The Role of Actr10 in Nervous System Development and Disease

Amy Herbert
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/art_sci_etds
Part of the Developmental Biology Commons, Genetics Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation
https://openscholarship.wustl.edu/art_sci_etds/1535

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
The Role of Actr10 in Nervous System Development and Disease
by
Amy Louise Herbert

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
degree of Doctor of Philosophy

May 2018
St. Louis, Missouri
# Table of Contents

List of Figures .................................................................................................................. v
List of Tables ..................................................................................................................... viii
Acknowledgments .......................................................................................................... ix
Abstract ............................................................................................................................... xiii

## Chapter 1: Introduction ................................................................................................. 1
  1.1 The myelin sheath ................................................................................................. 3
  1.2 Intrinsic factors guiding glial development ....................................................... 3
  1.3 Mechanical regulation of myelinating glial development and differentiation ...... 4
  1.4 Cytoskeletal regulation of myelination ............................................................... 6
  1.5 Activity-dependent control of myelination and myelin maintenance ............... 8
  1.6 Myelin and metabolism ................................................................................... 10
  1.7 Conclusion and outlook .................................................................................... 11
  1.8 Acknowledgements ............................................................................................ 12
  1.9 Figures ............................................................................................................... 13
  1.10 References ....................................................................................................... 17

## Chapter 2: A forward genetic screen in zebrafish to uncover new regulators of myelinated axon development ................................................................. 23
  2.1 An introduction to zebrafish screens ................................................................. 24
  2.2 Set-up of the “LM” screen .................................................................................. 27
  2.3 Whole genome sequencing identifies the causative lesions responsible for mutant phenotypes ......................................................... 29
  2.4 Figures ............................................................................................................... 34
  2.5 References ....................................................................................................... 40

## Chapter 3: Dynein/dynactin is necessary for anterograde transport of Mbp mRNA in oligodendrocytes and for myelination in vivo ....................................................... 44
  3.1 Significance ....................................................................................................... 46
  3.2 Abstract ............................................................................................................. 46
  3.3 Introduction ....................................................................................................... 47
Appendix B: Regulation of mitochondria-dynactin interaction and mitochondrial retrograde transport in axons

B.1 Abstract
B.2 Introduction
B.3 Results
B.4 Discussion
B.5 Conclusions
B.6 Figures
B.7 Materials and methods
B.8 Acknowledgements
B.9 Author contributions
B.10 References
List of Figures

Chapter 1: Introduction
Figure 1: Mechotransduction plays a critical role in Schwann cell development and differentiation................................................................. 13
Figure 2: Multiple factors fine tune the myelination potential of oligodendrocytes. ................. 15
Figure 3: Comparing and contrasting Schwann cell and oligodendrocyte development and differentiation.................................................................................................................................. 16

Chapter 2: A forward genetic screen in zebrafish to uncover new regulators of myelinated axon development
Figure 1: A forward genetic screen in zebrafish to identify regulators of myelination.............. 34
Figure 2: The screen uncovered myelin mutants with a variety of phenotypes.......................... 36
Figure 3: Whole genome sequencing is used to identify the genes responsible for mutant phenotypes. ...................................................................................................................................... 38
Figure 4: A complementation test confirms that actr10 is the gene responsible for the stl83 mutant phenotype.................................................................................................................. 39

Chapter 3: Dynein/dynactin is necessary for anterograde transport of Mbp mRNA in oligodendrocytes and for myelination in vivo
Figure 1: A forward genetic screen uncovers actr10 mutants. ................................................. 72
Figure 2: actr10 mutants have fewer myelinated axons in the ventral spinal cord. .................. 73
Figure 3: actr10nl15/nl15 mutants have fewer OPCs............................................................... 75
Figure 4: Cell division is delayed in actr10nl15/nl15 mutants................................................... 76
Figure 5: Transient expression of actr10 in oligodendrocytes partially suppresses myelination defects in actr10nl15/nl15 mutants. ........................................................................................................... 77
Figure 6: Dynein and dynactin are associated with Mbp mRNA granules............................... 79
Figure 7: Acute dynein inhibition arrests both anterograde and retrograde Mbp mRNA transport in cultured oligodendrocytes and perturbs mbp localization in zebrafish.............................. 81
Figure 8: Chronic dynein inhibition disrupts MBP protein translation in cultured oligodendrocytes and disrupts myelination in zebrafish................................................................. 82
Figure S1 (related to Fig. 1 and Fig. 2): nl15 and stl83 are alleles of actr10. ......................... 84
Figure S2 (related to Fig. 6): actr10nl15/nl15 mutants have similar phenotypes to dynactin and dynein mutants. ............................................................................................................................................ 86
Figure S3 (related to Fig. 5): Stable expression of actr10 in neurons suppresses myelination defects in actr10nl15/nl15 mutants. ................................................................. 88

