2018

The Role Of Molecular Motors In Peripheral Nerve Regeneration

Melissa D. Priest
University of Pennsylvania, melissa.d.priest@gmail.com

The Role Of Molecular Motors In Peripheral Nerve Regeneration

Abstract
Following injury, axons of the peripheral nervous system have retained the capacity for regeneration. While it is well established that injury signals require molecular motors for their transport from the injury site to the nucleus, whether kinesin and dynein motors play additional roles in peripheral nerve regeneration is not well understood. Here we use genetic mutants of motor proteins in a zebrafish peripheral nerve regeneration model to visualize and define in vivo roles for kinesin and dynein. We find that both kinesin-1 and dynein are required for zebrafish peripheral nerve regeneration. While loss of kinesin-1 reduced the overall robustness of axonal regrowth, loss of dynein dramatically impaired axonal regeneration and also reduced injury-induced Schwann cell remodeling. Chimeras between wild type and dynein mutant embryos demonstrate that dynein function in neurons is sufficient to promote axonal regrowth. Finally, by simultaneously monitoring actin and microtubule dynamics in regenerating axons we find that dynein appears dispensable to initiate axonal regrowth, but is critical to stabilize microtubules, thereby sustaining axonal regeneration. These results reveal two previously unappreciated roles for dynein during peripheral nerve regeneration, initiating injury induced Schwann cell remodeling and stabilizing axonal microtubules to sustain axonal regrowth.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Michael Granato

Keywords
peripheral nerve, regeneration, Schwann cells, zebrafish

Subject Categories
Cell Biology | Molecular Biology | Neuroscience and Neurobiology

THE ROLE OF MOLECULAR MOTORS IN PERIPHERAL NERVE REGENERATION

Melissa Ducommun Priest

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

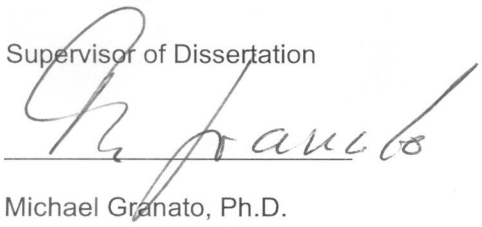
in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

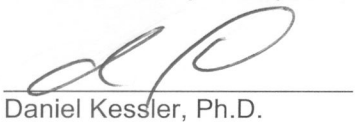
2018

Supervisor of Dissertation



Michael Granato, Ph.D.
Professor of Cell and Developmental Biology

Graduate Group Chairperson



Daniel Kessler, Ph.D.
Associate Professor of Cell and Developmental Biology

Dissertation Committee

Jonathan Raper, Ph.D., Professor of Neuroscience

Erika Holzbaur, Ph.D., Professor of Physiology

Steven Scherer M.D., Ph.D., Professor of Neurology

Wenqin Luo, M.D., Ph.D., Associate Professor of Neuroscience

DEDICATION

To the many amazing women in science and engineering that came before me—chief among these, my fiercely determined mother. You made it possible for me to pursue this path and never doubt the value I would have in this field.

ACKNOWLEDGMENT

The work detailed in this thesis would not have been possible without the mentorship, support, and friendship of many people over the past seven years. I would first and foremost like to thank my incredible mentor, Michael, for his guidance and support throughout graduate school. His high scientific standards and thoughtful feedback always pushed me to ask the best questions and answer them in the most rigorous way. While my project had many technical difficulties that discouraged me along the way, Michael never lost faith in my ability to persevere and produce great science.

I also have a long list of previous mentors to thank for preparing me for graduate school, including Daphne Preuss from the University of Chicago, Greg Bietel, Lonnie Shea, Laura DeLaporte and Hannah Tuinstra from Northwestern University, and Glen Lawson, Paula Schlax and Lee Abramson from Bates College. Thank you for the opportunities and guidance you all provided.

I would like to thank my thesis committee for all of the insight they have provided throughout my project. I would also like to thank my graduate group coordinator, Meagan Schofer, and the chair of DSRB during my time here, Steve Dinardo. These two work tirelessly to create a great environment and training program for DSRB students.

My labmates during my time in the Granato lab were one of the greatest highlights of my experience in graduate school. Not only are they all excellent scientists, but they were some of the friendly faces that helped keep me sane and motivated. Santanu, Marc, Roshan, Kurt, Juliane, Rajiv, Ali, Jesse, Katherine— you all welcomed me into the lab and put up with my many, many questions and showed me the ropes. Thank you for

being wonderful friends and making our lab environment so fun and supportive. Current Granato lab members—Melissa B, Owen, Lauren, Joy, Beth, Patti, Hannah, Elelbin, and Mafe—thank you for carrying the torch and keeping the lab environment as amazing as it has always been. And a special thank you to Jess, my bay mate for the last several years, who had to put up with me more than anyone else. Thanks for indulging all the questions, discussions, songs, dances, and tangential conversations. I was also lucky to have wonderful colleagues in our neighboring labs and other zebrafish labs, including the Mullins, Fisher, Pack, Raper and DiNardo labs.

I also had the support from many friends in my graduate program. Graduate school would not have been the same without you all walking alongside me during this experience. A special thanks to our game night crew (Will, Michael, Amanda, Liz, and Jeff) who made sure we kept up our friendship long after classes ended and as we all got busy finishing up grad school and moving on to other jobs. And also to some of my first friends in grad school, Eunsun and Xiu Li—it was always fun to catch up and commiserate and I'm glad we managed to find some time to keep in touch. My friends outside of graduate school were also crucial in supporting and motivating me over the past many years. Especially the Dragon and Flora & Jocelyn, who encouraged me in all of my pursuits and tried to remind me what a substantial and impressive undertaking this was.

I would especially like to thank my family for supporting me since the very beginning. My parents have always encouraged and enabled me to pursue my dreams, starting from my very first lab internship in high school that started me down this road. They are some

of my biggest supporters, finding even my smallest accomplishments in graduate school to be impressive and worthwhile. Their discussions always helped me see the bigger picture when I got lost and discouraged in the minutiae of my project. I have also been lucky to have the support of my wonderful in-laws over the past many years.

Finally, I would like to thank my incredible husband James for his endless love and support throughout graduate school. It was a difficult time and huge undertaking, and he always encouraged me and helped get me through the tough times, all while managing some huge undertakings of his own.

ABSTRACT

THE ROLE OF MOLECULAR MOTORS IN PERIPHERAL NERVE REGENERATION

Melissa Ducommun Priest

Michael Granato

Following injury, axons of the peripheral nervous system have retained the capacity for regeneration. While it is well established that injury signals require molecular motors for their transport from the injury site to the nucleus, whether kinesin and dynein motors play additional roles in peripheral nerve regeneration is not well understood. Here we use genetic mutants of motor proteins in a zebrafish peripheral nerve regeneration model to visualize and define *in vivo* roles for kinesin and dynein. We find that both kinesin-1 and dynein are required for zebrafish peripheral nerve regeneration. While loss of kinesin-1 reduced the overall robustness of axonal regrowth, loss of dynein dramatically impaired axonal regeneration and also reduced injury-induced Schwann cell remodeling. Chimeras between wild type and dynein mutant embryos demonstrate that dynein function in neurons is sufficient to promote axonal regrowth. Finally, by simultaneously monitoring actin and microtubule dynamics in regenerating axons we find that dynein appears dispensable to initiate axonal regrowth, but is critical to stabilize microtubules, thereby sustaining axonal regeneration. These results reveal two previously unappreciated roles for dynein during peripheral nerve regeneration, initiating injury induced Schwann cell remodeling and stabilizing axonal microtubules to sustain axonal regrowth.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1: INTRODUCTION

Peripheral nerve regeneration: a clinical concern

Peripheral nerve degeneration results from injury or disease and is termed peripheral neuropathy. In both of these instances, axons undergo a highly stereotyped degeneration program known as Wallerian degeneration. The degeneration of peripheral axons leads to sensory and/or motor loss as these neurons lose the connection to their functional targets. Some peripheral neuropathies are inherited genetic conditions, including the most common form called Charcot-Marie-Tooth disease (CMT), affecting 1 in 2,500 patients in the United States. Peripheral neuropathies can also be acquired, including causes such chemotherapy treatments, diabetes, and peripheral nerve injuries.

While axons of the peripheral nervous system (PNS) have much greater capacity for regrowth than those of the central nervous system (CNS), it is still estimated that fewer than 10 percent of patients recover full function after peripheral nerve injury (1). There are a number of challenges that peripheral nerves encounter during the process of regeneration. First, the distances across which axons must regrow are typically quite large in an adult human, and the speed at which peripheral axons regrow is not often sufficient to reach the appropriate target organs. Additionally, this slow regrowth leaves Schwann cells in the distal stump denervated for prolonged periods of time, and without trophic signals from innervating axons this results in Schwann cell loss.

In addition to the rate of regrowth, the direction of regrowth is also a concern for functional regeneration. During peripheral nerve regeneration, axons must navigate back

toward their original targets in an environment that is very different from that of the developing embryo, when axons initially find their targets. The ease of pathfinding depends on the nature of the injury–in crush injuries, the axons distal to the injury site degenerate but the nerve tube stays in place, leaving an existing pathway for the regrowing axons to follow back to their appropriate targets. In transection injuries, the axons, Schwann cells, and basal lamina of the nerve tube are all disrupted and an acellular transection gap must be traversed by axons in order for them to regrow in the proper direction. This pathfinding task is much more complex, requiring more active guidance of regrowing axons, and results in further reduced target innervation after this type of injury. Further studies elucidating the cellular and molecular mechanisms that promote axonal regrowth and guidance may reveal potential therapeutic targets for human peripheral neuropathies.

While the field of peripheral nerve regeneration is far too large to be covered in single section here, I have included a collection of reviews that discuss the current state of the field (Table 1.1).

Neuron-intrinsic mechanisms of nerve regeneration

In recent years, several key neuron-intrinsic regulators of axonal regrowth have been identified. Some of these signals are positive regulators, such as DLK, which has been identified as critical in promoting axonal regrowth in both C. elegans motor neurons (2) and the mouse sciatic nerve (3). In the central nervous system, several negative regulators have been identified as intrinsic, inhibitory factors in axonal regrowth. Neuron-specific deletion of either *PTEN* or *SOCS3* was found to improve axonal regrowth in the

optic nerve (4,5), and co-deletion of these two factors further increased axonal regrowth after injury (6). These experiments demonstrate that intrinsic axonal regrowth potential can be modulated by both activation of positive regulators, as well as downregulation of pathways that inhibit axonal regrowth.

Neuron-extrinsic mechanisms of nerve regeneration

In addition to neuron intrinsic mechanisms, there are critical extrinsic mechanisms that also promote nerve regeneration. The difference in regenerative capacity between axons of the CNS and axons of the PNS was once largely attributed to the intrinsic growth capacity of the neuron (7). Seminal experiments in which denervated peripheral nerve stumps were grafted into an injured spinal cord revealed that axons of the CNS have the capacity to regrow when surrounded by the environment of the PNS (7,8). This demonstrated that a combination of inhibitory factors in the CNS environment and growth promoting factors in the PNS environment contribute to the difference in regeneration observed in CNS and PNS axons.

Many of the extrinsic factors involved in regeneration come from surrounding cell types in the nerve. Schwann cells are the primary glia of the peripheral nervous system, residing in close proximity to axons. The majority of Schwann cells associate with a single axon, ensheathing the axon in tight wraps of myelin in a process known as myelination (9). Schwann cells provide neighboring axons with trophic support important for axonal maintenance and repair. Mice lacking Schwann cells exhibit early-onset axonal neuropathy, indicating a requirement for Schwann cells in axonal maintenance

(10). In addition to myelinating Schwann cells, there are also non-myelinating (Remak) Schwann cells (11) and perineural ensheathing glia (12) in peripheral nerves.

In response to injury, both myelinating and non-myelinating Schwann cells in the distal nerve stump dedifferentiate to a regeneration-promoting state and form bands of Bungner (13,14). Schwann cells in the bands of Bungner proliferate and provide growth-promoting factors, such as NGF, BDNF, and FGF, to regrowing axons (15-17). As regrowing axons enter the distal nerve stump, Schwann cells and their associated basal lamina serve as a scaffold to guide axons toward their original targets (18). Once these newly regrown axons arrive at their targets, Schwann cells stabilize and remyelinate them (19). In addition to promoting growth in the peripheral nervous system, Schwann cells transplanted into the central nervous system also promote axonal growth and remyelination, suggesting that Schwann cells have therapeutic applicability (20-22). Perineural glia have also recently been shown to play a role in nerve regeneration, helping to engulf axonal debris and bridging the transection gap to promote axonal regrowth (23).

In addition to non-neuronal cell types, extracellular molecules are other neuron-extrinsic factors that help promote nerve regeneration. Our lab previously identified a pathway by which glycosylated collagens help to specifically scaffold signaling molecules in order to direct axonal regrowth *in vivo* (24). This highlights the value of studying nerve regeneration in a system in which the behavior of both axons and Schwann cells can be experimentally modified and visualized in a whole organism context, as such axonal regrowth occurs through a fully *in vivo* environment.

Microtubules and associated motors in nerve maintenance and regeneration

Mutations in microtubule associated proteins and motors have widely been identified as causing neurodegenerative conditions, which highlights the importance of microtubules and associated transport functions in neuronal health (Table 1.2). Microtubules are a key component of the cytoskeleton, and these filaments consist of α-tubulin and ß-tubulin heterodimers arranged in a 13-protofilament lattice. Axonal microtubules have uniform polarity, with the minus ends toward the cell body and the plus ends toward the synaptic terminals (25). As is the case in other cell types, microtubules in the neuron are nucleated at the centrosome, from which they are transported in a dynein-dependent manner into the axon (26-28). Microtubule organization is critical to axon formation and stability–microtubules demonstrate increased stability in axons and it was also found that microtubule stabilization is sufficient to induce axon formation (29).

Microtubules serve not only to provide structural support to the cell, but also as tracks for active transport within the cell. Microtubule-associated motors move along microtubules in a polarity-dependent manner. Kinesins are a large family of microtubule-associated motors that move toward microtubule plus ends. Kinesin-1 is the conventional kinesin and is composed of two light chains and two heavy chains – the heavy chains make up the motor domain while the light chains are responsible for cargo binding and regulation (30). Cargos of kinesin include organelles, proteins and RNAs (31). Kinesin-1 is critical for neuronal maintenance, as mouse knockouts of various heavy chains cause neuronal loss and dysfunction (32,33). Furthermore, human mutations in the kinesin-1 heavy chain gene *KIF5A* have been found to cause a form of hereditary spastic paraplegia (34,35), as well as Charcot-Marie-Tooth type 2 (36).

In addition to its role in neuronal homeostasis, kinesin-1 is also involved in axonal outgrowth during both development and regeneration. During development, kinesin-1 binds and slides microtubules against one another, termed microtubule sliding (Figure 1.2). This activity had been found to drive major cell shape changes, and to specifically drive both dendritic and axonal extension in neurons (37,38). After axonal injury, it was found that kinesin-1-powered microtubule sliding is similarly required for axonal regrowth (39).

The complementary motor to kinesin is dynein, a minus end-directed motor protein that is responsible for all retrograde transport in neurons, carrying similar cargos such as mitochondria, signaling endosomes, and autophagosomes. Dynein is a large protein complex comprised of many subunits, with two homodimerizing heavy chains at the core of the complex. The heavy chain contains the microtubule binding domains, ATPase activity, as well as subunit interacting domains. The light and intermediate chains help confer cargo specificity and stabilization to the dynein complex (40). Previously, three mouse mutants with distinct dominant mutations in the singular dynein heavy chain, *dync1h1* (Loa, Cra and Swl), were identified in forward genetic screens as having progressive motor or sensory neuron loss (41,42). Moreover, human patients with heterozygous mutations in *DYNC1H1* experience progressive motor neuron loss, with multiple mutations now found to cause spinal muscular atrophy, lower extremity dominant (SMA-LED) and Charcot-Marie-Tooth disease (CMT) (43,44). These findings have underscored the importance of dynein-mediated transport in neuronal development and maintenance. Dynein has also been found to organize the cytoskeleton during

axonal outgrowth by transporting microtubules in a polarity-sorting manner to establish uniform microtubule polarity in the axon (45).

In addition to its role in neuronal maintenance, dynein has also been found to have a critical role in the neuron after injury. Following injury, one of the most important cellular changes within the neuron is the cell body response. This response consists of structural changes to organelles in the soma, as well as an increase in transcription and translation (46,47). The cell body response is elicited by both negative and positive injury signals. Negative injury signals result from a lack of target-derived trophic factors from the disconnected, distal axon. For example, retrogradely transported NGF decreases 10-fold after transection (48).

Conversely, positive injury signals are carried back to the cell body from the site of injury. One such signal is the phosphorylation of MAP kinases, Erk1 and Erk2. These activated MAPKs are carried retrogradely to the cell body linked to dynein through the intermediate filament vimentin (49). Once in the cell body, injury signals up-regulate regeneration associated genes, which promote axonal regrowth. These studies demonstrate the importance of retrograde signaling within the neuron following nerve injury.

While studies have previously demonstrated key roles for kinesin-1-powered microtubule sliding and dynein-dependent injury signaling after axonal injury, both of these motors bind many cargos and have multifaceted roles within the cell. This raises the possibility that kinesin-1 and dynein may have additional functions after injury. Furthermore, the studies of kinesin-1 and dynein function after axonal injury have lacked analysis of *in*

vivo dynamics in a multicellular environment, including the dynamics of regrowing axons themselves and the role of surrounding Schwann cells as my work demonstrates.

Zebrafish as a model to study nerve regeneration *in vivo*

In order to dissect the cellular and molecular mechanisms of peripheral nerve regeneration *in vivo*, we use the zebrafish larvae 5 days post-fertilization (dpf). At this stage, the larvae are optically clear which allows for *in vivo* imaging of nerves in the context of a live vertebrate animal. At 5 dpf, the nervous system is already fairly mature – motor axons have fully extended, formed functional connections with their muscle targets, and are myelinated by Schwann cells (50,51). Using combinations of transgenic lines in conjunction with live cell imaging, we can monitor interactions between relevant cell types, such as motor neurons and Schwann cells (Figure 1.3, 52). We use laser mediated axotomy to fully transect motor nerves, which is followed by Wallerian degeneration and functional regeneration, and combine this with the use of genetic mutants to assess the requirements of a cell type or signaling pathway. Using this approach, we previously described nerve-macrophage interactions *in vivo*, revealing a novel nerve scanning behavior (51). We have also used this approach to provide the first minute-by-minute account of interactions between motor axons and Schwann cells after injury *in vivo*, which revealed a requirement for Schwann cells in guiding axonal regrowth (52). Here, we use this system to assess the roles of the motor proteins kinesin-1 and dynein in peripheral nerve regeneration *in vivo* and reveal novel roles for dynein in the Schwann cell response to injury and microtubule modulation at the growth cone to promote sustained axonal regrowth.

