PI3K/AKT might regulate the DLK pathway. In this work, we have identified two additional regulators of the DLK pathway: *X11* and *rheb*. We have also tested the role of PI3K/AKT/mTOR in axonal growth *in vivo*, and have examined how the PI3K/AKT/mTOR pathway regulates the DLK pathway.

X11 and DLK/Wnd

The X11 protein family are multi-domain proteins composed of a conserved PTB domain and two C-terminal PDZ domains, which is conserved form worms to human. They are involved in regulating neuronal signaling, trafficking and plasticity through interactions with other proteins (Rogelj et al., 2006). In *Drosophila*, there are two paralogs of X11, *X11Lα* (CG5675) and *X11L*β (CG32677). mRNA expressions of both paralogs are restricted to differentiated neurons in the CNS (Gross et al., 2013). We have previously found that the *X11* genes control polarized localization of axonal membrane proteins *in vivo* (Gross et al., 2013). In this work, we have found that loss of *X11* in *Drosophila* leads to enhanced axonal regeneration after injury. We found that enhanced regeneration in *X11* mutants is due to the activation of the DLK/Wnd pathway because inhibition of this pathway completely suppresses the enhanced regeneration phenotypes. Furthermore, we found that X11 and DLK/Wnd colocalize at the Golgi and physically interact with each other. In *X11* mutants, we observed that DLK/Wnd no longer localizes to the Golgi, but instead localizes to the cytosol. After injury, we found that both Spectrin and X11 were

degraded. Furthermore, we showed that an increase in intracellular calcium is sufficient to trigger degradation of Spectrin and X11. Finally, we showed that in S2 cells, degradation of Spectrin and X11 is due to the calcium-activated protease CalpB. Taken together, our findings suggest that upon injury, a rise of intracellular calcium level may activate CalpB that leads to the degradation of Spectrin and X11. Degradation of X11 in turn leads to the activation of the DLK/Wnd pathway that ultimately leads to axonal regeneration. An interesting follow up to this study is to find out how mislocalization of DLK/Wnd from the Golgi to the cytosol leads to its activation. Another interesting follow up study would be to test whether *calpB* is required for axonal regeneration *in vivo*.

Rheb/mTOR and DLK/Wnd

Rheb, a small GTPase that belongs to the Ras superfamily, is a key upstream activator of the mTOR pathway that regulates cellular growth and protein synthesis (Aspuria and Tamanoi, 2004). We hypothesized that genes that lead to axonal growth during development might be able to enhance axonal regeneration after injury. Because *rheb* overexpression has previously been shown to cause overgrowth of the larval neuromuscular junctions (NMJs) during development, we tested if overexpression of *rheb* would lead to enhanced axonal regeneration. We found that *rheb* overexpression leads to enhanced axonal regeneration that is dependent on the DLK/Wnd pathway because inhibition of this pathway suppresses the enhanced regeneration. Interestingly, inhibition of the DLK/Wnd pathway suppresses the number of branches and boutons in the larval NMJs, but not the increased cell sizes caused by *rheb* overexpression, suggesting that cell size increase by *rheb* overexpression is regulated by a different pathway. Because

rheb has been shown to regulate both the mTORC1 pathway and the mTORC2 pathway, we have tested mutants of these pathways. We found that *rheb*-mediated axonal overgrowth requires mTORC1 pathway because mutant of a key downstream target of mTORC1, S6K, suppresses *rheb*-mediated axonal overgrowth, but not mutants of the mTORC2 pathway. This suggests that mTORC1 pathway plays a key role in axonal growth in *vivo*. Furthermore, we found that *rheb* overexpression leads to an increase of DLK/Wnd level in the motor neuron cell bodies while Hiw, an E3 ubiquitin ligase known to regulate the level of DLK/Wnd, controls the level of DLK/Wnd in the neuropil, where axons and synapses are found in the larval ventral nerve cord. This suggests that Rheb and Hiw regulates DLK/Wnd independently.

Finally, because *dlk/wnd* overexpression has previously been shown to prevent axonal degeneration in the motor neurons, we have tested whether *rheb* overexpression also prevents axonal degeneration, and we found that *rheb* overexpression prevents axonal degeneration in a DLK/Wnd-dependent manner. Taken together, we found that *rheb* overexpression enhances axonal growth and prevents axonal degeneration by increasing the level of DLK/Wnd through the mTORC1 pathway. Further studies will be needed in the future to understand how the PI3K/AKT/mTOR pathway is regulated after injury and why mature CNS has diminished PI3K/AKT/mTOR activities. Understanding how the DLK pathway and the PI3K/AKT/mTOR pathway, the two major pathways that determine the intrinsic growth capacity of neurons, are regulated will help develop new therapeutic strategies for people who suffer from CNS and PNS injuries. Past studies in *Drosophila* have led to major breakthroughs in our understanding of the molecular mechanisms of

axonal growth and guidance during development. We have just begun studying axonal regeneration and degeneration in flies. With the tools available and the ability to conduct genetic screens, *Drosophila* will continue to provide new insights into the molecular mechanisms that regulate axonal regeneration and degeneration in both the CNS and PNS.

REFERENCES

Abe, N., Borson, S.H., Gambello, M.J., Wang, F., and Cavalli, V. (2010). Mammalian target of rapamycin (mTOR) activation increases axonal growth capacity of injured peripheral nerves. J Biol Chem *285*, 28034-28043.

Aspuria, P.J., and Tamanoi, F. (2004). The Rheb family of GTP-binding proteins. Cell Signal *16*, 1105-1112.

Gross, G.G., Lone, G.M., Leung, L.K., Hartenstein, V., and Guo, M. (2013). X11/Mint genes control polarized localization of axonal membrane proteins in vivo. J Neurosci *33*, 8575-8586.

Rogelj, B., Mitchell, J.C., Miller, C.C., and McLoughlin, D.M. (2006). The X11/Mint family of adaptor proteins. Brain Res Rev *52*, 305-315.

Saijilafu, Hur, E.M., Liu, C.M., Jiao, Z., Xu, W.L., and Zhou, F.Q. (2013). PI3K-GSK3 signalling regulates mammalian axon regeneration by inducing the expression of Smad1. Nat Commun *4*, 2690.

Yiu, G., and He, Z. (2006). Glial inhibition of CNS axon regeneration. Nat Rev Neurosci *7*, 617-627.