Figure S4 (related to Fig. 6 and Fig. 7): Co-immunoprecipitation pulls out p135 in addition to p150Glued. ................................................................. 90

Figure S5 (related to Fig. 7): Effects of ciliobrevin D treatment on zebrafish and cultured oligodendrocytes. ................................................................. 91

Chapter 4: Investigating the role of ACTR10 in human disease

Figure 1: actr10nl15/nl15 and actr10sd183/sd183 mutants have reduced mbp expression in the PNS. .. 119

Figure 2: actr10nl15/nl15 and actr10sd183/sd183 mutants have axonal and myelin defects. ............ 120

Figure 3: actr10nl15/nl15 mutants exhibit swellings in the lateral line and neurofilament accumulation in neuromasts. .................................................. 122

Figure 4: actr10 mutants exhibit swelling in the large caliber Mauthner axon and neurofilament accumulation. .................................................................... 123

Figure 5: Generation of a humanized zebrafish strain with a patient specific actr10 mutation. 125

Figure 6: Transheterozygous actr10nl15/+ ; actr10CMT/+ zebrafish do not have an axonal swelling phenotype at 5 dpf. .................................................. 127

Figure 7: Actr10 knockdown in mouse DRG culture results in a regeneration defect. ............ 128

Appendix A: Whole genome sequencing-based mapping and candidate identification of mutations from fixed zebrafish tissue

Figure 1: The st49 allele is accurately mapped to chromosome 20 and gpr126 using gDNA extracted from fresh tissue. .......................................................... 154

Figure 2: The fh227 allele is accurately mapped to chromosome 12 and egr2b using gDNA extracted from fixed tissue. .................................................. 156

Figure 3: The stl64 phenotype is linked to chromosome 1 and is likely caused by a nonsense mutation in fbxw7. .......................................................... 158

Figure 4: The fbxw7stl64 allele phenocopies the fbxw7su56 allele. .................................................. 160

Appendix B: Regulation of mitochondria-dynactin interaction and mitochondrial retrograde transport in axons

Figure 1. actr10 mutants have swollen axon terminals. .......................................................... 200

Figure 2: Loss of Actr10 causes mitochondrial accumulation in swollen axon terminals. .... 201

Figure 3: TEM analysis of axon terminal swellings reveal mitochondrial accumulation. .... 203

Figure 4: Mitochondrial retrograde transport is specifically disrupted in actr10 mutants. .... 204

Figure 5: Actr10 functions cell autonomously in axons. .......................................................... 205
Figure 6: Dynein-dynactin localization and retrograde movement are intact in actr10 mutants. ................................................................. 206

Figure 7: Peroxisome and Lamp1 vesicle transport in actr10 mutants at 4 dpf. .................. 208

Figure 8: dync1h1 and p150a/b mutants fail to phenocopy actr10 mutants....................... 209

Figure 9: p150a and p150b expression in zebrafish embryos and larvae. ......................... 211

Figure 10: pJNK and Lamp1 accumulate in p150a/b pLL axon terminals ....................... 213

Figure 11: Actr10 is essential for mitochondria-dynactin interaction. ......................... 215

Figure 12: Drp1 functions with Actr10 in mitochondrial retrograde transport. ............ 216
Appendix A: Whole genome sequencing-based mapping and candidate identification of mutations from fixed zebrafish tissue

Table 1: WGS Coverage of the gpr126, egr2b and stl64 Pools................................. 162
Table 2: Number of Exonic SNPs and INDELS in the gpr126, egr2b and stl64 Pools. ........ 163
Acknowledgments

I would firstly like to acknowledge my thesis mentor, Kelly Monk. Kelly has been a source of support and encouragement throughout graduate school, from the beginning of my Ph.D., through my post-doctoral search. Kelly gave me great freedom to follow my research interests and never said no to an experiment I wanted to do. Even now that she is Co-Director of the Vollum Institute, Kelly continues to respond to my e-mails and questions instantly. I would not have become the scientist I am today without Kelly as a role model, and I know that she will continue to mentor me after I have left her lab.

I would also like to acknowledge the members of my thesis committee. Aaron DiAntonio, in addition to being a rigorous and careful scientist, has made me feel like a scientific colleague. Lila Solnica-Krezel has been a role model for me for how to be a leader in science. She has made the Department of Developmental Biology a great place to be a student, not only because of the excellent science, but also because of the collegial environment. I am grateful to Chuck Kaufman for allowing me to join his lab at the end of my graduate career. Chuck, like Kelly, has an open-door policy and I have enjoyed becoming a member of his lab as it is beginning. Finally, I would like to thank Jim Skeath. Jim is one of the main reasons I decided to attend Washington University for graduate school. Jim supports students throughout graduate school and the graduate programs at Washington University would not be as successful as they are without him. In addition to being a mentor and a role model, Jim is a friend.