Table 1.1: Useful reviews of peripheral nerve regeneration

Review	Notes
Mechanisms of Disease: what factors limit the success of peripheral nerve regeneration in humans? (53)	Summary of clinical challenges
Retrograde signaling in axonal regeneration (54)	Review of retrograde injury signaling
Intrinsic control of axon regeneration (55)	Review of neuron-intrinsic mechanisms of regrowth
The repair Schwann cell and its function in regenerating nerves (56)	Review of Schwann function after injury

Table 1.2: Human disease-causing mutations in microtubule-associated proteins

Gene	Disease	Reference
MAPT (microtubule-associated protein tau)	Alzheimer's disease	(57-60)
SPAST/SPG4 (spastin, microtubule severing protein)	Upper motor neuron diseases	(61,62)
DCTN1 (dynactin subunit 1)	Perry syndrome; lower motor neuron disease	(63,64)
KIF5A (kinesin heavy chain isoform 5A)	Hereditary spastic paraplegia (SPG10)	(34,65)
KIF1Bß (mitochondrial transport kinesin)	Charcot-Marie-Tooth Disease	(66)
DYNC1H1 (cytoplasmic dynein 1 heavy chain 1)	Charcot-Marie-Tooth 2O; Spinal muscular atrophy	(43,44)

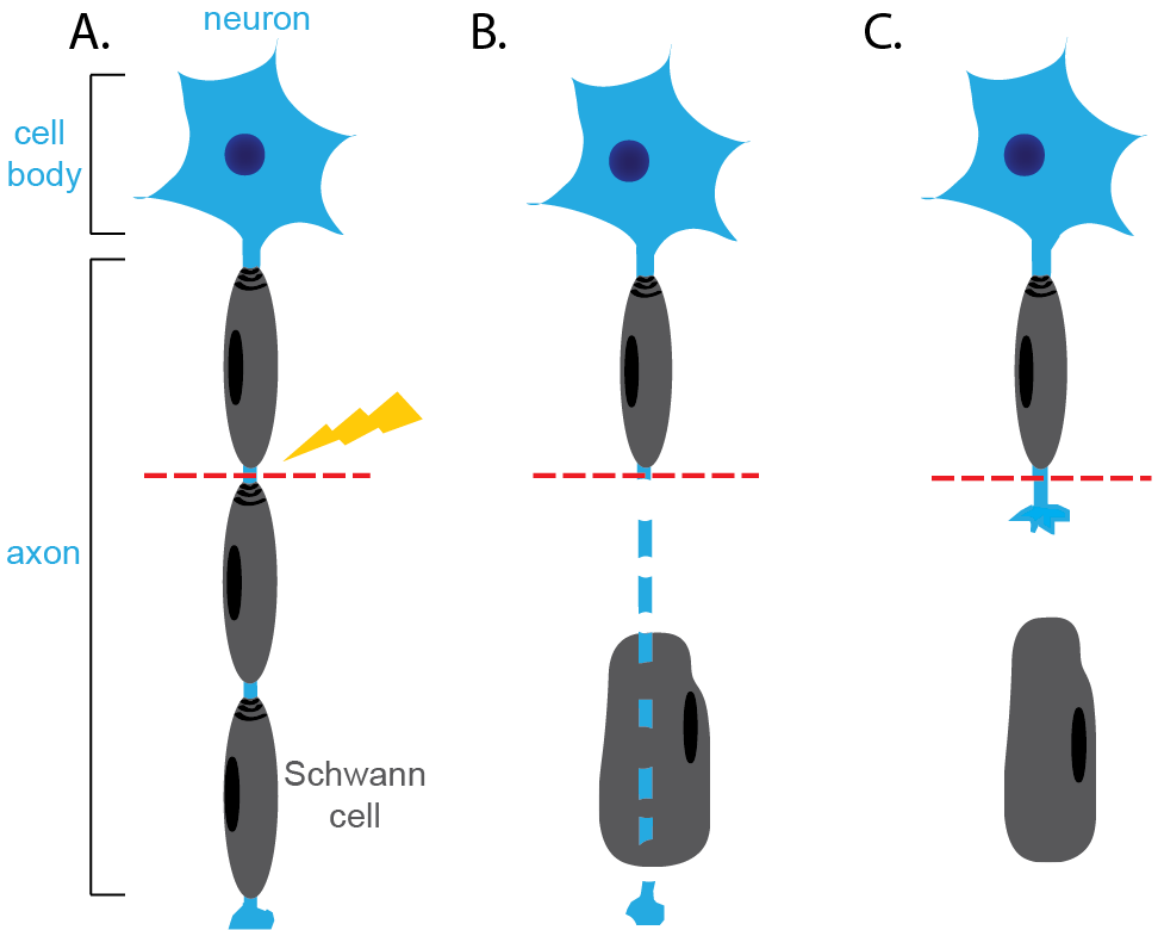


Figure 1.1: Schematic of peripheral nerve injury and regeneration. (A) Anatomy of an uninjured peripheral nerve. A long axon extends from the cell body of the neuron to synapse with a target cell (target not shown). The axon is ensheathed in myelinating Schwann cells. Red dashed line indicates site of transection. (B) Peripheral nerve after injury and axon fragmentation. Distal section of the axon fragments and distal Schwann cells dedifferentiate to a repair cell state. (C) Proximal axon section sprouts a growth cone that navigates back towards the denervated distal nerve and original target.

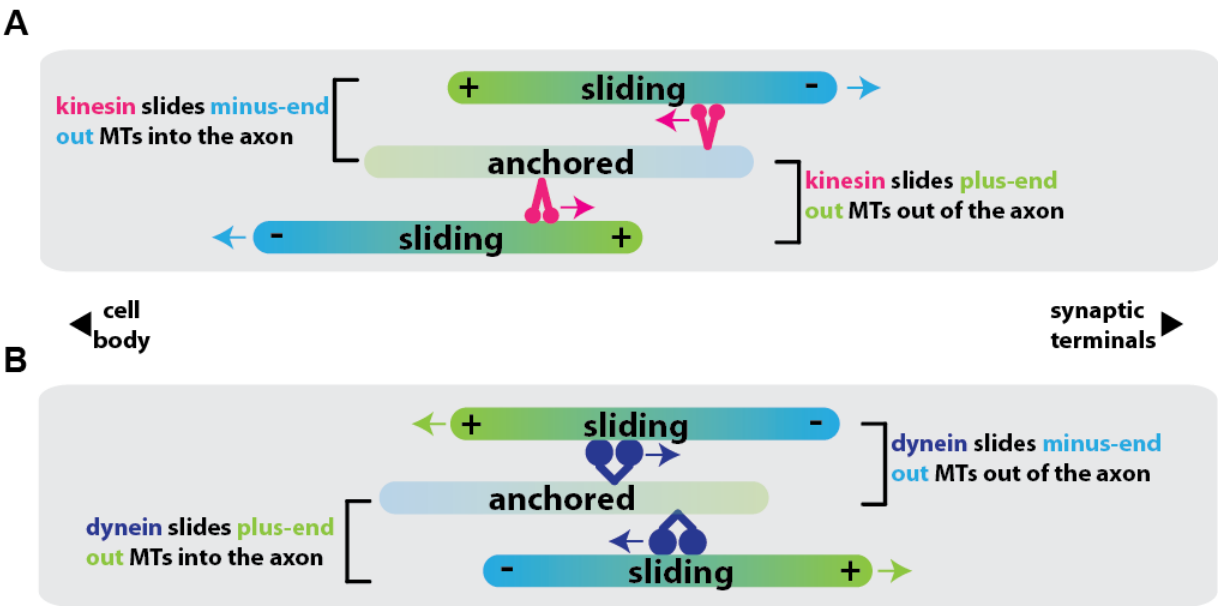


Figure 1.2: Schematic of microtubule sliding by motors kinesin-1 and dynein. (A) Kinesin-1 slides microtubules with their minus ends out into the axon. (B) Dynein slides microtubules with their plus ends out into the axon, and can also slide minus end out microtubules back towards the cell body.

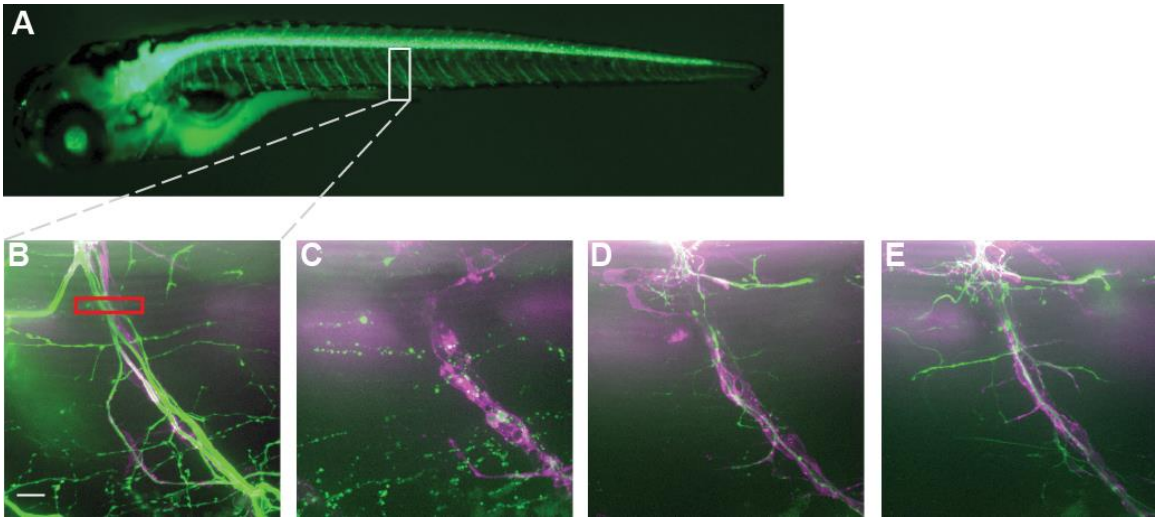


Figure 1.3: Zebrafish as a model for peripheral nerve regeneration *in vivo.* Adapted from Rosenberg et al., 2014 (A) 5 dpf zebrafish larvae with motor nerves labeled by *Tg(mnx1:GFP)* (green). White box magnified in panels B-E. (B-E) Motor nerve, with axons in green and Schwann cell membranes in magenta. Scale bar = 10 μm. Red box indicates site of transection. Motor nerve uncut (B), 6 hours post-transection (C), 24 hours post-transection (D), and 48 hours post-transection (E).

CHAPTER 2: KINESIN-1 AND DYNEIN IN NERVE REGENERATION

Axons of the mature peripheral nervous system have retained a remarkable ability for regeneration. Although simple in concept, peripheral nerve regeneration is a complex process that requires extrinsic as well as intrinsic mechanisms. Chief amongst the intracellular mechanisms that contribute to axonal regeneration are microtubule organization and dynamics as well as axonal transport. It has long been known that following injury the pool of dynamic microtubules at the lesion site, as well as axonal transport, increase (67-69). Given the central role of both microtubule dynamics and axonal transport in promoting axonal regeneration, factors that regulate both processes are prime candidates for regulating peripheral nerve regeneration.

The molecular motor proteins kinesin-1 and dynein are key regulators of both microtubule organization and axonal transport, and have both been implicated in peripheral nerve regeneration. Kinesin-1 is an anterograde motor that is essential for maintaining neuronal homeostasis by transporting cargos, including organelles and mRNA, from the cell body toward synaptic terminals. Kinesin-1 has also been shown to drive axonal outgrowth during development and after injury (38,39). Dynein has similarly been studied for its role in maintaining homeostasis by transporting cargo, however dynein moves cargo retrogradely towards the cell body. Dynein also plays an important role in axonal injury by trafficking injury signals, including components of JNK and ERK MAPK pathways, which are generated at the lesion site and actively transported to the cell body (70,71). There these injury signals initiate a regenerative response, characterized first by upregulation of regeneration-associated genes that prevent

neuronal cell death, and by initiating a genetic program that promotes regrowth of injured axons back to their original targets (72,73).

More recently it has become clear that in addition to its role in retrograde transport, dynein also functions in cytoskeletal organization and maintenance. For example, in C. elegans dynein regulates local microtubule dynamics in dendrites to promote microtubule stabilization (74). Additionally, in the axon dynein transports microtubules to establish and maintain microtubule polarity (28,45,75). Finally, besides its preeminent role in axonal homeostasis, dynein is also required for Schwann cell development and myelination (76). Yet despite dynein's well documented roles in both axons and glial cells, the effects of dynein on the cellular behaviors of regenerating axons and their associated glial cells in intact animals have not been examined.

In order to examine the diverse cellular functions of molecular motors in multiple cell types, we combined genetic mutants with live imaging of nerve regeneration in larval zebrafish, as previously described (51). This allowed us to study the real-time dynamics of regenerating axons and surrounding Schwann cells in a whole organism context. We find that the molecular motors kinesin-1 and dynein, albeit to different degrees, are both required for axonal regrowth in vivo and that dynein is also required to initiate injury-induced morphology changes in Schwann cells. We show that wild type neurons transplanted into otherwise dynein mutant animals are able to regrow robustly, indicating that neuronal dynein is sufficient to promote axonal regrowth. Finally, we find that dynein is dispensable for initiation of axonal regrowth but is required to stabilize microtubules in injured axons to generate persistent, long-range regrowth. These findings elucidate

previously unknown roles for dynein in the initiation of injury-induced Schwann cell behaviors, and identify a distinct role for dynein in promoting axonal regeneration through persistent axonal regrowth via microtubule stabilization.

Kinesin-1 and dynein are critical for peripheral nerve regeneration in vivo

To determine the in vivo roles of molecular motors in peripheral nerve regeneration, we first assessed regeneration in mutants lacking *kif5aa*, which encodes the neuron-specific Kif5A heavy chain of the conventional anterograde motor kinesin-1. We have previously shown that laser mediated transection of motor nerves in larval zebrafish initiates a Schwann cell dependent peripheral nerve regeneration program reminiscent of what is observed in adult vertebrate (52). Following their complete transection at 5 days post-fertilization (dpf), ventral motor nerves exhibit Schwann cell dependent functional regeneration by 48 hours post- transection (hpt) (Figure 2.1, A-B, 52). Prior to transection, *kif5aa*-/- motor nerves were indistinguishable from wild type nerves (Figure 2.1, C). By 48 hpt, motor axons in *kif5aa*-/- mutants had regrown across the full extent of the ventral myotome, although when compared to wild type siblings the number of fascicles that reached their ventral targets was reduced (Figure 2.1, D-E). Using a previously established semi-quantitative scoring index (for details see materials and methods and (52) we confirmed that compared to wild type siblings, motor axons in *kif5aa* mutants exhibited reduced regeneration ($p=0.0487$, Fisher's exact test).

We next assessed motor axon regeneration in genetic mutants for the dynein heavy chain gene (*dync1h1*) which encodes a core component of the retrograde motor dynein. These mutants survive through development due to maternally deposited dynein, which

persists until 4 dpf (77). This allows the fish to develop normally but have minimal levels of dynein at 5 dpf when we perform nerve transection assays. Importantly, this *dync1h1*^{hi3684} allele is a presumed null, unlike human mutations in *DYNC1H1* which have been found to be dominant gain of function alleles (43,44).

Prior to injury at 5 dpf, *dync1h1*-/- motor axons exhibit normal architecture, presumably due to the large maternal load sufficient to promote axonal development ((77), Figure 2.1, F). In contrast, following transection, motor axons in *dync1h1*-/- mutant animals frequently failed to extend beyond the transection site (Figure 2.1, G; quantified in Figure 2.1, H). Analysis of dynein heterozygotes revealed a less severe defect in axonal regrowth ($p=0.0745$), demonstrating a dose-dependent requirement for dynein in promoting axonal regrowth. The severity of the regeneration phenotype we observed in homozygous *dync1h1*-/- mutants was significantly stronger than that present in kif5aa-/- mutants ($p<0.0001$ and $p=0.0487$, respectively). This is consistent with the notion that other heavy chains of kinesin-1 as well as other kinesin family motors might compensate for the absence of *kif5aa* (78). In contrast, dynein is the sole protein responsible for microtubule-associated retrograde transport, and therefore the regeneration phenotype we observe in homozygous mutants likely represents a complete block of retrograde transport. We therefore focused on further defining the role of dynein in peripheral nerve regeneration.

Dynein mutant motor neurons persist in 5 dpf larvae

After finding that dynein mutants have impaired axonal regrowth when injured at 5 dpf, we next wondered whether this was truly a result of decreased regenerative capacity of

the dynein mutant axons, or whether there was a reduction in the number of motor neurons in a 5 dpf mutant larvae that caused fewer motor axons to regrow. It is possible that the lack of dynein in motor neurons is lethal to the cells, so we performed TUNEL staining in uninjured larvae at 5 dpf to determine whether there was increased cell death of motor neurons in the dynein mutants (Figure 2.2, A). In the wild type siblings, very few TUNEL positive cells were observed across the spinal cord, and no TUNEL positive cells were labeled in the ventral spinal cord where the motor neurons reside (Figure 2.2, B). In dynein mutant larvae, an increased number of TUNEL positive cells were observed in the spinal cord, indicating that there is some general increase in cell death in the mutants. To determine whether any of this cell death was in motor neurons, we quantified the number of TUNEL positive cells in the ventral spinal cord and found that this number was minimal (4 TUNEL+ cells across 36 hemisegments). Additionally, none of the TUNEL+ cells colocalized with the motor neuron label (*tg*(*mnx1:GFP*)), so we concluded that while the dynein mutants exhibited an increase in cell death at 5 dpf, this does not affect the motor neuron population (quantified in Figure 2.2, D). This indicates that rather than a lack of viable motor neurons to regrow axons, motor neurons persist in dynein mutant larvae at 5 dpf and the decreased regeneration observed in dynein mutants is in fact a defect specific to the process of axonal regrowth.

Dynein is required for injury-induced Schwann cell remodeling

In addition to its well-studied function in neurons, dynein is also required for proper differentiation and myelination of Schwann cells during development (14). Furthermore, in zebrafish lacking Schwann cells, regenerating axons sprout from the proximal nerve stump but fail to grow across the injury gap (20), somewhat reminiscent of the

phenotype we observe in dynein mutants. Given the importance of Schwann cells for peripheral nerve regeneration and the role of dynein in Schwann cell development, we sought to determine whether dynein is also required for the Schwann cell response to injury, characterized by stereotyped changes in Schwann cell morphology.

We have previously shown that before injury, Schwann cell membranes ensheathe individual motor axons, and that following post-injury axonal fragmentation, Schwann cell membranes reorganize, changing from a smooth, tube-like appearance to a more rounded and granular morphology (20), indicative of their transition to an activated, dedifferentiated state—known as the repair cell state—that promotes axonal regeneration. Previous studies revealed that in dynein mutants, Schwann cells development prematurely arrests at the promyelinating stage (14). We therefore first wanted to determine whether immature Schwann cells are able to respond appropriately to injury. For this we examined a mutant for the G-protein coupled receptor GPR126, in which Schwann cells also arrest at the promyelinating stage (21), similar to what has been reported for *dync1h1* mutants. Analysis of gpr126 mutants revealed that Schwann cells respond to injury by extending their membranes dramatically compared to their pre-injury state, indistinguishable from wild type Schwann cells (Figure 2.3, A-D). This demonstrates that developmentally arrested Schwann cells are still able to respond appropriately to nerve injury.

Having determined that promyelinating Schwann cells are competent to respond appropriately to nerve injury, we next examined the behavior of *dync1h1*-/- mutant Schwann cells. Unlike wild type and gpr126 mutant Schwann cells, we find that following

nerve transection *dync1h1*-/- mutant Schwann cells fail to initiate any morphological changes, and instead retain their pre-injury morphology and membrane position for the duration of the imaging period (up to five hours), arguing against a delay in onset but rather for a complete lack to initiate a Schwann cell injury response (Figure 2.3, E-F). To quantify this phenotype, we measured the changes in Schwann cell width following nerve transection as a simpler proxy for the complex changes in Schwann cell morphology (Figure 2.3, G). This revealed that while wild type and *gpr126*-/- Schwann cells significantly increase in width after injury, *dync1h1*-/- Schwann cells show no significant change. Thus, while *dync1h1*-/- mutant axons initiate fragmentation following injury, their associated Schwann cells fail to respond, consistent with the idea that dynein is critical for injury-induced Schwann cell remodeling.

Neuronal dynein is sufficient to promote axonal regrowth

Our results reveal injury-induced phenotypes in two cell types after injury in dynein mutants, and we therefore wondered whether dynein functions in neurons or Schwann cells to promote axonal regrowth. To determine the cell type in which dynein functions to promote axonal regrowth, we generated blastula stage chimeras (27) that contained wild type motor neurons and axons in otherwise *dync1h1*-/- larvae (Figure 2.4, A-B). Control transplantations have previously shown that wild type cells transplanted into wild type embryos generate motor neurons that are morphologically and functionally unaffected by transplantation (22). Following development and subsequent transection in a *dync1h1*-/- environment, wild type axons were able to regenerate robustly for the first 9 hours after sprouting (Figure 2.4, C-F), in a manner indistinguishable from wild type axons in a fully

wild type environment. This indicates that restoring dynein specifically in neurons in a dynein mutant is sufficient to promote axonal regrowth, demonstrating a neuron-intrinsic role for dynein during peripheral nerve regeneration. While technical considerations prevented us from visualizing Schwann cells in chimeric nerves, it is unlikely that wild type axonal regrowth would rescue dynein mutant Schwann cell response to injury, as these processes are temporally distinct—Schwann cells respond to injury between 2 and 4 hours post transection, while axons do not begin to regrow until about 9 hours after transection.

Interestingly, we found that *dync1h1*-/- axons that had wild type axons in the same nerve regrew more robustly than *dync1h1*-/- axons in nerves with no transplanted cells (14.23 ± 2.06 μm growth in *dync1h1*-/- larvae without transplants, see below; 39.33 ± 4.72 μm growth in *dync1h1*-/- larvae with transplants; Figure 2.4, F). In several instances, we observed *dync1h1*-/- axons growing along previously extended wild type axons (Figure 2.4, G-I). This indicates that the presence of wild type axonal regrowth is able to partially rescue the *dync1h1*-/- axonal regrowth defects. Thus, while dynein acts intrinsically in neurons to promote axonal regrowth, it may also play a role in inter-neuronal interactions during regeneration.

Dynein stabilizes axonal growth during regeneration

We next asked how dynein promotes axonal regeneration within peripheral nerves. Peripheral nerve regeneration is a dynamic process composed of several defined stages, starting with growth cones emerging from the proximal stump and probing the injury gap environment. This is followed by stabilization of axonal regrowth across the

injury gap and along the correct trajectory, and finally rapid, sustained axonal regrowth towards their original targets (23). We used live cell imaging after nerve transection to quantify axonal dynamics in dynein mutants and determine which of these stages require dynein. In wild type siblings, we observed growth cones emerging from the proximal stump extending (3.54 events per 8 hours) and retracting (1.08 events per 8 hours) repeatedly, consistent with the idea that these growth cones are probing the injury gap for a path towards their original targets (Figure 2.5, A-B). We found that *dync1h1*-/- axons exhibit similar frequencies of axonal extensions and retractions (Figure 2.5, C-D), suggesting that they probe the injury gap as actively as their wild type siblings (Figure 2.5, E).

We next examined the second stage of axonal regeneration when axons become stabilized and then extend toward their original targets. To quantify this process, we measured the overall displacement of regenerating growth cones over the first ~8 hours after sprouting began. We found that the majority of regenerating wild type axons grew beyond the transection site within 8 hours of sprouting (Figure 2.5, F), travelling an average of 41.49 μm (SEM ± 5.84) over this time period. In contrast, regenerating *dync1h1*-/- axons rarely extended beyond the transection site (Figure 2.5, G), travelling an average of 14.23 μm (SEM ± 2.06) and never exceeding 21.94 μm in growth. Moreover, quantification of growth cone displacement at 8 hours post transection revealed that compared to regenerating wild type axons, *dync1h1*-/- axons exhibited a significant decrease in axonal extension (Figure 2.5, H). Combined these results argue that rather than initiating growth cone sprouting and short range axonal extensions, dynein predominantly acts early during axonal regeneration to stabilize regenerating

axons thereby promoting persistent, long-range regrowth.