There have also been mentors outside my thesis committee who have helped me during my graduate career. I would like to thank Irving Boime and Craig Micchelli in particular. Having Irv as a mentor in the Department has been a privilege and I have appreciated his guidance and
interest in my career as a scientist. I have also enjoyed hallway chats with and drive-by mentorship by Craig Micchelli. I would also like to thank everyone in the Department of Developmental Biology, particularly Andrew Yoo, Shin Imai and Doug Covey, who have always stopped me in the hallway to say hello and to ask how I am doing. Furthermore, the office staff, including Connie H, Connie C, and Connie A, Toni Hill, and Kati Riebold have been helpful in taking care of the managerial aspects of science. Outside Washington University, my collaborators Meng-meng Fu, Ben Barres, Katie Drerup, and Alex Nechiporuk were instrumental in contributing and shaping my published research manuscripts. I would also like to acknowledge the funding sources that have made this dissertation possible: a generous fellowship from Phil and Sima Needleman and an F31 Ruth L. Kirschstein National Research Service Award grant.

I would like to thank all members of the Monk lab, past and present. The Monk lab was a tight knit group and I am grateful to have worked alongside colleagues who were not only motivated and excited about science, but also kind and helpful. Sarah Ackerman and Breanne Harty both took a leadership role during the forward genetic screen, and I learned a lot in my first year in the lab working alongside them. Nick Sanchez was always willing to help me with bioinformatics or to chat about science or current events. All three post-docs, Amit Mogha, Mitch D’Rozario, and Sarah Petersen were knowledgeable, helpful, and showed me how to be a successful post-doc. Rebecca Cunningham has become a good friend and I have enjoyed many interesting discussions with her. I would also like to thank our technician, Charleen Johnson, and the fish facility staff.

Finally, I would like to thank my many friends and family outside of graduate school. My friends from high school, Margot, Liz, Cassie, Emily, Amelia, and Parul, and my friends from
college, Lia, Aparna, Julia, Diane and Kim, helped me to succeed in college and in graduate school. Even though we have lived far apart for many years, we talk almost every day and I am grateful for their encouragement and friendship. Thank you to Kim in particular for being my best friend for so many years.

I am lucky to be part of a family of scientists that have always supported me. Both my parents are academic scientists, and have inspired and encouraged me throughout my life, and especially during graduate school. My sister Lisa is also a fellow scientist who travels to the Arctic circle for her research, and has always been a loyal friend. Finally, I have to thank my fellow graduate student and partner Matt McCoy. Matt has made me a better person and a better scientist. This dissertation would not have been possible without Matt’s support.

Amy Louise Herbert

Washington University in St. Louis

May 2018
Dedicated to my family.
ABSTRACT OF THE DISSERTATION

The Role of Actr10 in Nervous System Development and Disease

by

Amy Louise Herbert

Doctor of Philosophy in Biology and Biomedical Sciences

Developmental, Regenerative, and Stem Cell Biology

Washington University in St. Louis, 2018

Professor Kelly Monk, Chair

Professor Jim Skeath, Co-Chair

The vertebrate nervous system requires myelinating glia for the fast propagation of action potentials, as well as for vital trophic support to axons. Myelinating glia produce myelin, which is a lipid-rich, multi-lamellar sheath that surrounds axons and allows for rapid electrical signaling. In the central nervous system (CNS), myelin is produced by oligodendrocytes, while in the peripheral nervous system (PNS), Schwann cells perform this function. Although glia have historically been understudied compared to neurons, recent research has uncovered critical roles for glia in nervous system development and disease. Disruption to myelin or to the glial cells that generate myelin can have severe consequences for human health, as demonstrated by the debilitating symptoms of multiple sclerosis (MS) and Charcot-Marie-Tooth disease (CMT). In order to improve patient health, it is necessary to determine the etiology of demyelinating diseases, which in turn requires a comprehensive understanding of glial cell development and myelination. The scientific advances made in our understanding of myelinating glial cell development will be discussed in Chapter 1 of this dissertation. Although great progress has been
achieved, our understanding of the molecular mechanisms that regulate myelination is incomplete.

The zebrafish has emerged as an important model organism for studying myelin. In particular, the ability to perform forward genetic screens in zebrafish has greatly increased our understanding of the individual genes involved in myelination in both the CNS and the PNS. Although several myelin-related forward genetic screens have been previously performed in zebrafish, these screens were not done to saturation, potentially leaving essential genes unidentified. Our lab therefore performed a large scale forward genetic screen to uncover new players in myelin development. The screen was a collaborative effort between students in the Monk lab and members of the Solnica-Krezel lab. The myelin screen was highly successful, uncovering 31 mutants. The set-up and outcome of the screen is described in more detail in Chapter 2.