Dynein stabilizes microtubules to promote persistent regrowth

Dynein has recently been shown to play a critical role in generating and maintaining microtubule organization, both processes central to axonal growth (10,11,24,25). To determine whether dynein regulates microtubule dynamics in axons during regeneration, we used a transgenic line that simultaneously labels actin and microtubules in motor neurons (*mnx1:Gal4; UAS:lifeact-GFP-v2a-EB3-RFP*). Growth cone extension occurs in three stages: first, protrusion driven by F-actin, then engorgement driven by microtubule-based transport of organelles and vesicles, and finally consolidation in which the growth cone contracts and stabilizes to form a cylindrical axon shaft (26). In regenerating wild type axons, filopodia extend at the growth cone and microtubules follow behind, stabilizing and consolidating newly formed protrusions (Figure 2.6, A- D). The majority of regenerating *dync1h1*-/- axons (n=30/37) displayed one of two phenotypes characteristic for microtubule disruption. In 59 percent (n=22/37) we observed filopodia extension followed briefly by microtubule extension (Figure 2.6, E-F) and then arrest at the engorgement stage before finally retracting (Figure 2.6, G-H). In 22 percent (n=8/37) of regenerating *dync1h1*-/- axons, microtubules faithfully followed filopodia extending at growth cones. However, rather than consolidating in the proximal growth cone, they adopted a looped conformation at the leading edge of the growth cone, leading to stalling and retraction (Figure 2.6, I-M). This demonstrates that lack of dynein leads to loss of microtubule organization at regenerating growth cones and stalling of regenerating axons early during the regeneration process. Combined, these findings

support a model by which dynein plays a critical role in regulating microtubule dynamics, thereby stabilizing growth of regenerating axons as they initiate their trajectory across the injury gap and towards their original targets. Our data reveal a critical role for dynein in promoting axonal extension via microtubule stabilization, as well as a previously uncharacterized role in initiating Schwann cell response to injury.

Dynein is required for cytoskeletal maintenance in axons

Given that dynein regulates microtubule dynamics in the axon during regeneration, we wondered if dynein was also required for long term maintenance of the axonal cytoskeleton. While the homozygous *dync1h1* mutants are only viable until 8 dpf, the heterozygotes are fully viable into adulthood. This gave us the opportunity to examine axons in animals with a reduction in functional dynein levels and determine how this effects the cytoskeleton long-term.

In collaboration with Clara Franzini-Armstrong, we fixed *dync1h1* heterozygotes at 2 months of age (wild type n=2 fish, *dync1h1*+/- n=2 fish) and performed electron microscopy on cross sections of ventral motor nerves. Dissections, fixation and sectioning were all performed in parallel across samples. In the wild type siblings, we found nicely organized cytoskeletal components within most axons. Elongated intermediate filaments were highly ordered and microtubules were apparent in the majority of axon sections. In the *dync1h1* heterozygotes, most axons contained disordered intermediate filaments and many sections lacked apparent microtubules (Figure 2.7, quantified in Figure 2.8). This suggests that dynein is required to maintain cytoskeletal structure in motor axons.

There are several key experiments that should follow up on these preliminary findings – first, the initial experiment should be repeated to confirm that the disrupted cytoskeleton in *dync1h1* heterozygotes is not simply an artifact from fixation and sectioning. While this is unlikely due to the number of samples in the first experiment, it would also be an extremely surprising finding that adult axons can function with a severely disrupted cytoskeleton. Mutations in various cytoskeletal proteins have been found to cause severe neurodegeneration in humans, indicating that cytoskeletal structure is critical to neuronal maintenance and function (79).

Another critical question regarding the cytoskeletal defects observed in *dync1h1* heterozygote adults is whether the cytoskeletal structure degenerates over time, or whether it is improperly established during development. To distinguish between these possibilities, electron microscopy should be performed on *dync1h1* heterozygote larvae at between 3-5 dpf, after the axonal cytoskeleton is established but likely before any cytoskeletal degeneration could occur. If the axonal cytoskeleton is intact and properly organized in the heterozygous larvae, this would suggest that the cytoskeleton develops properly despite reduced dynein levels but subsequently degenerates over time, indicating that dynein is required for cytoskeletal maintenance. If the axonal cytoskeleton is already disordered in the heterozygote larvae, this would indicate that dynein is required to establish the cytoskeleton rather than to maintain it.

Dynein promotes axonal degeneration after injury

Wallerian degeneration is both a highly stereotyped and highly conserved process, occurring in organisms from insects to mammals. Axonal degeneration is initially marked

by membrane beading and swelling, followed by rapid breakdown of the cytoskeleton. After the axon fragments, surrounding cells, such as Schwann cells and macrophages in the peripheral nervous system, engulf and digest cellular debris. While debris clearance is dependent on surrounding cell types, axon fragmentation is a neuron-intrinsic event (80). It was originally thought that the loss of trophic support following injury caused axonal degeneration. However, expression of a novel fusion protein in a spontaneous mouse mutant, Wld^S, was found to protect injured axons from degeneration (81). This suggests that axonal death is an active process, rather than a passive, wasting process resulting from loss of trophic factors. Furthermore, a more recent study found that a loss of function mutation in the protein dSarm results in delayed axonal degeneration. This finding demonstrates that there are specific pro-death signals involved in axonal degeneration (82). However, axonal death is distinct from apoptotic death as it is caspase-independent and localized to the axon, leaving the soma intact (83).

The cytoskeletal events that occur during axonal degeneration have been well characterized. Cytoskeletal breakdown is initiated by both the ubiquitin-proteasome system (UPS) and calpain-mediated proteolysis (84,85). These processes are calcium-dependent and therefore upregulated after injury due to an initial increase in intracellular calcium levels. Inhibiting UPS and calpain either directly or through calcium chelation delays cytoskeletal breakdown, although it does not prevent initial beading and swelling of the axonal membrane (83,84,86). This indicates that there must be additional mechanisms of cellular breakdown involved in initiating axon fragmentation that are still unknown.

To determine whether the motor protein dynein is involved in the process of axonal degeneration, we transected nerves in 5 dpf dynein mutant larvae and performed timelapse imaging of the distal nerve stump beginning shortly after injury. We found that compared to their wild type siblings, dynein mutant larvae exhibited a dramatic increase in the time to first axonal fragmentation (Figure 2.9). Given that the time to axonal fragmentation increases in the absence of dynein, this demonstrates that dynein promotes axonal degeneration *in vivo.* There are several possibilities of how dynein may promote axonal degeneration. Interestingly, when axonal fragmentation begins, the entire length of the axon fragments simultaneously (87), suggesting spatial and temporal coordination of the fragmentation process. As dynein carries signaling endosomes throughout the axon, it is possible that some of these active death signals required for Wallerian degeneration may be transported by dynein to properly localize throughout the axon after injury and coordinate the initiation of fragmentation.

Alternatively, it is possible that dynein coordinates some of the mechanical breakdown required for the process of fragmentation and degeneration. Dynein is required for axonal autophagy as it transports autophagosomes, which are intracellular vesicles that form a double membrane that engulfs cytoplasmic contents and delivers them to lysosomes for degradation. In neurons, autophagosomes initially form and sequester cytoplasmic contents at the distal end of the axon and move retrogradely as they mature and ultimately fuse with lysosomes, which are then termed autolysosomes (88). Retrograde transport by dynein is critical in mediating encounters between autophagosomes and lysosomes to allow for fusion and acidification of autolysosomes (89). Though there is evidence for increased autophagy in degenerating neurons

(90,91), the transport and function of autophagosomes during axonal degeneration remains unclear. Given that dynein mutants exhibit delayed degeneration after injury and autophagosomes are a direct cargo for dynein, future experiments should examine autophagy in dynein mutant axons to determine whether dynein-dependent transport of autophagosomes contributes to axonal degeneration *in vivo.*

Taken together, the studies in this chapter reveal for the first time an *in vivo* requirement for the motor proteins kinesin-1 and dynein in nerve regeneration. We also identified novel roles for dynein in the process of nerve regeneration, including involvement in axonal extension via modulation of microtubule dynamics and initiation of Schwann cell morphology changes after injury. Preliminary studies suggest involvement of dynein in cytoskeletal maintenance of axons as well as an active role in the initiation of axonal fragmentation after injury. Here we have identified that *in vivo,* dynein promotes axonal degeneration, regrowth, and maintenance.

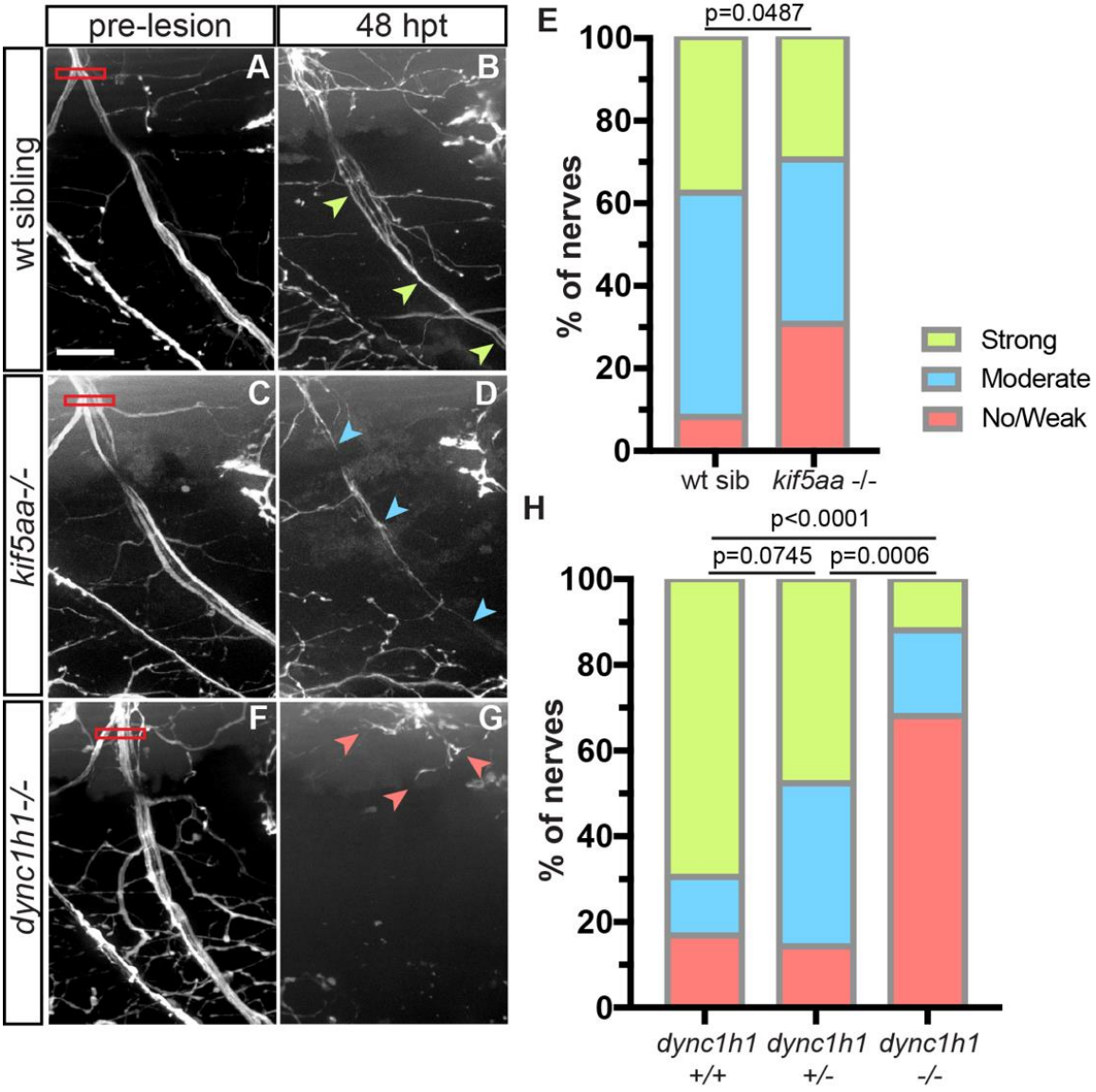

pre-lesion
48 hpt
wt sibling
kif5aa-/-
dync1h1-/-
A
B
C
D
F
G
E
p=0.0487
100
80
60
40
20
0
% of nerves
wt sib
kif5aa -/-
Strong
Moderate
No/Weak
H
p<0.0001
p=0.0745
p=0.0006
100
80
60
40
20
0
% of nerves
dync1h1 +/+
dync1h1 +/-
dync1h1 -/-

Figure 2.1: Dynein and Kinesin-1 are required for peripheral nerve regeneration *in vivo.* (A) Wild type motor nerve pre-lesion (red box, transection site; scale bar = 20 μm). (B) By 48 hpt, several fascicles have regrown fully across the ventral myotome (green arrowheads, regrown axons, strong regeneration). (C) *kif5aa*-/- motor nerve pre-lesion. (D) At 48 hpt, some axons have extended across the myotome (blue arrowheads, regrown axons, moderate regeneration). (E) Quantification of *kif5aa* mutant regeneration at 48 hpt (wild type siblings, n=66 nerves; *kif5aa*-/-, n=20 nerves, p=0.0487, Fisher's exact test).

(F) *dync1h1*-/- motor nerve pre-lesion. (G) By 48 hpt, regrowing axons have extended slightly but failed to reach the ventral extend of the myotome (red arrowheads, stalled axons, no/weak regeneration). (H) Quantification of *dync1h1* mutant regeneration at 48 hpt (*dync1h1*+/+, n=59 nerves; *dync1h1*+/-, n=21 nerves; *dync1h1*-/-, n=25 nerves; p=0.007; p=0.0006; p<0.0001, respectively, Fisher's exact test).

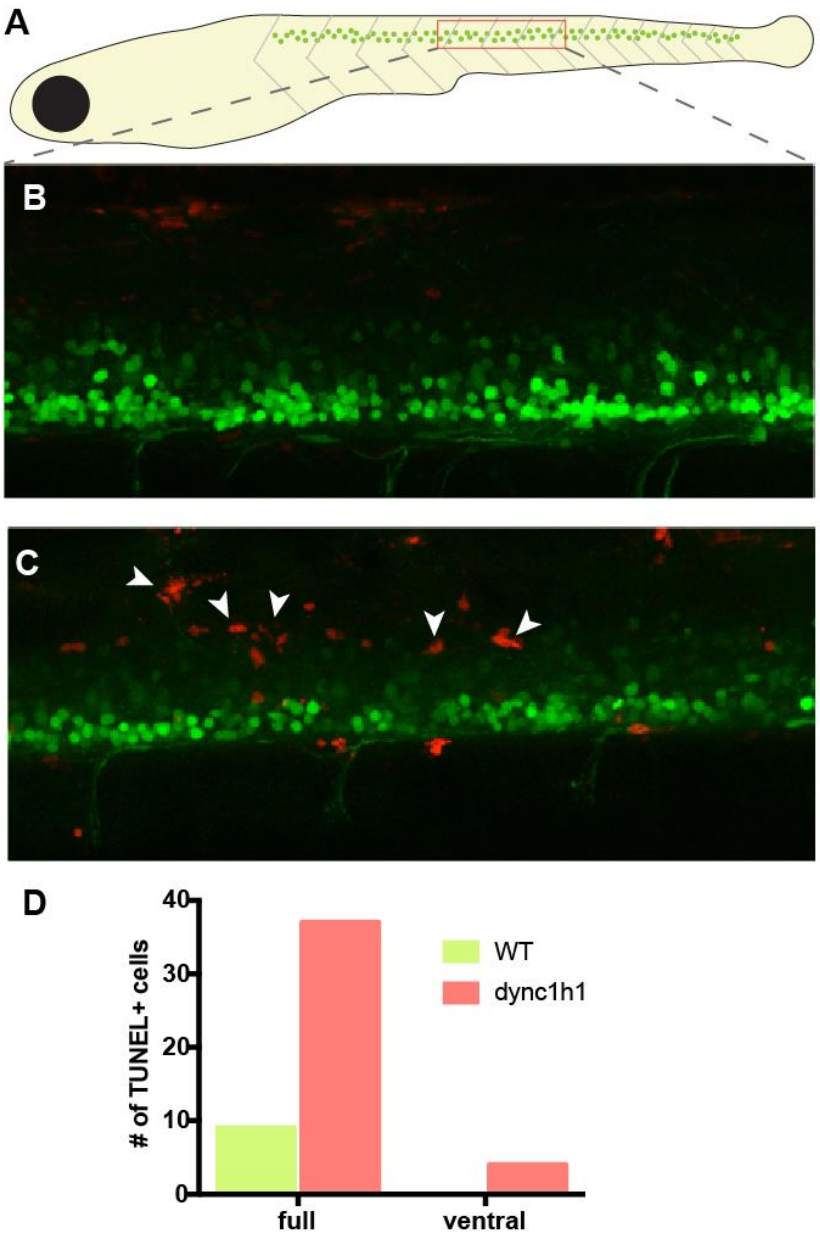

A
B
C
D
40
30
20
10
0
# of TUNEL+ cells
WT
dync1h1
full
ventral

Figure 2.2: Dynein mutant motor neurons persist in 5 dpf larvae. (A) Schematic of 5 dpf larvae. Motor neuron cell bodies shown in green, red box indicates area imaged for TUNEL quantification. (B-C) TUNEL staining of spinal cord of uninjured larvae at 5 dpf. Motor neuron cell bodies labeled in green (mnx1:GFP), TUNEL + cells labeled in red. (B) Wild type larvae and (C) *dync1h1*-/- larvae. (D) Quantification of TUNEL + cells in the full spinal cord and ventral spinal cord across 36 hemisegments (n= 3 larvae).

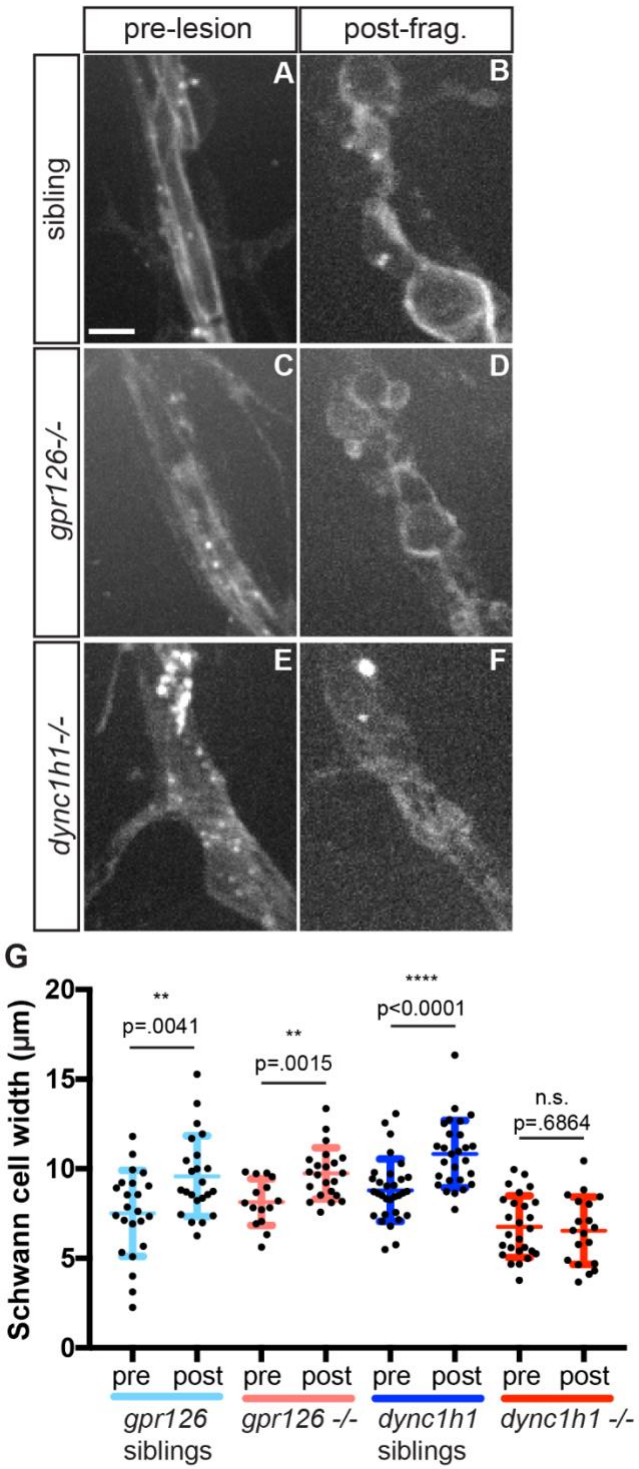

pre-lesion
post-frag.
sibling
gpr126-/-
dync1h1-/-
A
B
C
D
E
F
G
Schwann cell width (µm)
20
15
10
5
0
**
p=.0041
**
p=.0015
****
p<0.0001
n.s.
p=.6864
pre
post
pre
post
pre
post
pre
post
gpr126 siblings
gpr126 -/-
dync1h1 siblings
dync1h1 -/-

Figure 2.3: Dynein is required for injury-induced Schwann cell morphology changes. (A-F) Schwann cells in 5 dpf larvae labeled by *Tg*(*sox10*:*mRFP*). (A) Prior to injury, wild type Schwann cells have smooth, straight membranes that are tightly associated with the axonal track (scale bar = 5 μm). (B) After axonal fragmentation, Schwann cell membranes change morphology and widen to accommodate axonal debris. (C) Prior to injury, *gpr126*-/- Schwann cells are loosely associated with axons as they do not myelinate. (D) After axonal fragmentation, *gpr126*-/- Schwann cells are able to change morphology and widen. (E) Prior to injury, *dync1h1*-/- Schwann cells are loosely associated with axons as they also do not myelinate. (F) After axonal fragmentation, *dync1h1*-/- Schwann cell membranes maintain an elongated conformation and do not dramatically change morphology, indicating a disrupted injury response. (G) Quantification of Schwann cell width pre- and post-fragmentation in *gpr126* and *dync1h1* mutants.

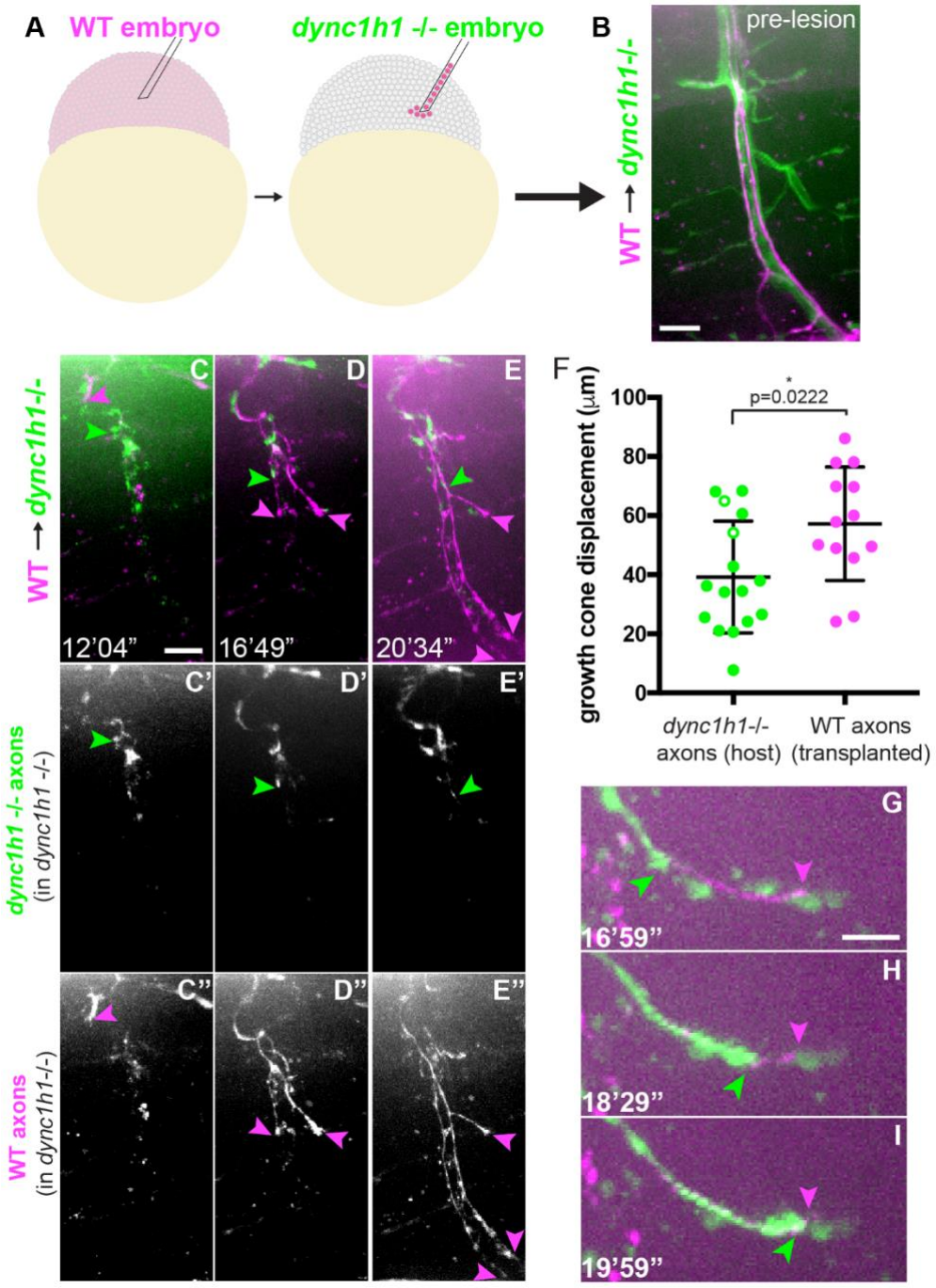

A
WT embryo
dync1h1 -/- embryo
B
pre-lesion
WT → dync1h1-/-
C
D
E
12'04"
16'49"
20'34"
C'
D'
E'
dync1h1 -/- axons
(in dync1h1 -/-)
C"
D"
E"
WT axons
(in dync1h1-/-)
F
growth cone displacement (μm)
p=0.0222
dync1h1-/-
axons (host)
WT axons
(transplanted)
G
16'59"
H
18'29"
I
19'59"

Figure 2.4: Neuronal dynein is sufficient to promote axonal regrowth. (A) ~10 rhodamine-labeled cells were transplanted from wild type blastulas to *dync1h1*-/- blastulas. (B) At 5 dpf, nerves contained wild type neurons (transplanted cells labeled by rhodamine-dextran, magenta) in a *dync1h1*-/- larva (host motor neurons labeled by *Tg*(*mnx1:GFP*), green; scale bar = 10 μm). (C-E) After transection, wild type axons (magenta arrowheads) are able to regrow robustly in the *dync1h1*-/- embryo, while *dync1h1*-/- host axons regrow significantly less (green arrowheads; scale bar = 10 μm). (F) Quantification of growth cone displacement in *dync1h1*-/- host axons and transplanted wild type axons. Open circles indicate *dync1h1*-/- mutant axons that grew along transplanted wild type axons. (G-I) Some *dync1h1*-/- axons demonstrated improved regeneration in the presence of wild type axons in the same nerve. Here, a *dync1h1*-/- axon (green arrowheads) follows along a previously regrown wild type axon (magenta arrowheads; scale bar = 5 μm).

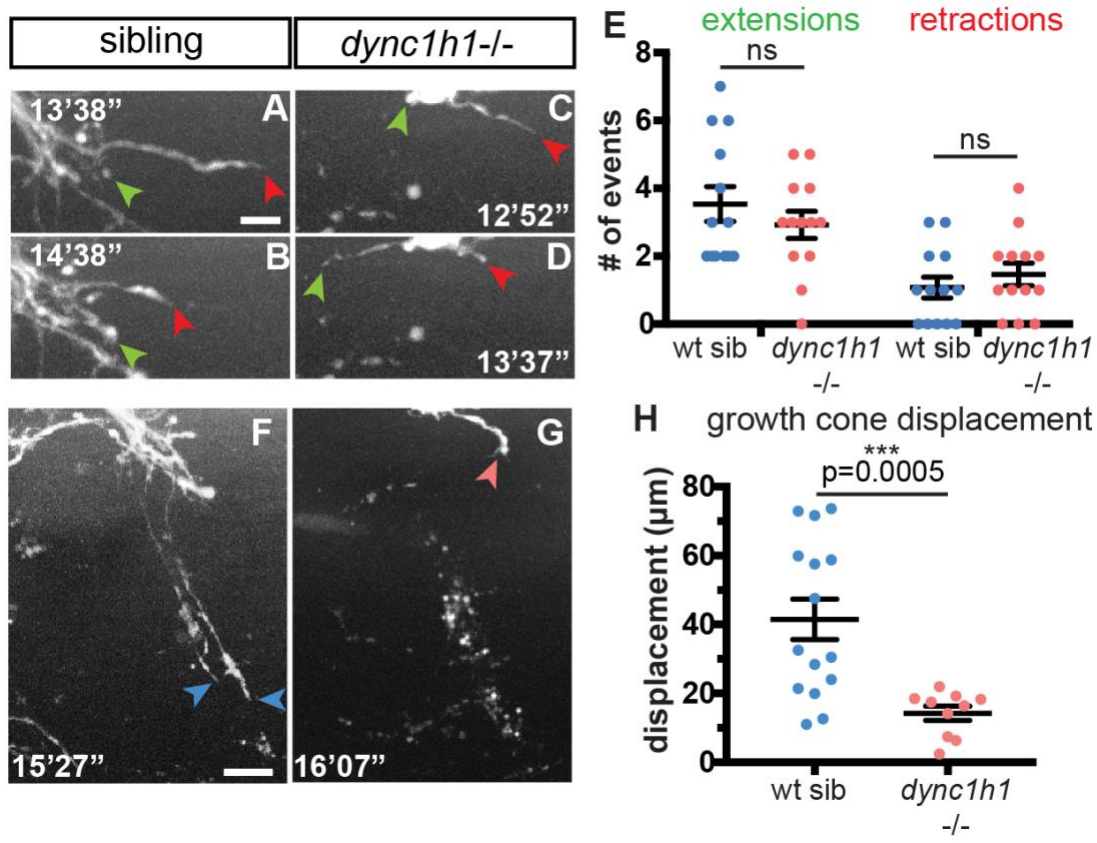
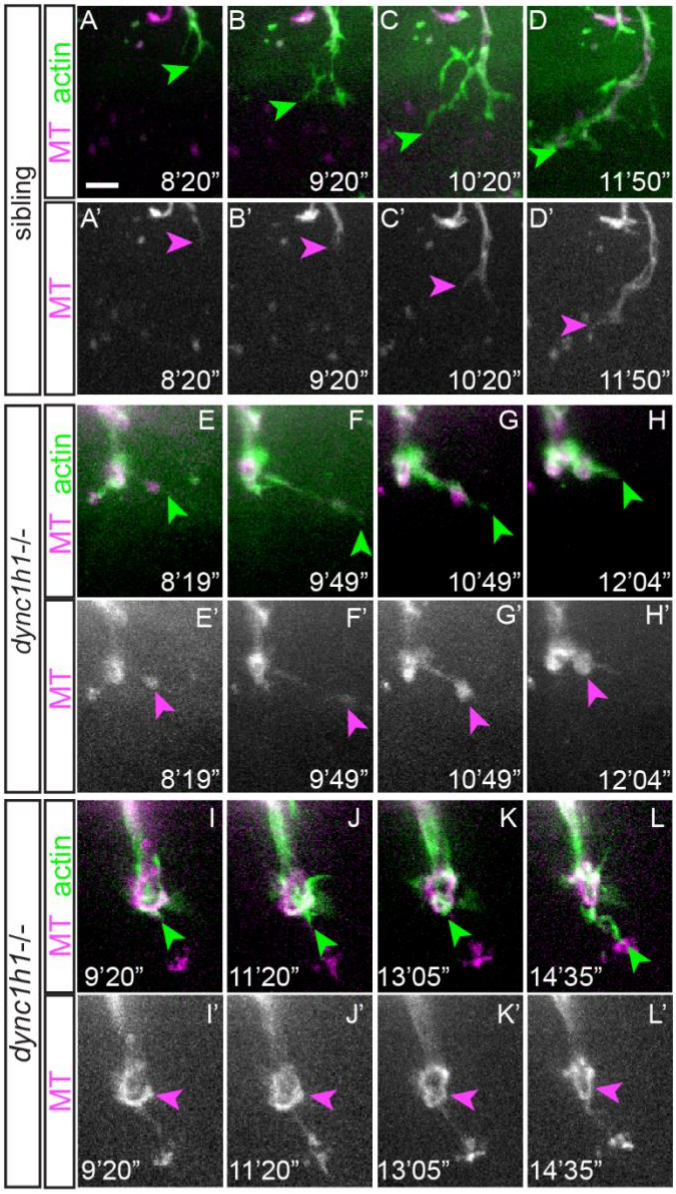


Figure 2.5: Dynein stabilizes axonal extensions during regeneration. (A-B) In wild type animals, regenerating axons begin probing the environment by extending and retracting (green and red arrowheads, respectively; scale bar = 5 µm). (C-D) *dync1h1-/-* axons also extend and retract after injury. (E) Quantification of extension and retraction events in wild type siblings (n=13 axons) and *dync1h1-/-* axons (n=13 axons). (F-G) Measurement of overall growth cone displacement from transection site ~16 hpt in wild type siblings (F; blue arrowheads, growth cones; scale bar = 10 µm) and *dync1h1-/-* (G; red arrowheads, growth cones). (H) Quantification of growth cone displacement ~16 hpt (wild type siblings, n=15; *dync1h1-/-*, n=10; p=0.0005, unpaired t-test).


sibling
dync1h1-/-
dync1h1-/-
MT actin
MT
A
B
C
D
A'
B'
C'
D'
8'20"
9'20"
10'20"
11'50"
E
F
G
H
E'
F'
G'
H'
8'19"
9'49"
10'49"
12'04"
I
J
K
L
I'
J'
K'
L'
9'20"
11'20"
13'05"
14'35"

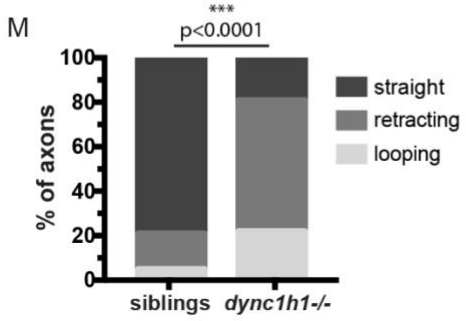

M
***
p<0.0001
100
80
60
40
20
0
% of axons
straight
retracting
looping
siblings
dync1h1-/-

Figure 2.6: Dynein stabilizes microtubules to promote persistent regrowth. (A-D) Regenerating wild type axons first extend actin protrusions then extended microtubules, leading to stable growth (scale bar=5 μm; green arrowheads, actin; magenta arrowheads, microtubules). (E-H) *dync1h1*-/- axons extend actin protrusions followed by microtubule growth that arrests during growth cone engorgement and leads to axon retraction (G,H). (I-L) *dync1h1*-/- axons extend actin protrusions but microtubules form aberrant loop structures (magenta arrowheads), leading to axonal retraction. (M) Quantification of microtubule organization in regrowing axons of *dync1h1* mutants (siblings, n=19 axons; *dync1h1*-/-, n=37 axons; $p<0.0001$, Fisher's exact test).

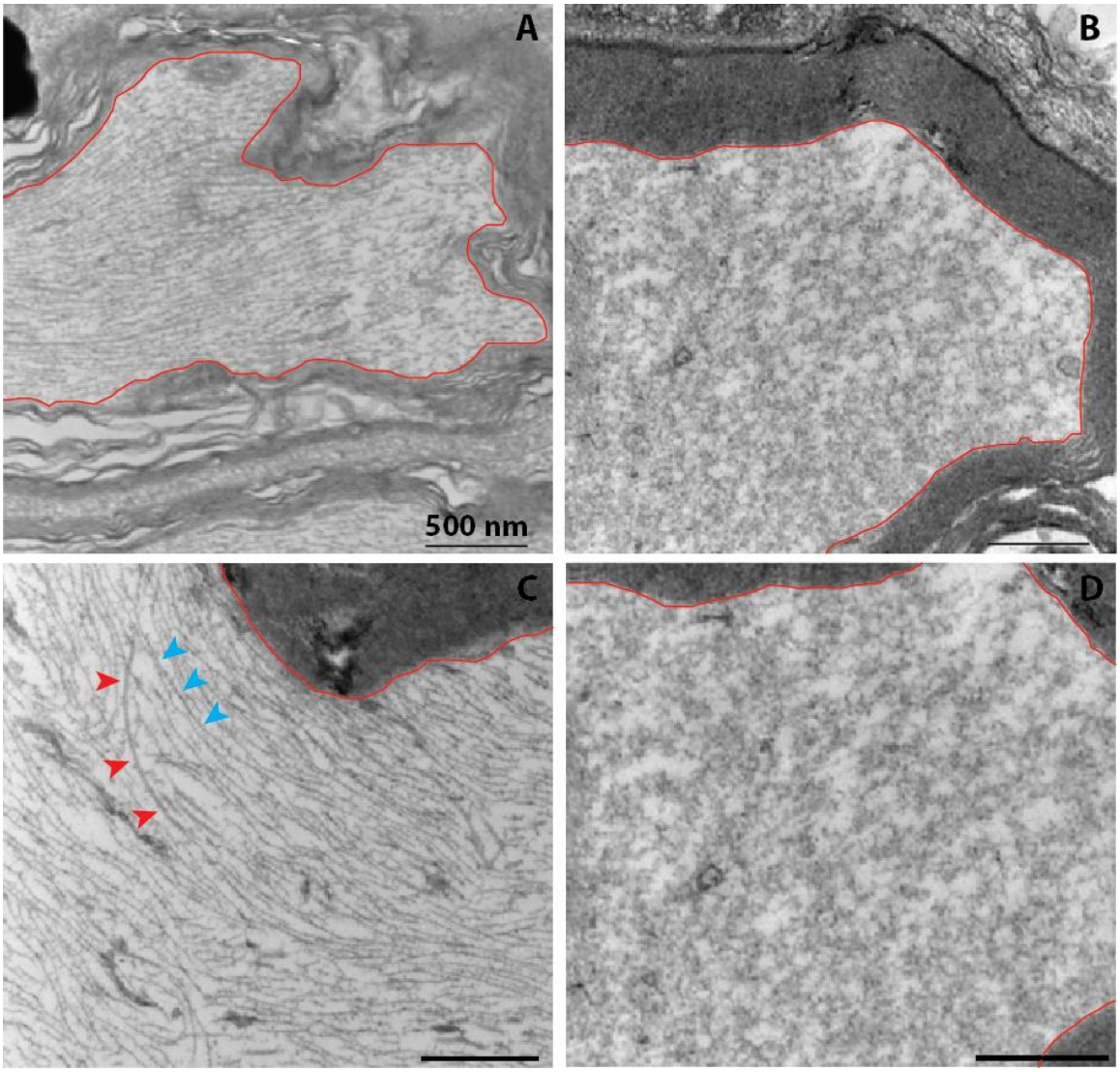


Figure 2.7: Dynein maintains cytoskeletal structure in motor axons of adult zebrafish. (A-D) Cytoskeletal structure of large caliber axon (red outline), scale bar = 500 nm. Wild type sibling (A,C) and *dync1h1*+/- (B,D). Red arrows, microtubules; blue arrows, intermediate filaments.

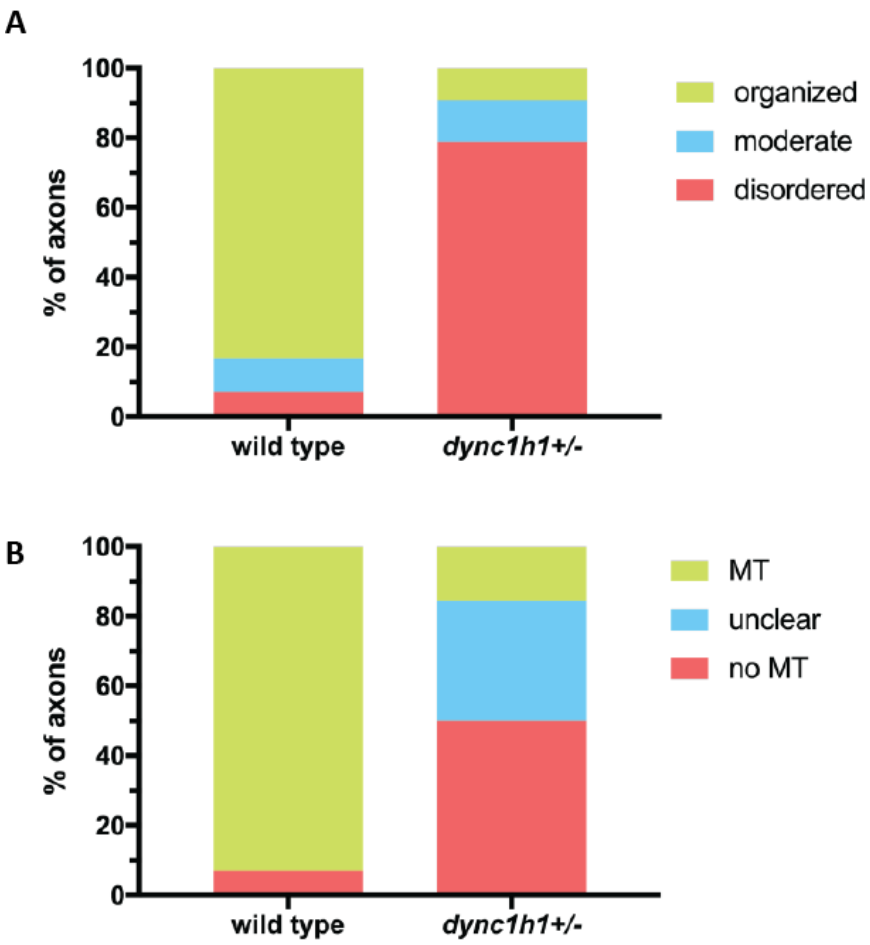


Figure 2.8: Quantification of cytoskeletal structure in motor axons of adult *dync1h1*+/- zebrafish. (A) Quantification of intermediate filament organization in wild type (n=43 axons) and *dync1h1+/-* (n=33 axons) motor axons. (B) Quantification of microtubules present in wild type (n=43 axons) and *dync1h1+/-* (n=33 axons) axons.

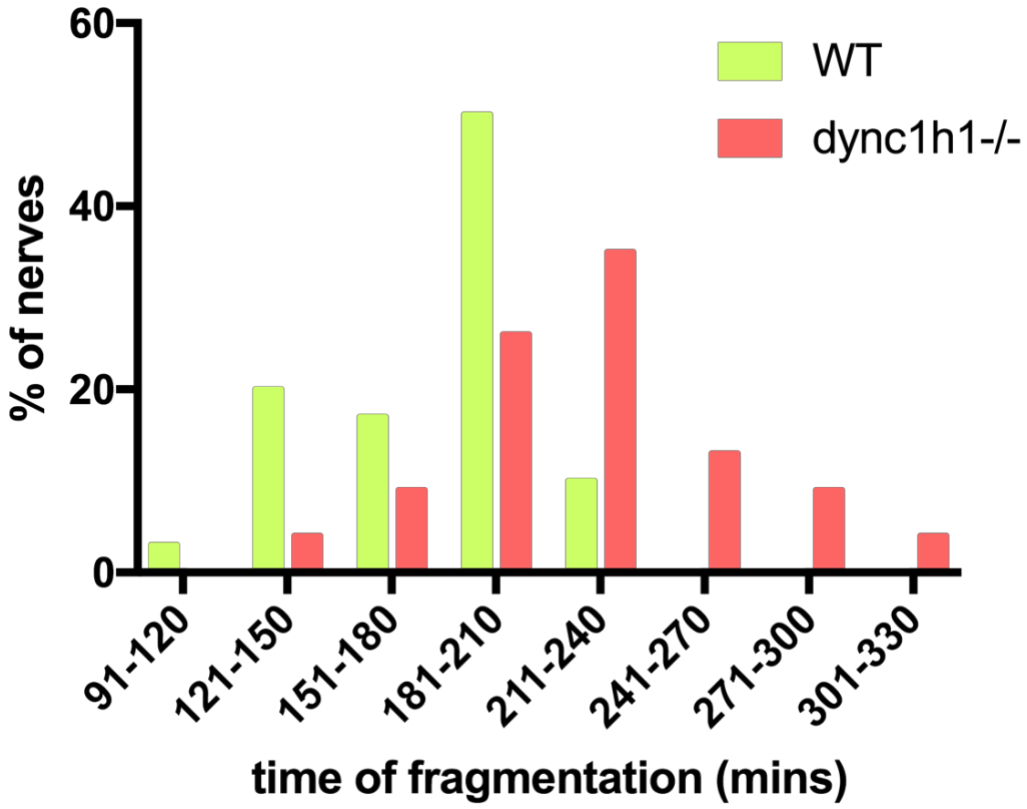


Figure 2.9: Dynein promotes axonal degeneration after injury. Time to first axonal fragmentation after injury in wild type and *dync1h1-/-* mutant axons.

CHAPTER 3: NOVEL *IN VIVO* IMAGING APPROACHES

In vivo imaging of cytoskeletal dynamics in Schwann cells

Schwann cells are critical in promoting axonal regrowth and nerve regeneration. Following nerve injury, Schwann cells distal to the lesion site respond dynamically by breaking down their myelin and dramatically altering their morphology. These morphological changes are accompanied by changes in transcriptional profile – Schwann cells dedifferentiate to a state similar to the immature Schwann cell, termed the repair cell, during regeneration. In this pro-regenerative state, Schwann cells can engulf axonal debris, migrate, proliferate, and ultimately remyelinate regrown axons. Previous work from our lab found that Schwann cells are required for proper axonal regrowth *in vivo* (92). Furthermore, studies transplanting denervated Schwann cells from peripheral nerves into CNS injury sites found that Schwann cells are able to promote axonal regrowth of CNS axons (7). Despite their therapeutic potential, many questions remain regarding the morphological and molecular changes in Schwann cells that promote neural repair. Though factors have been identified that initiate Schwann cell dedifferentiation after injury (93-95), a comprehensive description of the changes in Schwann cell morphology and cytoskeletal organization *in vivo* is missing, mainly due to the technical challenges of imaging regeneration in live intact animals. Similarly, if and to what extent changes in the Schwann cell cytoskeleton are critical for axonal regeneration is currently unknown.

Current understanding of the Schwann cell cytoskeleton comes predominantly from immunohistochemistry in cultured Schwann cells (96-99). While this has provided some

structural information, static images lack dynamic information and indication of microtubule polarity. Furthermore, the morphology of Schwann cells in culture can vary widely, and these Schwann cells lack association with axons which is a primary determinant of morphology in vivo.

In order to visualize the Schwann cell cytoskeleton *in vivo*, I specifically examined microtubules which are critical in directing and maintaining cell shape. I expressed fluorescently labeled EB3, a microtubule plus-end binding protein which marks the growing ends of microtubules, in Schwann cells. This allowed me to observe microtubule dynamics in Schwann cells live, in real time and importantly also relative to their associated axons, which are critical to the complex, three-dimensional morphology of Schwann cells *in vivo.* The *in vivo* Schwann cell cytoskeleton has not previously been described, even in cells before injury, due to the technical challenges of visualizing such components in a live animal. I similarly encountered many technical challenges in this pursuit, the details of which I will report here.

The major challenge in visualizing microtubules in Schwann cells of zebrafish larvae was expressing this marker in Schwann cells at all. Initially, I tried to use the *sox10(7.2kb*) promoter to drive expression of EB3 in Schwann cells transiently so that I could observe individual Schwann cells using the mosaic labeling that results from transient expression. When injecting the construct (*sox10(7.2kb*):*EB3-GFP*) and looking for transient expression, I frequently saw expression in other cell types labelled by sox10, as this is a broad marker of neural crest lineage. Across the thousands of fish I have

examined for transient Schwann cell expression using the *sox10(7.2kb)* promoter, I still to this day have never seen a Schwann cell labeled transiently.

My next approach was to generate stable transgenic lines using the *sox10(7.2kb)* promoter, as this promoter has previously been used to successfully generate lines labeling Schwann cells in zebrafish (100). I injected the *sox10(7.2kb)*:*EB3-GFP* construct and raised ~100 embryos to adulthood. I then screened for lines that labeled microtubules in Schwann cells and identified two separate lines. The first, line 4, labeled microtubules at a high enough expression level to easily see comets but not so high as to fill the cell with free EB3 (Figure 3.1A). Unfortunately, when I tried to raise this identified larvae (and several of its siblings with similar expression patterns) to adulthood to maintain the line, they all died. It is possible that even with levels of EB3 that were ideal for visualizing microtubules, this still may have negatively impacted microtubule dynamics in the neural crest cells of these individuals, causing lethality.

The second line, line 8, had a slightly lower level of EB3 expression, although comets could still be easily visualized before injury (Figure 3.1B). An important point here for the purpose of visualizing microtubules after injury is that we use a laser commonly used for FRAP analysis to perform our nerve transections. This laser causes photobleaching of GFP, which with cytosolic GFP can recover over time as more GFP diffuses from parts of the axon that were not cut. It seems, however, that the photobleaching of EB3-GFP was much more severe and long-lasting, preventing the visualization of microtubules in Schwann cells for a few hours after injury, exactly when the critical morphological changes are occurring in Schwann cells.

I was able to successfully raise two identified line 8 larvae to adulthood, and outcrossed them to a wildtype stain to maintain the line. Unfortunately, when these adults were then incrossed, the level of labeled EB3 in Schwann cells was too low to image and quantify. This raises another important point about generating transgenic lines in zebrafish: using the Tol2 transposase greatly increases the rate of transgenesis, however it does not always generate the most reliable stable lines. With Tol2 transgenesis, concatamers of the transgene can be formed, meaning that multiple copies of the desired gene may integrate into the genome. While this can be helpful for expression levels initially, this also means that with subsequent outcrossing, some of these copies are lost, and expression level can decrease.

Given the difficulty of identifying stable transgenic lines with an EB3 expression level suitable for imaging before and after injury, I turned to another Schwann cell promoter, Claudin K. Claudin K is a myelin-associated protein and thus labels only myelinating Schwann cells and oligodendrocytes. Using the CldnK promoter, I was able to see good transient expression of EB3 in Schwann cells (Figure 3.1C). The expression pattern tends to be clonal for a single nerve, so if one Schwann cell was labeled in a nerve, it also usually labeled all of the Schwann cells in that nerve. I also compared this structure to acetylated tubulin staining in 5 dpf fixes larvae (Figure 3.1D) and found the live images to provide a more defined structure.

While CldnK was a much more reliable promoter and allowed me to successfully express many proteins both transiently and in stable transgenic lines, the most ideal levels of EB3 for live imaging in Schwann cells came from expression using stable lines

generated with the Gal4/UAS system. I crossed a *sox10:Gal4* stable line and with a *UAS:EB3-GFP* line and this yielded bright labeling in most Schwann cells (Figure 3.1E). Interestingly, levels of EB3-GFP occasionally varied greatly between Schwann cells of the same nerve, allowing for some single cell resolution. The levels of EB3-GFP were strong enough to prevent photobleaching after laser transection, which allowed me to image cytoskeletal dynamics after injury (Figure 3.1F).

Going forward, these lines can be used to visualize dynamics of the Schwann cell cytoskeleton first in wild type larvae, both before and after nerve injury. This will determine how the cytoskeleton changes after injury, as Schwann cells transition from their mature, myelinating state into a repair cell state. The lines have also already been crossed into a dynein mutant background which will allow us to assess if and how the Schwann cell cytoskeleton differs in dynein mutant nerves, both before and after injury. One unique challenge for assessing the Schwann cell cytoskeleton *in vivo* is the complex cellular morphology. This dramatically increases the complexity of automatically tracking and measuring individual EB3 comets, as many more comets are present simultaneously than in a single section of an axon. I have previously used the Imaris software to automate the tracking of EB3 particles, and have had moderate success with the automatic “track particles” feature. It is likely that a more specific protocol will be needed to accurately track and measure the microtubule dynamics in Schwann cells. This preliminary data represents the first visualization of the Schwann cell cytoskeleton in a live, whole organism context.

In vivo imaging of nerve regeneration in midlarval zebrafish

The early larval zebrafish is an excellent system for studying nerve regeneration, as the larvae are optically clear at 5 dpf, allowing for *in vivo* imaging of cellular interactions after injury. Motor nerves in 5 dpf larvae demonstrate stable branching patterns and a degree of myelination roughly equal to that of a one week postnatal mouse (87,101,102). While this stage of larval development has many of the hallmarks of a mature motor nerve, it would also be useful to assess nerve regeneration in an even more mature animal. In our current experimental paradigm, nerves are transected in 5 dpf larvae and regeneration is assessed 48 hours later, when the larvae are 7 dpf. The ability to image older larvae would also allow us to assess nerve regeneration beyond 48 hours after transection. This would be useful for examining aspects of nerve regeneration that take longer than 48 hours, such as Schwann cell remyelination.

As larvae mature from the early to the mid-larval stages, this is accompanied by substantial growth in terms of both length and thickness. At 5 dpf, the average length of a larva is 4 mm and by 15 dpf, the length increases to an average of 15 mm. This predominantly affects the number of fish that can be imaged simultaneously in a single imaging dish (Figure 3.2A). The greater consideration for imaging, however, is the thickness of the fish. As the fish grows, there is more tissue between the microscope objective and the spinal motor nerves, making it more difficult to image clearly. The larvae also have a more fully inflated swim bladder in the midlarval stages, making the trunk section substantially thicker than the tail section.

To determine how late the spinal motor nerves of midlarval zebrafish can be imaged using standard spinning disk confocal live imaging techniques, I raised larvae expressing *mnx1:GFP* to 21 dpf and imaged periodically from 5 dpf forward. I imaged motor nerves in both the midtrunk section (Figure 3.2B, box 1) as well as the tail (Figure 3.2B, box 2). I also transected these nerves at each timepoint to see in the laser could penetrate the tissue sufficiently in each location.

At all timepoints (14, 16 and 19 dpf) nerves were successfully imaged and transected in both the midtrunk section as well as the tail (Figures 3.3, 3.4, 3.5). One challenge of nerve regeneration as an animal grows is that the distance across which an axon must regrow becomes much larger. The motor nerves of the trunk in the midlarval stages are much greater in length while the motor nerves of the tail are much shorter, and therefore a less useful tool to study more mature nerve regeneration. Surprisingly nerves in the midtrunk could be successfully imaged out to 19 days, after which point the full depth of the nerve could no longer be observed or imaged. This demonstrates that regeneration studies can be performed using the spinal motor nerves of the trunk in midlarval zebrafish until 19 dpf.

Repeated nerve lesioning *in vivo*

Another useful experimental paradigm enabled by the ability to image longer into the midlarval stages is a repeated lesioning model. The idea of conditioning lesions has been studied extensively in DRG neurons, which contain both CNS and PNS projecting axons. It has been found that if the PNS projection is first injured and allowed to recover, then the CNS projection is subsequently injured, the CNS axon will then regrow much

more robustly than typical CNS regeneration (103). This is thought to be caused by cell body changes in transcription that occur after the PNS injury, thus priming the cell body for regeneration and aiding in the regrowth of the CNS projection when it is subsequently injured. Conditioning lesions have also been seen to promote growth after subsequent lesions in the mouse rat peripheral nerves (104,105). Typically, a conditioning crush will be performed several days before the test crush, after which increased regeneration is observed.

It is currently unknown whether conditioning lesions may promote subsequent regrowth of motor axons in the zebrafish. Furthermore, lesioning a nerve repeatedly may cause extra stress to the system and provide a sensitized condition in which to assay regeneration. In this case, if a particular gene has a milder contribution to axonal regrowth, we may be unable to see an effect on regeneration in our standard single transection assay but may see an effect in the repeated lesioning paradigm after transecting the same nerve multiple times.

In order to determine whether motor nerves in larval zebrafish can regenerate in response to multiple lesions, I transected nerves in 5 dpf larvae, allowed them to regrow for 6 days, then transected the nerves again (Figure 2.6, A-D). I then assayed regrowth in response to the second lesion (Figure 2.6, E-H). I altered the site of transection from our standard single lesion assay, cutting more distally so that there would be sufficient regrowth to lesion a second time. This did in fact lead to robust regrowth 5 days after the first lesion, and again 4 days after the second lesion. Given that the animal grows quite dramatically in overall size throughout the course of this experiment (5 dpf, ~4 mm long

to 15 dpf, ~15 mm long), it is difficult to qualitatively compare the appearance of the nerve regrowth and determine whether regeneration is improved after a conditioning lesion. Experiments following up on this preliminary result should compare this regrowth to control animals that receive a single lesion at 11 dpf, without the conditioning lesion at 5 dpf. These initial results demonstrate that larval zebrafish can survive, be imaged and have nerves transected throughout a 10 day assay in which both a conditioning lesion and test lesion are administered.

Taken together, these experiments demonstrate an expanded potential of the zebrafish for assessing nerve regeneration *in vivo,* allowing us to examine later time points than before, as well as visualize subcellular components in Schwann cells.

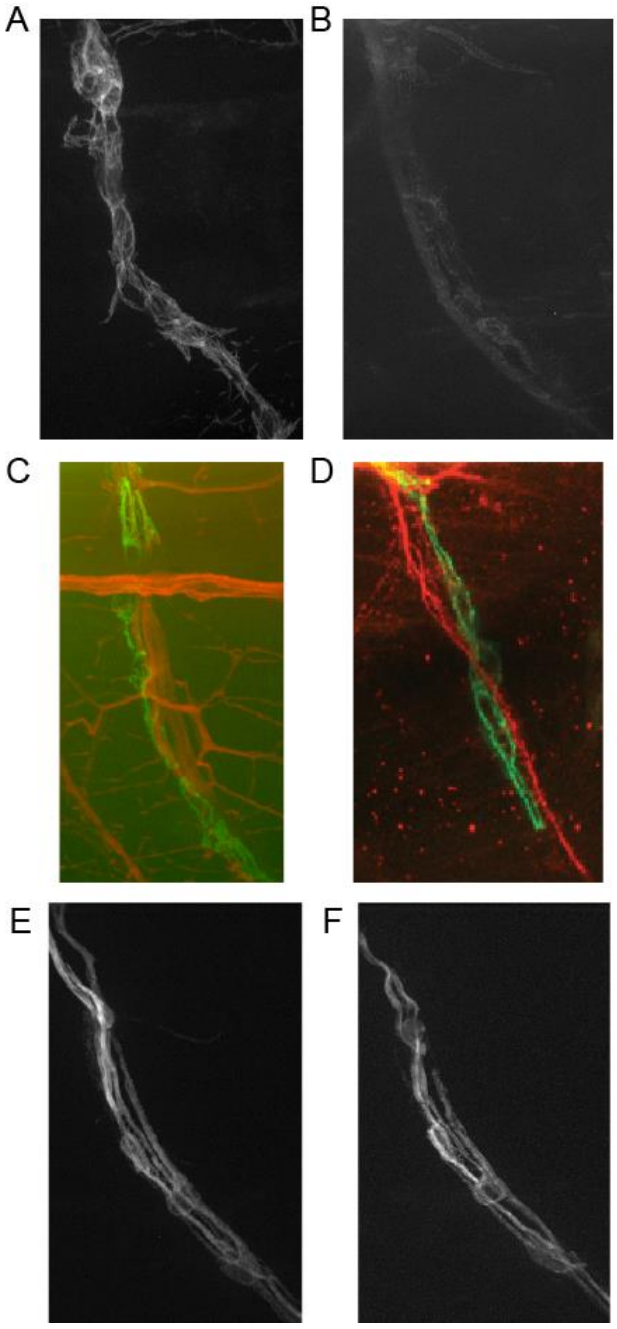

A
B
C
D
E
F

Figure 3.1: Microtubule structure in Schwann cells *in vivo*. Schwann cell cytoskeletal structure in zebrafish larvae. (A) *Tg(sox10:EB3-GFP)* line 4, uninjured motor nerve of 2 dpf larvae; images taken every 5 seconds for 2 minutes and time projected (B) *Tg(sox10:EB3-GFP)* line 8, uninjured motor nerve 5 dpf larvae; images taken every 5 seconds for 2 minutes and time projected (C) *Tg(NBT:dsRed)* labels axons in red, injected *CldnK:EB3-GFP* labels microtubule plus ends in green, uninjured motor nerve of 5 dpf larvae; images taken every 10 mins for 5 hours and time projected. (D) Acetylated tubulin (green, microtubules) and znp1 (red, motor axons) stains in fixed 5 dpf larvae). (E-F) *Tg(sox10:Gal4;UAS:EB3-GFP)* in 5 dpf larvae, images taken every 10 seconds for 2 minutes and time projected; in uninjured motor nerve (E) and 10 minutes post transection (F).

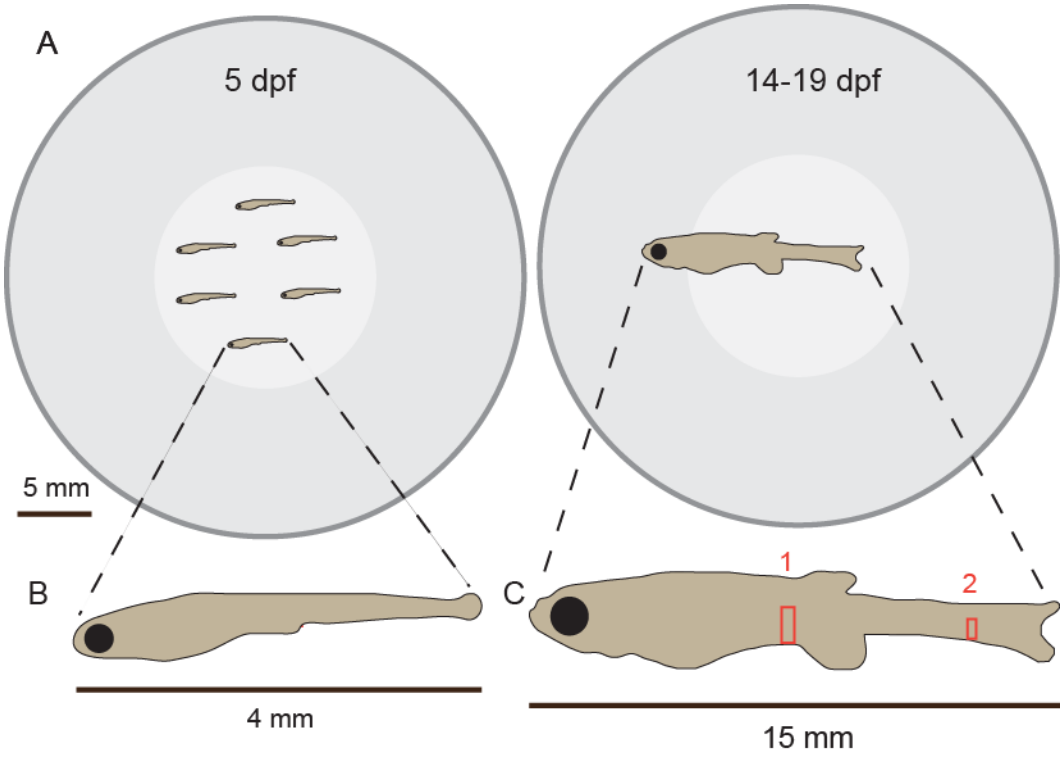


Figure 3.2: Schematic of mounting and imaging early and mid-larval zebrafish. (A) Placement of early (5 dpf) and midstage (14-19 dpf) larvae in glass bottom imaging dishes. (B) Enlargement of 5 dpf larvae; red box indicates imaging region of the ventral motor nerve in the midtrunk section, the standard nerve for transection in our regeneration studies. (C) Enlargement of a ~14-19 dpf larvae; red box 1 indicates ventral nerve imaging region in the midtrunk section, red box 2 indicates ventral motor nerve imaging region in the tail section.

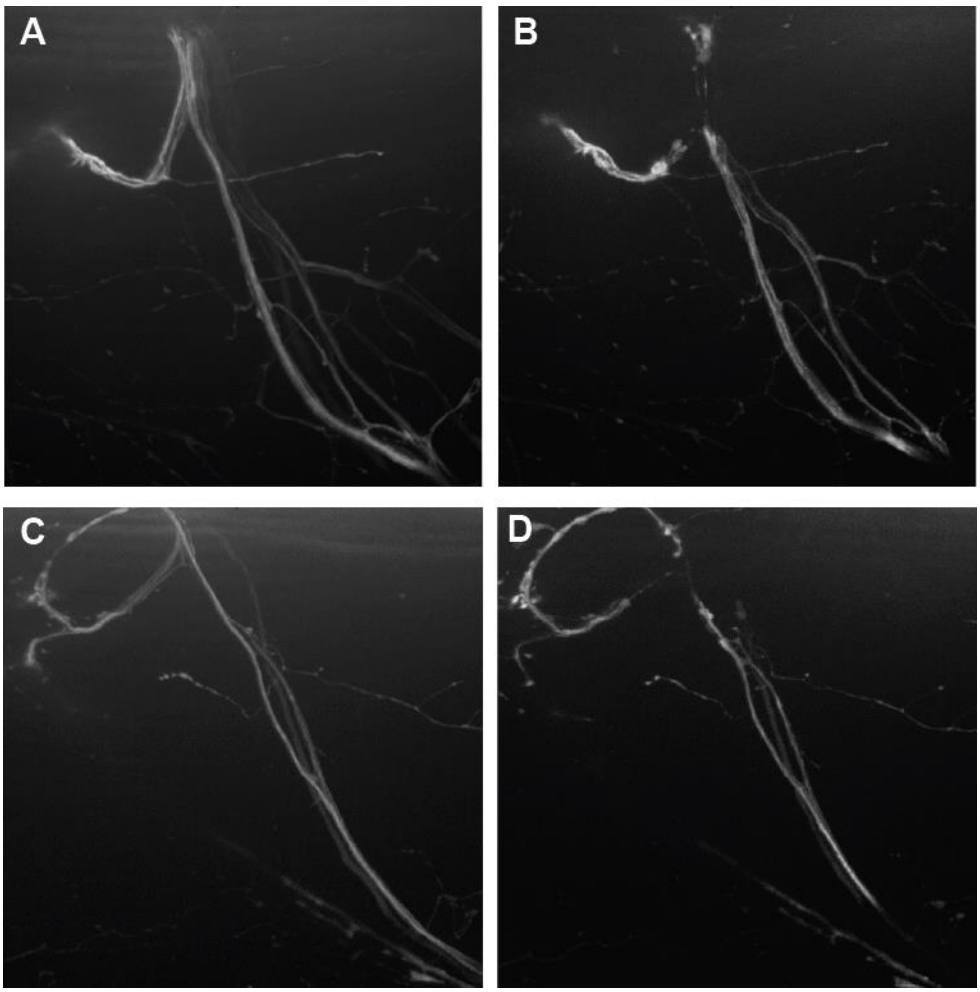


Figure 3.3: Midlarval imaging in 14 dpf zebrafish. *Tg(Hb9:GFP)* labeling ventral motor nerves. (A-B) Motor nerve in mid-trunk section before (A) and immediately after (B) laser transection. (C-D) Motor nerve in tail section before (C) and immediately after (D) laser transection. Motor nerves can be imaged and transected successfully in both areas.

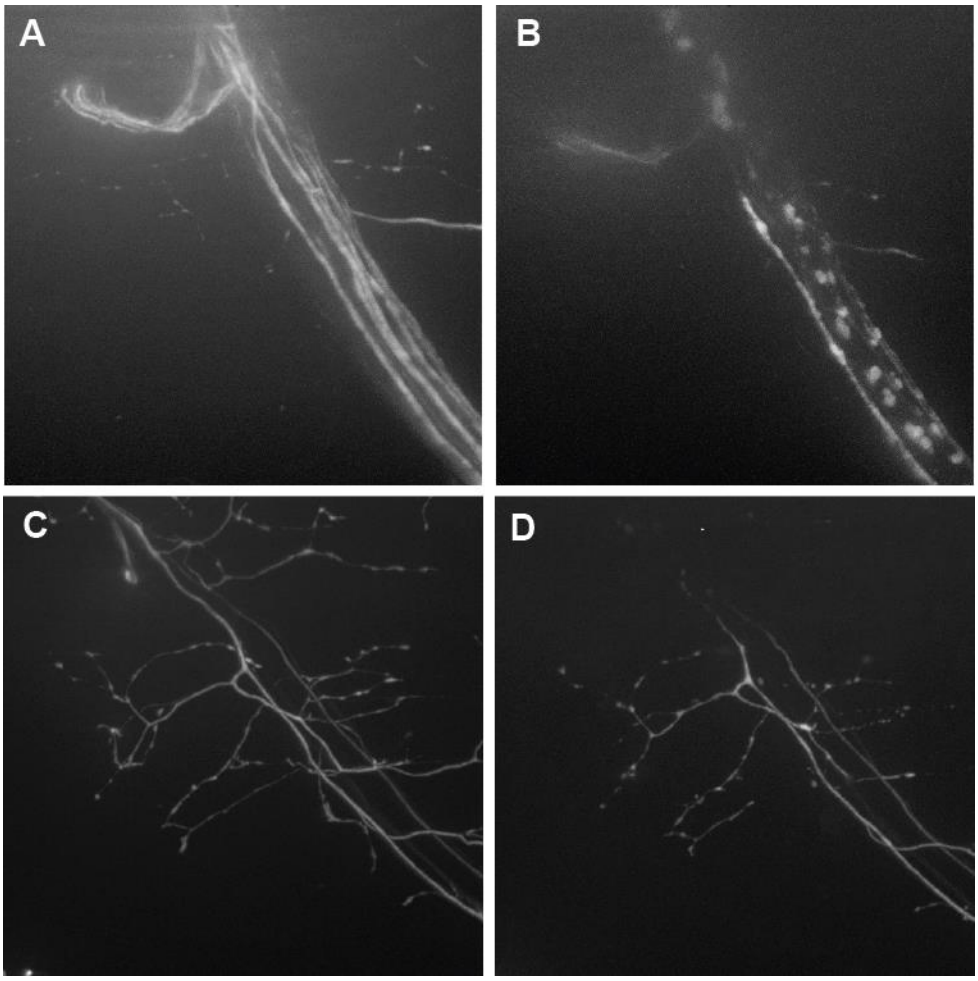


Figure 3.4: Midlarval imaging in 16 dpf zebrafish. *Tg(Hb9:GFP)* labeling ventral motor nerves. (A-B) Motor nerve in mid-trunk section before (A) and immediately after (B) laser transection. (C-D) Motor nerve in tail section before (C) and immediately after (D) laser transection. Motor nerves can be imaged and transected successfully in both areas.

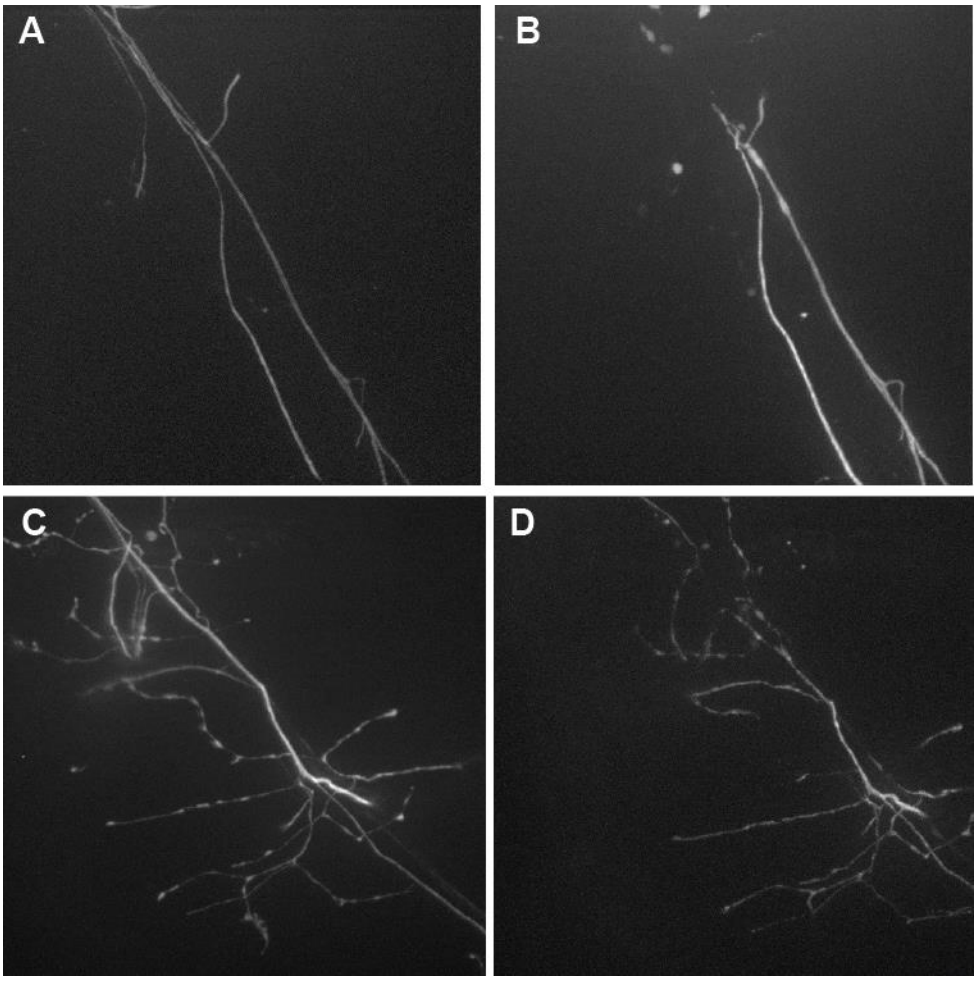


Figure 3.5: Midlarval imaging in 19 dpf zebrafish. *Tg(Hb9:GFP)* labeling ventral motor nerves. (A-B) Motor nerve in mid-trunk section before (A) and immediately after (B) laser transection. (C-D) Motor nerve in tail section before (C) and immediately after (D) laser transection. Motor nerves can be imaged and transected successfully in both areas.

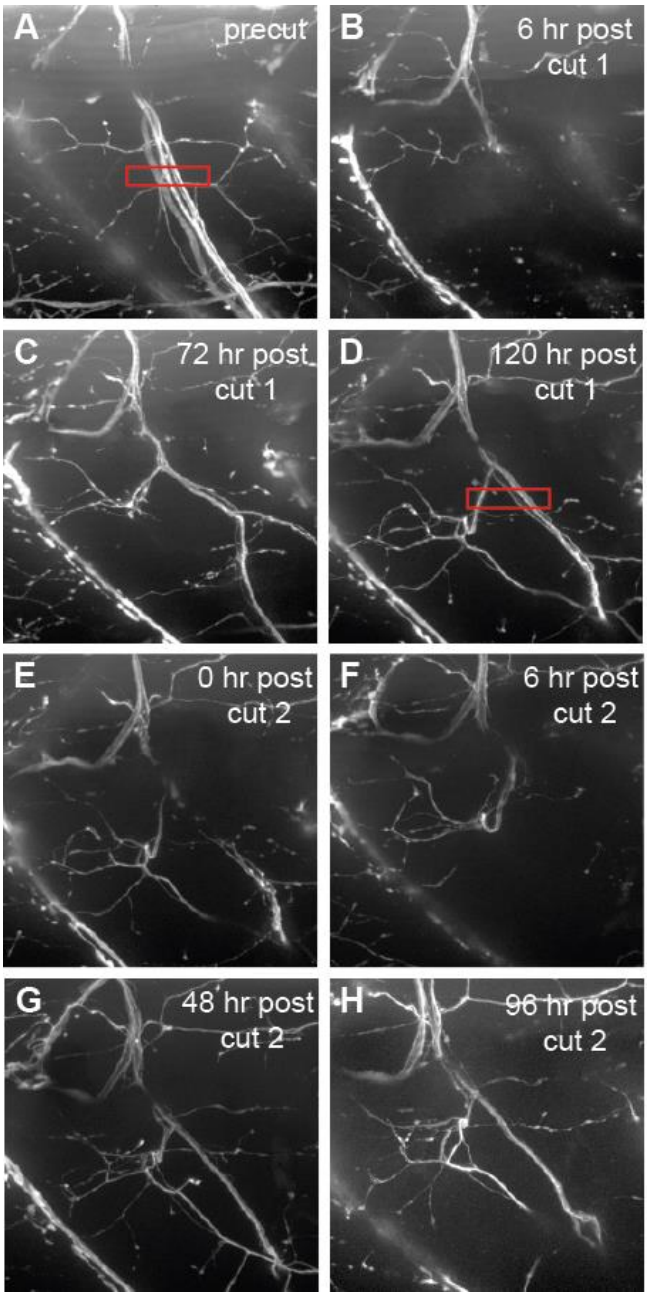

A
precut
B
6 hr post
cut 1
C
72 hr post
cut 1
D
120 hr post
cut 1
E
0 hr post
cut 2
F
6 hr post
cut 2
G
48 hr post
cut 2
H
96 hr post
cut 2

Figure 3.6 Repeated nerve lesioning in zebrafish larvae. Wild type larvae, motor axons labeled with *Tg(mnx1:GFP).* (A) Pre-lesion motor nerve at 5 dpf, red box shows site of transection. (B) Motor nerve at 5 dpf, 6 hours after first lesion. (C) Motor nerve at 8 dpf, 72 hours after first lesion. (D) Motor nerve at 11 dpf, 120 hours after first lesion; red box shows site of second transection. (E) Motor nerve at 11 dpf, 0 hours after second lesion. (F) Motor nerve at 11 dpf, 6 hours after second lesion. (G) Motor nerve at 13 dpf, 48 hours after second lesion. (H) Motor nerve at 15 dpf, 96 hours after second lesion.

CHAPTER 5: MATERIALS AND METHODS

Ethics statement

All experiments were conducted according to an Animal Protocol fully approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) on January 24, 2014, protocol number 803446. Veterinary care is under the supervision of the University Laboratory Animal Resources (ULAR) of the University of Pennsylvania.

Zebrafish genetics and transgenes

All transgenic lines were maintained in the Tübigen or Tupfel long fin genetic background and raised as previously described(106). Transgenic lines used are listed in Table 5.1. Mutant lines used are listed in Table 5.2. Genotyping primers are listed in Table 5.3.

Table 5.1: Transgenic lines.

Transgenic Lines	Population labeled	Source
Tg(mnx1:GFP)^{ml2}	Spinal motor neurons	(107)
Tg(sox10(7.2):mRFP)^{vu234}	Schwann cell membranes	(12)
Tg(UAS:lifeact-GFP-v2a-EB3-RFP)	Microtubule plus ends (red) and actin (green)	Bremer et. al., in preparation

Tg(sox10:EB3-GFP) line 4	Microtubule plus ends	generated
Tg(sox10:EB3-GFP) line 8	Microtubule plus ends	generated
Tg(Xla.Tubb:dsRed)	Pan-neuronal	(108)

Table 5.2: Mutant lines.

Mutant	Mutation	Source
dync1h1^{hi3684Tg}	Viral intronic insertion, presumed null from nonsense-mediated decay	(109)
gpr126^{stl47}	Indel causing frameshift and early truncation	(110)
kif5Aa^{sa7168}	Point mutation in essential splice site donor	(111)

Table 5.3: Genotyping primers.

Mutant	Genotyping primers	Reference
dync1h1^{hi3684Tg}	F1: 5'-AAACCTACAGGTGGGGTCTTTC-3' F2: 5'-CATGAACGTGGCGCTGGTAC-3' R: 5'-GFTACAACTACGAGCAAGTCAACC-3'	(109)
gpr126^{stl47}	F: 5'-GTCTTTGTCTCTGTCGATGC-3' R: 5':-GCTTGTAACTGATATGGAAGCC-3'	(110)
kif5Aa^{sa7168}	F: 5'-TGGAGAAACGTCTTCGTTCTACG-3' R1: 5'-GTGTGTGAATGTGAATGCAGTGCACAGTGT-3' R2: 5'-GTGTGTGAATGTGAATGCAGTGCACCAGCGT-3'	(111)

Nerve transection and live imaging

Nerve transection and live imaging were performed as previously described (51,52).

Axon growth extent quantification

Axon growth extent quantification was performed as previously described (52).

Transected nerves in which axons failed to regrow or did not extend through the entire

length of the ventral myotome are categorized as “no/weak regeneration.” Nerves with at least one fascicle that extended through the entire length of the ventral myotome are categorized as “moderate regeneration.” Finally, nerves with two or more fascicles extending through the entire length of the ventral myotome are categorized as “strong regeneration.”

Axon extension and retraction quantification

Axons were imaged every 15 minutes from ~7 to ~16 hpt. Extensions and retractions were defined as growth or retraction of >1 μm between timelapse frames and number of extension and retraction events was counted. Continued movements of the same direction in a subsequent frame were not counted as new events. Measurements were performed on each visibly distinct axon in a nerve.

Growth cone displacement quantification

Axons imaged at ~16 hpt were measured by drawing a line from the spinal cord exit point to the growth cone. Measurements were performed on each visibly distinct axon in a nerve.

Schwann cell width quantification

Axons and Schwann cells were imaged before transection and every 15 minutes from ~1 to ~5 hpt. Schwann cell width was measured at the widest point in pre- and post-transection images. Using ImageJ, a line was drawn from one edge of the Schwann cell membrane to the other in an orientation perpendicular to the motor nerve and was measured in microns.

Cell transplantation for chimera analysis

Cell transplantations were performed as previously described (32). Wild type cells were transplanted into *dync1h1*-/- embryos in areas known to develop into motor neurons. Larvae were screened at 5 dpf to identify nerves that contained transplanted motor neurons and no other transplanted cell types along the path of the ventral motor nerve. Transection, imaging, and quantification of growth cone displacement in identified nerves were performed as described above.

Image processing

Image stacks were compressed into maximum intensity projections (MIPs) in Slidebook 6 then processed using ImageJ and Photoshop to normalize brightness and contrast.

Statistical analysis

Fisher's exact and Student's t tests were performed on all applicable datasets.

CHAPTER 6: DISCUSSION

Molecular motors Kinesin-1 and Dynein are required for nerve regeneration *in vivo*

Microtubule-associated motors are critical for cellular maintenance, transporting important cargos throughout a cell and also helping to modulate the dynamics of the microtubules that they travel along. Previous studies have demonstrated a role for both the anterograde motor kinesin-1 and the retrograde motor dynein in establishing microtubule organization in developing axons. Additionally, many previous studies have found that dynein is critical in transporting signaling complexes from the axon to the cell body after injury. While this role for dynein in axonal regrowth has previously been described, kinesin-1 and dynein have many cellular cargos and functions, raising the possibility that these motors may be involved in nerve regeneration in multiple capacities. Furthermore, studies of the dynein-dependent retrograde injury signal have focused exclusively on the requirement for dynein in the axon. While neurons are known to be particularly dependent on active transport for cellular maintenance due to their length and highly polarized morphology, kinesin-1 and dynein are also critical in many other cell types. As such, we sought to evaluate the role of kinesin-1 and dynein in a multicellular context, using live-imaging in the zebrafish larvae to assess nerve regeneration in a live, whole organism. We took advantage of homozygous zebrafish mutants for the *kif5aa* and *dync1h1* genes, since their motor nerves developed normally due to maternal contribution. By transecting these nerves and imaging the regeneration 48 hours later, we showed for the first time a requirement for both kinesin-1 and dynein in peripheral nerve regeneration *in vivo.*

Dynein stabilizes axonal extensions during regeneration

Nerve injury induces a local signaling cascade that leads to the production of axon intrinsic signals at the lesion site (112). There is overwhelming evidence that dynein is critical to transport these injury signals from the lesion site to the cell body where they initiate a neuronal injury response (113-115). We find that in presumptive dynein null mutants, injured neurons robustly respond to the injury and within ~8-10 hours, regenerating axons sprout from the proximal stump, indistinguishable from what we observe in wild type animals. This raises the question whether axonal sprouting can occur independently of dynein-dependent injury dependent signals, or whether in our zebrafish model dynein-mediated retrograde transport is less important to mount an injury response. One clear difference between rodent models and our model is the distance between the injury site and the neuronal cell bodies. In rodent sciatic nerve models lesions are introduced millimeters away from neuronal cell bodies (115), while in larval zebrafish – due to the smaller animal size – lesions are generated about 10-50 µm away from neuronal cell bodies (51) Thus, it is conceivable that due to the almost 100-fold reduction in distance between lesion site and cell body, injury signal propagation from the lesion site to the cell body is less dependent on dynein function. Although it remains unclear how injury signal propagation can occur independent of dynein, this provided us with the unique opportunity to examine dynein's role in peripheral nerve regeneration beyond its role in injury signal transport.

Endpoint analyses at 48 hpt uncovered a clear role for dynein in peripheral nerve regeneration, with clear effects on both axonal regrowth and Schwann cell injury

response (Figures 2.1 and 2.3). Using live-imaging to visualize the early stages of the regeneration process, we found that dynein promotes the stabilization and growth of long-range axonal projections, providing compelling evidence that apart from its well-documented role in retrograde injury signal transport, dynein also plays a critical role in sustaining axonal regrowth.

Dynein is required for Schwann cell remodeling after injury

In addition to the axonal regrowth defects observed in dynein mutants, simultaneously visualizing the cellular behavior of both axons and Schwann cells revealed that loss of dynein also prevented injury-induced Schwann cell remodeling. The transition of Schwann cells from their fully differentiated state to a repair cell state is a well-documented and integral aspect of peripheral nerve regeneration (95,116), accompanied by dramatic morphological changes to the Schwann cell, as the cell breaks down its myelin and extends its membrane to engulf axonal debris (117,118). Dynein regulates several steps of membrane trafficking, including ER to Golgi transport, as well as endosomal trafficking (119), so it is conceivable that dynein plays a direct, cell-autonomous role in this process. Alternatively, the inability of Schwann cells to initiate the remodeling process might be a consequence of strongly reduced axonal regrowth, and future experiments will be required to test a possible Schwann cell-specific role for dynein in the remodeling process. Of these two possibilities, however, it seems most likely that dynein is playing a role specifically in the Schwann cell to promote remodeling after injury. This is due to the timing of Schwann cell remodeling, which occurs at the time of axonal fragmentation, hours before axonal regrowth begins. It is, however,

possible that axon-Schwann cell communication before injury is critical to prime Schwann cells to respond to injury, and this communication may be disrupted by the absence of dynein and a potential impairment in signaling endosome transport, etc.

The absence of Schwann cell remodeling in dynein mutants also reveals novel insights into the mechanism of Schwann cell injury response. It has previously been thought that the morphological changes occurring in Schwann cells after injury result simply from the loss of axonal tension as the distal axons fragment. However, we observe in dynein mutants that even as distal axons fragment following injury, the Schwann cells do not respond with characteristic morphological changes. This indicates that mechanical forces alone are insufficient to induce the morphology changes observed in Schwann cells after injury, and suggests that molecular and genetic mechanism drive this repair cell transition both transcriptionally and morphologically.

This raises another interesting question regarding the relationship between Schwann cell differentiation state and morphology changes after injury – does a change in Schwann cell morphology after injury necessarily indicate that the transcriptionally regulated repair cell transition has occurred, and vice versa? One way to address this question is to assess the transcriptional state of dynein mutant Schwann cells after injury. While we know that dynein mutant Schwann cells do not respond morphologically to injury, it is possible that the appropriate transcriptional response towards the repair cell state still occurs. The repair cell state is characterized by both dedifferentiation and activation processes. Difficulties arise when trying to assess the repair cell transition transcriptionally in dynein mutants, however, as these mutants also have defects that

prevent full differentiation during development. As a result, many of the markers of the repair cell transition are developmental marker that become re-expressed during the transition to the repair cell state, such as Sox2 (120). Unfortunately, these markers never actually become downregulated in the 5 dpf dynein mutant Schwann cells as the animal matures. Thus, it will be critical to use transcriptional markers that are unique to the repair cell state and are not simply developmental markers that are re-expressed after injury, but rather distinct markers of Schwann cell activation after injury. One candidate marker is c-Jun and its downstream targets, which are expressed at low levels in Schwann cells developmentally but become highly expressed after injury (95,116).

In addition to further characterizing the repair cell transition by assessing transcriptional changes that occur in Schwann cells after injury, it will also be useful to examine cytoskeletal changes that occur after injury in both wild type and mutant animals. Using the tools and techniques developed in Chapter 3, we can first assess the cytoskeletal changes that drive Schwann cell remodeling in wild type animals. We can subsequently assess the cytoskeleton in dynein mutant Schwann cells and determine whether any cytoskeletal changes are initiated after injury, though the gross morphology does not change appreciably. Together, these experiments will help determine to what extent the cytoskeletal and morphological changes in Schwann cells after injury dictate the transition to the repair cell state.

Neuronal dynein is sufficient to promote axonal regrowth after injury

Given that dynein mutants exhibit defects in axonal regrowth and Schwann cell morphology, we performed chimeric analysis experiments to determine in which cell type

dynein is required to promote nerve regeneration *in vivo*. These experiments revealed that dynein function in injured neurons is sufficient to sustain axonal regeneration. Importantly in our chimera experiments, of the roughly 60 axons contributing to an individual motor nerve (121), on average only 1-3 transplanted wild type axons were present. This low level of chimerism was critical to evaluate regrowth capacity of individual wild type axons. This also revealed that the presence of individual wild type axonal regrowth facilitated regrowth of individual, neighboring dynein deficient axons (Figure 2.4, G-I). At the same time, the low level of chimerism precluded us from asking whether neuronal dynein restored all aspects of peripheral nerve regeneration, including injury-induced Schwann cell remodeling. Thus, while neuronal dynein plays a critical role in sustaining axonal regrowth, we cannot exclude the possibility that dynein function in Schwann cells also contributes to peripheral nerve regeneration.

Dynein promotes axonal regeneration by modulating microtubule dynamics

Cytoskeletal dynamics are critical to growth cone formation (122), axonal outgrowth during development (123), and axonal regeneration (124). Previous studies have revealed that microtubule stabilization promotes axonal regrowth after injury both *in vitro* and *in vivo* (125-127). Interestingly, studies of C. elegans dynein heavy chain mutants recently revealed that dynein acts locally in dendrites to stabilize microtubules (74). This raised the possibility that dynein may also act locally in regenerating axons to stabilize microtubules. We assessed cytoskeletal dynamics during regeneration using a transgene that allowed us to visualize actin and microtubules simultaneously in live, regenerating axons. This revealed that while actin dynamics were grossly unaffected in dynein mutant axons, microtubules often appeared unstable and disordered, with some

axons exhibiting looping microtubule configurations reminiscent of those seen in the dendrites of C. elegans dynein heavy chain mutants (74). Thus, our results provide compelling evidence that besides its well-documented role in retrograde transport, dynein also promotes microtubule stability critical for growth cone advancement (128), providing a potential mechanism for the rapid and sustained extension observed during wild type axonal regrowth, and deficient in dynein mutants (Figure 2.6).

Dynein is also known to play a role in microtubule sliding (129), providing an alternative mechanism through which dynein may modulate axonal microtubule dynamics during regeneration. This may be a direct effect of dynein specifically interacting with microtubules, as it has previously been shown that dynein slides microtubules in a polarity-sorting manner during axon outgrowth. It is conceivable that dynein has this same role during axonal regrowth after injury and that in the absence of dynein, the polarity of microtubules in the axon is not established properly and eventually leads to retraction of the axonal extension. This idea is consistent with the axonal extension phenotype we observe in the dynein mutants, in which axonal extension proceeds for a short distance before becoming destabilized and retracting.

Another possibility is that dynein may affect microtubule sliding indirectly via modulation of kinesin-1, as these motors have been shown to transport each other directly (130). Some studies in culture have shown that severe disruption of the anterograde motor kinesin-1 can disrupt dynein-dependent transport, and vice versa. Further studies will be required to determine whether dynein's role in promoting axonal outgrowth specifically affects microtubule sliding, and whether this effect is through a direct microtubule

interaction or the modulation and transport of the anterograde motor, kinesin-1, which has previously been found to power microtubule sliding during axonal outgrowth.

One remaining question regarding the role of dynein in modulating microtubule dynamics after injury is whether dynein is a direct effector of microtubule dynamics, or whether microtubule dynamics are simply a downstream effector of the retrograde injury signals activated after injury. As discussed previously, we have not yet been able to determine whether critical retrograde injury signals arrive at the cell body in the absence of dynein. While the scale of the injury model in the zebrafish suggests that activated retrograde injury signals can likely reach the cell body without active transport by dynein, it is still possible that critical signals, such as p-JNK, are disrupted in the dynein mutant. It has previously been shown that microtubules are downstream effectors of JNK signaling to promote neurite outgrowth (131,132). In order to determine whether dynein is directly affecting microtubule dynamics, it will be important to determine whether critical retrograde injury signals such as p-ERK and p-JNK are detected in the cell bodies of dynein mutant motor neurons after injury. Experiments are underway to visualize p-JNK and p-ERK via antibody staining, and I have also obtained a construct for fluorescently tagged p-JNK that can be used to visualize p-JNK in motor neurons after injury (133). Experiments to test the direct interaction between dynein and microtubules in the regrowing axon will be challenging, as dynein interacts with microtubules in its microtubule binding domain for all cargo transport functions, as well as microtubule sliding. An interesting experiment to further probe dynein's role in modulating microtubule dynamics during axonal regrowth is to use taxol to stabilize microtubules in dynein mutant larvae. If the critical role for dynein in promoting axonal regrowth is in fact

modulation of microtubule dynamics, we would expect that stabilization of microtubules by taxol could rescue the axonal regrowth defects observed in dynein mutant nerves.

If it is confirmed that dynein is directly affecting microtubule dynamics in the regrowing axon after injury, the nature of its interaction with microtubules should be examined. One possibility is that dynein impacts microtubule stability, while another is that dynein is critical for establishing appropriate microtubule polarity during axon outgrowth, as in development. To distinguish between these functions, microtubule polarity should be examined in regrowing axons. If dynein is indeed acting to slide microtubules in a polarity-dependent manner, we would expect to see disrupted microtubule polarity in the regrowing axons of dynein mutants. These experiments will help determine the specific nature of dynein's role in modulating axonal microtubule dynamins after injury.

Dynein in cytoskeletal maintenance: insight for human disease

Multiple mutations in the dynein heavy chain, *DYNC1H1*, have been found to cause neurodegenerative conditions in human patients, indicating a critical role for the motor dynein in neuronal homeostasis and maintenance. First, a dominant point mutation in the homodimerization domain of *DYNC1H1* was found to cause axonal Charcot-Marie-Tooth disease in a large family pedigree (44). Additionally, two dominant mutations in the tail domain of *DYNC1H1* have been found to cause spinal muscular atrophy (SMA) (43), and biochemical analysis revealed that these mutations disrupted complex stability and function.

Thus far, insights into the disease mechanism of these human mutations have come from biochemical analysis *in vitro* as well as endpoint analysis of mouse mutants. The

heterozygous *dync1h1* mutants may provide another unique entry point into studying the role of dynein in neuronal maintenance *in vivo*. The zebrafish *dync1h1*^{hi3684} mutation is an intronic viral insertion in the motor domain of the dynein heavy chain, which is expected to disrupt function and stability of the dynein complex (77). These heterozygous dynein mutants are fully viable into adulthood, however our preliminary results have revealed that cytoskeletal organization of the axon is severely disrupted in these mutants (Figures 2.7, 2.8). This cytoskeletal disruption suggests a potential mechanism for the axonal degeneration observed as a result *DYNC1H1* mutations in humans.

Interestingly, there are no obvious defects in the motor function of the heterozygous dynein mutant zebrafish, despite their disrupted cytoskeletal structure. One possibility is that these cytoskeletal defects do not disrupt axonal function, however this seems unlikely since relatively minor mutations in cytoskeletal proteins can lead to severe axonal degeneration in human and mouse mutants (Table 1.1). Another possibility is that these adult heterozygotes have motor defects that are imperceptible without a closer method of study. To determine whether *dync1h1* heterozygotes have any functional motor deficits, we can examine the startle response at larval, juvenile, and adult stages as previously described by and developed in our lab. This will reveal whether there is any developmental deficit in these animals, and additionally determine whether motor function degenerates over time.

To complement these studies, we can also perform electron microscopy on motor nerves at each of these stages and quantify the number of axons in a given nerve. This

should be another indication of whether axonal degeneration is occurring in these animals, as we would expect the number of axons per nerve to decrease over time as a result of degeneration. As mentioned previously, we can also examine cytoskeletal structure in the axons at each of these stages to determine whether the cytoskeleton is disrupted during development or degenerates over time, and additionally correlate this to any functional deficits or axonal loss that is observed.

To further assess the cytoskeletal structure in axons of dynein heterozygotes, we can use the fluorescently tagged cytoskeleton line described in Chapter 2 (Figure 2.6; *Tg(mnx1:Gal4)* x *Tg(UAS:EB3-RFP-lifeact-GFP)*) combined with the midlarval imaging techniques described in Chapter 3. This will allow us to monitor the axonal cytoskeleton from the time of axon outgrowth in development through the midlarval stages, and should reveal whether the axonal cytoskeleton in dynein heterozygotes is established correctly in development, and also whether it degenerates over time. These experiments may provide new insights into the disease mechanism of degenerative diseases caused by dynein mutations, and may reveal that in addition to directing transport, dynein helps establish and/or maintain cytoskeletal structure in axons.

Dynein promotes axonal degeneration after injury

While studies in human and mouse have revealed a role for dynein in neuronal maintenance at homeostasis, our experiments revealed an additional role for dynein in the active process of Wallerian degeneration. Following nerve injury, we observed that dynein mutant axons in the distal stump show an extended lag phase prior to axonal fragmentation, indicating that dynein promotes Wallerian degeneration. Previous studies

of axonal degeneration have found that upon nerve crush or transection, cargos traveling retrogradely in the axon accumulate just distal to the site of injury as they can no longer pass into the proximal axon segment. This phenomenon has often been considered from the perspective of the cell body and proximal axon – that this accumulation of cargos distally subsequently results in a lack of these cargos and signals in the proximal nerve segment. Given that dynein affects the timing of axonal fragmentation in the distal stump, it seems it may have a more active function in the distal part of the nerve than previously thought.

One possibility is that cargo accumulation at the end of the distal nerve segment somehow induces Wallerian degeneration. In the absence of dynein, we would expect that cargo distribution is already disrupted and the change after injury would be less pronounced. Given that Wallerian degeneration is a process initiated by active death signals in the axon, it is also possible that dynein-mediated transport is required for the distribution of relevant signals through the axon to coordinate axonal fragmentation. Notably, when axonal fragmentation occurs, the entire length of the axon fragments at the same time, suggesting tight temporal and spatial regulation of the fragmentation process. Given dynein's key role in controlling the spatial distribution of critical cellular cargos, it is tempting to speculate that dynein's role in promoting Wallerian degeneration is related to this function.

One specific cargo of dynein that may be particularly critical for Wallerian degeneration is the autophagosome. In neurons, autophagosomes initiate distally and enclose cellular contents to be degraded. They then complex with dynein and travel retrogradely where

they fuse with lysosomes and degrade their contents. The process of autophagy is likely disrupted in that axons of dynein mutants, and this could be one explanation for the disrupted axonal fragmentation that is observed. It is conceivable that in addition to engulfment by macrophages and Schwann cells, the axonal debris generated upon fragmentation is also in part degraded by the neuron itself. This would process would likely occur prior to fragmentation while the axons are still intact, and could represent one functional benefit of the lag phase observed in Wallerian degeneration. Further studies will be required to determine whether autophagosome distribution and function is observed in dynein mutant axons. Additionally, it will be important to determine whether specifically disrupting autophagy affects the timing of axonal fragmentation after injury.

The preliminary studies described here are the first demonstration that the molecular motor dynein promotes the process of Wallerian degeneration following injury *in vivo.* Follow up studies will reveal more about the specific mechanism by which dynein controls the precise timing of axonal fragmentation.

Conclusions

The work in this thesis demonstrates novel roles for the motor proteins kinesin-1 and dynein in peripheral nerve regeneration *in vivo.* It identifies additional mechanisms, beyond the transport of retrograde injury signals, by which dynein promotes axonal regrowth. It also provides preliminary evidence that dynein promotes cytoskeletal maintenance in axons as well as degeneration after injury, and suggests potential mechanisms of these roles. Taken together, these studies reveal previously unknown functions of dynein in peripheral nerve de- and regeneration.

BIBLIOGRAPHY

1. Witzel C, Rohde C, Brushart TM. Pathway sampling by regenerating peripheral axons. J Comp Neurol. 2005;485(3):183–90.

2. Hammarlund M, Nix P, Hauth L, Jorgensen EM, Bastiani M. Axon regeneration requires a conserved MAP kinase pathway. Science. American Association for the Advancement of Science; 2009 Feb 6;323(5915):802–6.

3. Shin JE, Cho Y, Beirowski B, Milbrandt J, Cavalli V, DiAntonio A. Dual Leucine Zipper Kinase Is Required for Retrograde Injury Signaling and Axonal Regeneration. Neuron. Elsevier; 2012;74(6):1015–22.

4. Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, et al. Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science. 2008 Nov 7;322(5903):963–6.

5. Smith PD, Sun F, Park KK, Cai B, Wang C, Kuwako K, et al. SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron. 2009 Dec 10;64(5):617–23.

6. Sun F, Park KK, Belin S, Wang D, Lu T, Chen G, et al. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature. Nature Publishing Group; 2011 Nov 6;:1–5.

7. David S, Aguayo AJ. Axonal elongation into peripheral nervous system “bridges” after central nervous system injury in adult rats. Science. 1981;214(4523):931–3.

8. Richardson PM, McGuinness UM, Aguayo AJ. Axons from CNS neurons regenerate into PNS grafts. Nature. 1980 Mar 20;284(5753):264–5.

9. Simons M, Trotter J. Wrapping it up: the cell biology of myelination. Curr Opin Neurobiol. 2007 Oct;17(5):533–40.

10. Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature. 1997 Oct 16;389(6652):725–30.

11. Bhatheja K, Field J. Schwann cells: origins and role in axonal maintenance and regeneration. Int J Biochem Cell Biol. 2006;38(12):1995–9.

12. Kucenas S, Takada N, Park H-C, Woodruff E, Broadie K, Appel B. CNS-derived glia ensheath peripheral nerves and mediate motor root development. Nat Neurosci. 2008 Feb;11(2):143–51.

13. Lobsiger CS, Taylor V, Suter U. The early life of a Schwann cell. Biol Chem.

2002 Feb;383(2):245–53.

14. Thomas PK. The cellular response to nerve injury. 3. The effect of repeated crush injuries. Journal of Anatomy. Blackwell Publishing; 1970 May 1;106(Pt 3):463.

15. Saika T, Senba E, Noguchi K, Sato M, Yoshida S, Kubo T, et al. Effects of nerve crush and transection on mRNA levels for nerve growth factor receptor in the rat facial motoneurons. Brain Res Mol Brain Res. 1991 Jan;9(1-2):157–60.

16. Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol. Rockefeller Univ Press; 1992;119(1):45–54.

17. Grothe C, Nikkhah G. The role of basic fibroblast growth factor in peripheral nerve regeneration. Anatomy and embryology. Springer; 2001;204(3):171–7.

18. Ide C, Tohyama K, Yokota R, Nitatori T, Onodera S. Schwann cell basal lamina and nerve regeneration. Brain Res. 1983 Dec 12;288(1-2):61–75.

19. Oaklander AL, Spencer PS. Cold Blockade of Axonal Transport Activates Premitotic Activity of Schwann Cells and Wallerian Degeneration. J Neurochem. 1988 Feb;50(2):490–6.

20. BLAKEMORE WF. Remyelination of CNS axons by Schwann cells transplanted from the sciatic nerve. Nature Publishing Group; 1977.

21. Guest JD, Rao A, Olson L, Bunge MB, Bunge RP. The ability of human Schwann cell grafts to promote regeneration in the transected nude rat spinal cord. Experimental Neurology. 1997 Dec;148(2):502–22.

22. Kromer LF, Cornbrooks CJ. Transplants of Schwann cell cultures promote axonal regeneration in the adult mammalian brain. Proc Natl Acad Sci USA. 1985 Sep;82(18):6330–4.

23. Lewis GM, Kucenas S. Perineurial glia are essential for motor axon regrowth following nerve injury. J Neurosci. Society for Neuroscience; 2014 Sep 17;34(38):12762–77.

24. Isaacman-Beck J, Schneider V, Franzini-Armstrong C, Granato M. The lh3 Glycosyltransferase Directs Target-Selective Peripheral Nerve Regeneration. Neuron. 2015 Nov;88(4):691–703.

25. Heidemann SR, Landers JM, Hamborg MA. Polarity orientation of axonal microtubules. The Journal of Cell Biology. The Rockefeller University Press;

1981 Dec;91(3 Pt 1):661–5.

26. Baas PW. The neuronal centrosome as a generator of microtubules for the axon. Curr Top Dev Biol. 1996;33:281–98.

27. Baas PW. Microtubules and axonal growth. Curr Opin Cell Biol. 1997 Feb;9(1):29–36.

28. Ahmad FJ, Echeverri CJ, Vallee RB, Baas PW. Cytoplasmic dynein and dynactin are required for the transport of microtubules into the axon. The Journal of Cell Biology. 1998 Jan 26;140(2):391–401.

29. Witte H, Bradke F. The role of the cytoskeleton during neuronal polarization. Curr Opin Neurobiol. 2008 Oct;18(5):479–87.

30. Vale RD, Fletterick RJ. The design plan of kinesin motors. Annu Rev Cell Dev Biol. 1997;13:745–77.

31. Hirokawa N, Noda Y, Tanaka Y, Niwa S. Kinesin superfamily motor proteins and intracellular transport. Nat Rev Mol Cell Biol. Nature Publishing Group; 2009 Oct;10(10):682–96.

32. Xia C-H, Roberts EA, Her L-S, Liu X, Williams DS, Cleveland DW, et al. Abnormal neurofilament transport caused by targeted disruption of neuronal kinesin heavy chain KIF5A. The Journal of Cell Biology. Rockefeller University Press; 2003 Apr 14;161(1):55–66.

33. Kanai Y, Okada Y, Tanaka Y, Harada A, Terada S, Hirokawa N. KIF5C, a novel neuronal kinesin enriched in motor neurons. Journal of Neuroscience. 2000 Sep 1;20(17):6374–84.

34. Reid E, Kloos M, Ashley-Koch A, Hughes L, Bevan S, Svenson IK, et al. A Kinesin Heavy Chain (KIF5A) Mutation in Hereditary Spastic Paraplegia (SPG10). The American Journal of Human Genetics. 2002 Nov;71(5):1189–94.

35. Fichera M, Giudice Lo M, Falco M, Sturnio M, Amata S, Calabrese O, et al. Evidence of kinesin heavy chain (KIF5A) involvement in pure hereditary spastic paraplegia. Neurology. 2004 Sep 28;63(6):1108–10.

36. Crimella C, Baschirotto C, Arnoldi A, Tonelli A, Tenderini E, Airoldi G, et al. Mutations in the motor and stalk domains of KIF5A in spastic paraplegia type 10 and in axonal Charcot-Marie-Tooth type 2. Clin Genet. Wiley/Blackwell (10.1111); 2012 Aug;82(2):157–64.

37. Jolly AL, Kim H, Srinivasan D, Lakonishok M, Larson AG, Gelfand VI. Kinesin-1 heavy chain mediates microtubule sliding to drive changes in cell shape.

Proceedings of the National Academy of Sciences. National Academy of Sciences; 2010 Jul 6;107(27):12151–6.

38. Winding M, Kelliher MT, Lu W, Wildonger J, Gelfand VI. Role of kinesin-1-based microtubule sliding in Drosophila nervous system development. Proceedings of the National Academy of Sciences. National Academy of Sciences; 2016 Aug 23;113(34):E4985–94.

39. Lu W, Lakonishok M, Gelfand VI. Kinesin-1-powered microtubule sliding initiates axonal regeneration in Drosophila cultured neurons. Fehon R, editor. Mol Biol Cell. 2015 Apr 1;26(7):1296–307.

40. Pfister KK, Shah PR, Hummerich H, Russ A, Cotton J, Annuar AA, et al. Genetic analysis of the cytoplasmic dynein subunit families. PLoS Genet. 2006 Jan;2(1):e1.

41. Hafezparast M. Mutations in Dynein Link Motor Neuron Degeneration to Defects in Retrograde Transport. Science. 2003 May 2;300(5620):808–12.

42. Chen X-J, Levedakou EN, Millen KJ, Wollmann RL, Soliven B, Popko B. Proprioceptive sensory neuropathy in mice with a mutation in the cytoplasmic Dynein heavy chain 1 gene. Journal of Neuroscience. 2007 Dec 26;27(52):14515–24.

43. Harms MB, Ori-McKenney KM, Scoto M, Tuck EP, Bell S, Ma D, et al. Mutations in the tail domain of DYNC1H1 cause dominant spinal muscular atrophy. Neurology. 2012 May 29;78(22):1714–20.

44. Weedon MN, Hastings R, Caswell R, Xie W, Paszkiewicz K, Antoniadi T, et al. Exome Sequencing Identifies a DYNC1H1 Mutation in a Large Pedigree with Dominant Axonal Charcot-Marie-Tooth Disease. The American Journal of Human Genetics. 2011 Aug;89(2):308–12.

45. Rao AN, Patil A, Black MM, Craig EM, Myers KA, Yeung HT, et al. Cytoplasmic Dynein Transports Axonal Microtubules in a Polarity-Sorting Manner. CellReports. ElsevierCompany; 2017 Jun 13;19(11):2210–9.

46. Watson WE. Observations on the nucleolar and total cell body nucleic acid of injured nerve cells. J Physiol (Lond). 1968 Jun;196(3):655–76.

47. Cragg BG. What is the signal for chromatolysis? Brain Res. 1970 Sep;23(1):1–21.

48. Raivich G, Hellweg R, Kreutzberg GW. NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. Cell. 1991 Jul;7(1):151–64.

49. Perlson E, Jeong G-B, Ross JL, Dixit R, Wallace KE, Kalb RG, et al. A Switch in Retrograde Signaling from Survival to Stress in Rapid-Onset Neurodegeneration. jneurosciorg.

50. Eisen JS. Determination of primary motoneuron identity in developing zebrafish embryos. Science. 1991 Apr 26;252(5005):569–72.

51. Rosenberg AF, Wolman MA, Franzini-Armstrong C, Granato M. In vivo nerve-macrophage interactions following peripheral nerve injury. Journal of Neuroscience. 2012 Mar 14;32(11):3898–909.

52. Rosenberg AF, Isaacman-Beck J, Franzini-Armstrong C, Granato M. Schwann cells and deleted in colorectal carcinoma direct regenerating motor axons towards their original path. J Neurosci. Society for Neuroscience; 2014 Oct 29;34(44):14668–81.

53. Höke A. Mechanisms of Disease: what factors limit the success of peripheral nerve regeneration in humans? Nat Clin Pract Neurol. Nature Publishing Group; 2006 Aug;2(8):448–54.

54. Rishal I, Fainzilber M. Retrograde signaling in axonal regeneration. Experimental Neurology. 2010 May;223(1):5–10.

55. He Z, Jin Y. Intrinsic Control of Axon Regeneration. Neuron. 2016 May 4;90(3):437–51.

56. Jessen KR, Mirsky R. The repair Schwann cell and its function in regenerating nerves. J Physiol (Lond). Wiley/Blackwell (10.1111); 2016 Jul 1;594(13):3521–31.

57. Wagner U, Utton M, Gallo JM, Miller CC. Cellular phosphorylation of tau by GSK-3 beta influences tau binding to microtubules and microtubule organisation. Journal of Cell Science. 1996 Jun;109 (Pt 6):1537–43.

58. Patrick GN, Zukerberg L, Nikolic M, la Monte de S, Dikkes P, Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature. Nature Publishing Group; 1999 Dec 9;402(6762):615–22.

59. Seitz A, Kojima H, Oiwa K, Mandelkow E-M, Song Y-H, Mandelkow E. Single-molecule investigation of the interference between kinesin, tau and MAP2c. EMBO J. European Molecular Biology Organization; 2002 Sep 16;21(18):4896–905.

60. LaPointe NE, Morfini G, Pigino G, Gaisina IN, Kozikowski AP, Binder LI, et al. The amino terminus of tau inhibits kinesin-dependent axonal transport:

implications for filament toxicity. J Neurosci Res. Wiley-Blackwell; 2009 Feb;87(2):440–51.

61. Roll-Mecak A, Vale RD. Structural basis of microtubule severing by the hereditary spastic paraplegia protein spastin. Nature. Nature Publishing Group; 2008 Jan 17;451(7176):363–7.

62. Salinas S, Proukakis C, Crosby A, Warner TT. Hereditary spastic paraplegia: clinical features and pathogenetic mechanisms. The Lancet Neurology. Elsevier; 2008 Jan 12;7(12):1127–38.

63. Farrer MJ, Hulihan MM, Kachergus JM, Dächsel JC, Stoessl AJ, Grantier LL, et al. DCTN1 mutations in Perry syndrome. Nature Genetics. Nature Publishing Group; 2009 Feb;41(2):163–5.

64. Puls I, Jonnakuty C, LaMonte BH, Holzbaur ELF, Tokito M, Mann E, et al. Mutant dynactin in motor neuron disease. Nature Genetics. Nature Publishing Group; 2003 Apr;33(4):455–6.

65. Ebbing B, Mann K, Starosta A, Jaud J, Schöls L, Schüle R, et al. Effect of spastic paraplegia mutations in KIF5A kinesin on transport activity. Human Molecular Genetics. 2008 May 1;17(9):1245–52.

66. Zhao C, Takita J, Tanaka Y, Setou M, Nakagawa T, Takeda S, et al. Charcot-Marie-Tooth Disease Type 2A Caused by Mutation in a Microtubule Motor KIF1Bβ. Cell. 2001 Jun;105(5):587–97.

67. Oblinger MM, Lasek RJ. Axotomy-induced alterations in the synthesis and transport of neurofilaments and microtubules in dorsal root ganglion cells. Journal of Neuroscience. 1988 May;8(5):1747–58.

68. McQuarrie IG, Jacob JM. Conditioning nerve crush accelerates cytoskeletal protein transport in sprouts that form after a subsequent crush. J Comp Neurol. Wiley-Blackwell; 1991 Mar 1;305(1):139–47.

69. Misgeld T, Kerschensteiner M, Bareyre FM, Burgess RW, Lichtman JW. Imaging axonal transport of mitochondria in vivo. Nat Meth. 2007 Jun 10;4(7):559–61.

70. Cavalli V. Sunday Driver links axonal transport to damage signaling. The Journal of Cell Biology. 2005 Feb 28;168(5):775–87.

71. Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, Fainzilber M. Vimentin-Dependent Spatial Translocation of an Activated MAP Kinase in Injured Nerve. Neuron. 2005 Mar;45(5):715–26.

72. Abe N, Cavalli V. Nerve injury signaling. Curr Opin Neurobiol. 2008

Jun;18(3):276–83.

73. Hanz S, Fainzilber M. Retrograde signaling in injured nerve ? the axon reaction revisited. J Neurochem. 2006 Oct;99(1):13–9.

74. Yogev S, Maeder CI, Cooper R, Horowitz M, Hendricks AG, Shen K. Local inhibition of microtubule dynamics by dynein is required for neuronal cargo distribution. Nat Commun. Nature Publishing Group; 2017 Apr 6;8:1–14.

75. Zheng Y, Wildonger J, Ye B, Zhang Y, Kita A, Younger SH, et al. Dynein is required for polarized dendritic transport and uniform microtubule orientation in axons. Nat Cell Biol. 2008 Aug 31;10(10):1172–80.

76. Langworthy MM, Appel B. Schwann cell myelination requires Dynein function. Neural Dev. 2012;7:37.

77. Insinna C, Baye LM, Amsterdam A, Besharse JC, Link BA. Analysis of a zebrafish dync1h1 mutant reveals multiple functions for cytoplasmic dynein 1 during retinal photoreceptor development. Neural Dev. 2010;5:12.

78. Miki H, Setou M, Kaneshiro K, Hirokawa N. All kinesin superfamily protein, KIF, genes in mouse and human. Proceedings of the National Academy of Sciences. National Academy of Sciences; 2001 Jun 19;98(13):7004–11.

79. McMurray CT. Neurodegeneration: diseases of the cytoskeleton? Cell Death Differ. 2000 Oct;7(10):861–5.

80. Glass JD, Brushart TM, George EB, Griffin JW. Prolonged survival of transected nerve fibres in C57BL/Ola mice is an intrinsic characteristic of the axon. J Neurocytol. Kluwer Academic Publishers; 1993 May;22(5):311–21.

81. Lunn ER, Perry VH, Brown MC, Rosen H, Gordon S. Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve. European Journal of Neuroscience. 1989 Jan;1(1):27–33.

82. Osterloh JM, Yang J, Rooney TM, Fox AN, Adalbert R, Powell EH, et al. dSarm/Sarm1 Is Required for Activation of an Injury-Induced Axon Death Pathway. Science. 2012 Jul 26;337(6093):481–4.

83. Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC. Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. J Neurosci. Soc Neuroscience; 2000;20(4):1333–41.

84. Zhai Q, Wang J, Kim A, Liu Q, Watts R, Hoopfer E, et al. Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration.

Neuron. Elsevier; 2003;39(2):217–25.

85. Zimmerman UJ, Schlaepfer WW. Characterization of a brain calcium-activated protease that degrades neurofilament proteins. Biochemistry. 1982 Aug 17;21(17):3977–82.

86. George EB, Glass JD, Griffin JW. Axotomy-induced axonal degeneration is mediated by calcium influx through ion-specific channels. J Neurosci. 1995 Oct;15(10):6445–52.

87. Rosenberg AF, Wolman MA, Franzini-Armstrong C, Granato M. In vivo nerve-macrophage interactions following peripheral nerve injury. Journal of Neuroscience. 2012 Mar 14;32(11):3898–909.

88. Maday S, Wallace KE, Holzbaur ELF. Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. The Journal of Cell Biology. 2012 Feb 20;196(4):407–17.

89. Kimura S, Noda T, Yoshimori T. Dynein-dependent Movement of Autophagosomes Mediates Efficient Encounters with Lysosomes. Cell Struct Funct. 2008;33(1):109–22.

90. Wang QJ, Ding Y, Kohtz DS, Kohtz S, Mizushima N, Cristea IM, et al. Induction of autophagy in axonal dystrophy and degeneration. Journal of Neuroscience. 2006 Aug 2;26(31):8057–68.

91. Yang Y, Fukui K, Koike T, Zheng X. Induction of autophagy in neurite degeneration of mouse superior cervical ganglion neurons. European Journal of Neuroscience. 2007 Nov 13;26(10):2979–88.

92. Rosenberg AF, Isaacman-Beck J, Franzini-Armstrong C, Granato M. Schwann cells and deleted in colorectal carcinoma direct regenerating motor axons towards their original path. J Neurosci. Society for Neuroscience; 2014 Oct 29;34(44):14668–81.

93. Harrisingh MC, Perez-Nadales E, Parkinson DB, Malcolm DS, Mudge AW, Lloyd AC. The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation. EMBO J. Nature Publishing Group; 2004;23(15):3061–71.

94. Zanazzi G, Einheber S, Westreich R, Hannocks MJ, Bedell-Hogan D, Marchionni MA, et al. Glial Growth Factor/Neuregulin Inhibits Schwann Cell Myelination and Induces Demyelination. The Journal of Cell Biology. 2001 Mar 19;152(6):1289–300.

95. Arthur-Farraj PJ, Latouche M, Wilton DK, Quintes S, Chabrol E, Banerjee A, et al. c-Jun Reprograms Schwann Cells of Injured Nerves to Generate a Repair

Cell Essential for Regeneration. Neuron. 2012 Aug;75(4):633–47.

96. Carey DJ, Stahl RC, Cizmeci-Smith G, Asundi VK. Syndecan-1 expressed in Schwann cells causes morphological transformation and cytoskeletal reorganization and associates with actin during cell spreading. jcbrupressorg.

97. Barber SC, Mellor H, Gampel A, Scolding NJ. S1P and LPA trigger Schwann cell actin changes and migration. European Journal of Neuroscience. Wiley Online Library; 2004;19(12):3142–50.

98. Kidd G, Andrews SB, Trapp BD. Axons regulate the distribution of Schwann cell microtubules. J Neurosci. 1996 Feb 1;16(3):946–54.

99. Muranen T, Grönholm M, Lampin A, Lallemand D, Zhao F, Giovannini M, et al. The tumor suppressor merlin interacts with microtubules and modulates Schwann cell microtubule cytoskeleton. Human Molecular Genetics. 2007 Jul 15;16(14):1742–51.

100. Carney TJ, Dutton KA, Greenhill E, Delfino-Machín M, Dufourcq P, Blader P, et al. A direct role for Sox10 in specification of neural crest-derived sensory neurons. Development. The Company of Biologists Ltd; 2006 Dec;133(23):4619–30.

101. Schlaepfer WW, Myers FK. Relationship of myelin internode elongation and growth in the rat sural nerve. J Comp Neurol. Wiley-Blackwell; 1973 Jan 15;147(2):255–66.

102. Peters A, Muir AR. The relationship between axons and Schwann cells during development of peripheral nerves in the rat. Q J Exp Physiol Cogn Med Sci. 1959 Jan;44(1):117–30.

103. Richardson PM, Verge VM. Axonal regeneration in dorsal spinal roots is accelerated by peripheral axonal transection. Brain Res. 1987 May 19;411(2):406–8.

104. Navarro X, Kennedy WR. The effect of a conditioning lesion on sudomotor axon regeneration. Brain Res. 1990 Feb 19;509(2):232–6.

105. Sjöberg J, Kanje M. Effects of repetitive conditioning crush lesions on regeneration of the rat sciatic nerve. Brain Res. 1990 Oct 15;530(1):167–9.

106. Mullins MC, Hammerschmidt M, Haffter P, Nusslein-Volhard C. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Curr Biol. 1994 Mar 1;4(3):189–202.

107. Flanagan-Steet H, Fox MA, Meyer D, Sanes JR. Neuromuscular synapses can

form in vivo by incorporation of initially aneural postsynaptic specializations. Development. Oxford University Press for The Company of Biologists Limited; 2005 Oct;132(20):4471–81.

108. Peri F, Nüsslein-Volhard C. Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. Cell. 2008 May 30;133(5):916–27.

109. Amsterdam A, Nissen RM, Sun Z, Swindell EC, Farrington S, Hopkins N. Identification of 315 genes essential for early zebrafish development. Proc Natl Acad Sci USA. National Acad Sciences; 2004;101(35):12792.

110. Petersen SC, Luo R, Liebscher I, Giera S, Jeong S-J, Mogha A, et al. The Adhesion GPCR GPR126 Has Distinct, Domain-Dependent Functions in Schwann Cell Development Mediated by Interaction with Laminin-211. Neuron. 2015 Feb;85(4):755–69.

111. Kettleborough RNW, Busch-Nentwich EM, Harvey SA, Dooley CM, de Bruijn E, van Eeden F, et al. A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature. 2013 Apr 25;496(7446):494–7.

112. Zhang XP, Ambron RT. Positive injury signals induce growth and prolong survival in Aplysia neurons. J Neurobiol. 2000 Nov 5;45(2):84–94.

113. Schmied R, Ambron RT. A nuclear localization signal targets proteins to the retrograde transport system, thereby evading uptake into organelles in aplysia axons. J Neurobiol. 1997 Aug;33(2):151–60.

114. Wellmann H, Kaltschmidt B, Kaltschmidt C. Retrograde transport of transcription factor NF-kappa B in living neurons. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2001 Apr 13;276(15):11821–9.

115. Hanz S, Perlson E, Willis D, Zheng J-Q, Massarwa R, Huerta JJ, et al. Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron. 2003 Dec 18;40(6):1095–104.

116. Fontana X, Hristova M, Da Costa C, Patodia S, Thei L, Makwana M, et al. c-Jun in Schwann cells promotes axonal regeneration and motoneuron survival via paracrine signaling. The Journal of Cell Biology. 2012 Jul 9;198(1):127–41.

117. Glenn TD, Talbot WS. Signals regulating myelination in peripheral nerves and the Schwann cell response to injury. Curr Opin Neurobiol. 2013 Jul.

118. Zochodne DW. The challenges and beauty of peripheral nerve regrowth. Vol. 17, Journal of the peripheral nervous system : JPNS. Wiley/Blackwell (10.1111); 2012. pp. 1–18.

119. Palmer KJ, Hughes H, Stephens DJ. Specificity of cytoplasmic dynein subunits in discrete membrane-trafficking steps. Holzbaur E, editor. Mol Biol Cell. 2009 Jun;20(12):2885–99.

120. Jessen KR, Mirsky R. Negative regulation of myelination: relevance for development, injury, and demyelinating disease. Glia. 2008.

121. Myers PZ. Spinal motoneurons of the larval zebrafish. J Comp Neurol. Wiley-Blackwell; 1985 Jun 22;236(4):555–61.

122. Tanaka E, Sabry J. Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell. 1995 Oct 20;83(2):171–6.

123. Brandt R. Cytoskeletal mechanisms of axon outgrowth and pathfinding. Cell Tissue Res. 1998 May;292(2):181–9.

124. Blanquie O, Bradke F. Cytoskeleton dynamics in axon regeneration. Curr Opin Neurobiol. 2018 Mar 12;51:60–9.

125. Ruschel J, Hellal F, Flynn KC, Dupraz S, Elliott DA, Tedeschi A, et al. Axonal regeneration. Systemic administration of epothilone B promotes axon regeneration after spinal cord injury. Science. American Association for the Advancement of Science; 2015 Apr 17;348(6232):347–52.

126. Hellal F, Hurtado A, Ruschel J, Flynn KC, Laskowski CJ, Umlauf M, et al. Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. Science. American Association for the Advancement of Science; 2011 Feb 18;331(6019):928–31.

127. Sengottuvel V, Leibinger M, Pfreimer M, Andreadaki A, Fischer D. Taxol facilitates axon regeneration in the mature CNS. J Neurosci. Society for Neuroscience; 2011 Feb 16;31(7):2688–99.

128. Grabham PW, Seale GE, Bennecib M, Goldberg DJ, Vallee RB. Cytoplasmic Dynein and LIS1 Are Required for Microtubule Advance during Growth Cone Remodeling and Fast Axonal Outgrowth. Journal of Neuroscience. 2007 May 23;27(21):5823–34.

129. Adames NR, Cooper JA. Microtubule interactions with the cell cortex causing nuclear movements in Saccharomyces cerevisiae. The Journal of Cell Biology. 2000 May 15;149(4):863–74.

130. Ligon LA, Tokito M, Finklestein JM, Grossman FE, Holzbaur ELF. A direct interaction between cytoplasmic dynein and kinesin I may coordinate motor activity. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2004 Apr 30;279(18):19201–8.

131. Kawauchi T, Chihama K, Nabeshima Y-I, Hoshino M. The in vivo roles of STEF/Tiam1, Rac1 and JNK in cortical neuronal migration. EMBO J. EMBO Press; 2003 Aug 15;22(16):4190–201.

132. Lu W, Fox P, Lakonishok M, Davidson MW, Gelfand VI. Initial neurite outgrowth in Drosophila neurons is driven by kinesin-powered microtubule sliding. Curr Biol. 2013 Jun 3;23(11):1018–23.

133. Drerup CM, Nechiporuk AV. JNK-interacting protein 3 mediates the retrograde transport of activated c-Jun N-terminal kinase and lysosomes. Moens C, editor. PLoS Genet. Public Library of Science; 2013;9(2):e1003303.