One of the mutants identified in the screen was found to be the result of a mutation in the gene \textit{actin related protein 10} (\textit{actr10}). Actr10 (or Arp11) is a component of the dynactin complex, which is necessary for retrograde transport of cargo by cytoplasmic dynein. Two alleles of \textit{actr10} zebrafish mutants, \textit{actr10}^\textit{stl83} (the allele originally identified in the screen) and \textit{actr10}^\textit{nl15} (a presumptive null generously shared by the Nechiporuk lab) exhibited reduced myelin in the CNS and in the PNS, as well as a punctate expression of myelin basic protein (\textit{mbp}) in the hindbrain. Mbp has important roles in myelin compaction as well as in initiating wrapping of myelin around axon segments. Initial characterization of \textit{actr10}^\textit{nl15/nl15} mutants revealed a reduction in oligodendrocyte precursor cells, fewer myelinated axons by ultrastructural analysis, and increased cell division in mutant oligodendrocytes. Moreover, the punctate \textit{mbp} phenotype was reminiscent of another zebrafish mutant in the anterograde kinesin
motor kif1b. Importantly, mbp mRNA is transported and translated locally at the developing myelin sheath. I hypothesized that dynein/dynactin regulates anterograde transport of mbp mRNA in oligodendrocytes. To test this, I collaborated with another lab and found that indeed, mbp mRNA transport was arrested/perturbed in both rat oligodendrocyte cell culture and zebrafish in response to dynein inhibition, demonstrating a previously unknown role for dynein/dynactin in mbp transport. This published work can be found in its entirety in Chapter 3.

In addition to myelin defects, actr10 zebrafish mutants exhibited axonal swellings in both the CNS and in the PNS. Electron microscopy revealed neurofilament accumulation in the axons of mutant animals, which is a hallmark of many neurodegenerative disorders. We therefore wondered whether ACTR10 might have a role in human disease. In collaboration with a neurologist at Washington University, several patients diagnosed with amyotrophic lateral sclerosis (ALS), distal myopathy and CMT were also found to have mutations in ACTR10. Using genome editing technologies in zebrafish, we generated a line of zebrafish containing the CMT2 patient ACTR10 mutant single nucleotide polymorphism (SNP), thereby generating a patient specific disease zebrafish model. Current work is ongoing to characterize the zebrafish mutant and future experiments could include drug screens to identify compounds that may ameliorate CMT mutant phenotypes. Generation of the this CMT zebrafish line and future directions for this project are described in Chapter 4.

From a forward genetic screen to identify novel regulators of myelination to generating patient specific mutations in zebrafish, my dissertation has involved a broad range of genetic and molecular techniques in the study of nervous system development in general, and myelinating glial development in particular. The identification of Actr10 as a player in oligodendrocyte development and myelination, as well as a potential regulator of a major human demyelinating
disorder, demonstrates the power of zebrafish to address both basic and biomedical questions directly relevant to human patients.
Chapter 1: Introduction
Preface:

Portions of this chapter have been reproduced and adapted from the following published manuscript:

1.1 The myelin sheath

In the vertebrate nervous system, the fast conduction of action potentials is potentiated by the myelin sheath, a multi-lamellar, lipid-rich structure that also provides vital trophic and metabolic support to axons. Myelin is elaborated by the plasma membrane of specialized glial cells, oligodendrocytes in the central nervous system (CNS) and Schwann cells (SCs) in the peripheral nervous system (PNS). The diseases that result from damage to myelin or glia, including multiple sclerosis and Charcot-Marie-Tooth disease, underscore the importance of these cells for human health. Therefore, an understanding of glial development and myelination is crucial in addressing the etiology of demyelinating diseases and developing patient therapies. In this review, we discuss new insights into the roles of mechanotransduction and cytoskeletal rearrangements as well as activity dependent myelination and axonal maintenance by glia. Together, these discoveries advance our knowledge of myelin and glia in nervous system health and plasticity throughout life.

1.2 Intrinsic factors guiding glial development

Although both cells produce myelin to insulate and support axons, oligodendrocytes and SCs differ early in their genesis. Oligodendrocytes originate from neuroepithelial precursors, whereas SCs are derived from the neural crest. Furthermore, one oligodendrocyte can myelinate multiple axon segments, but one SC myelinates only a single axon segment (Fig. 1, Fig. 2). This is achieved through a process called radial sorting in which cytoplasmic processes from immature SCs extend into axon bundles and “select” an axon segment (1). SC development is mediated by a host of transcription factors and signaling molecules, including Sox10, which persists throughout development and differentiation, activating other transcription factors (1). In
pro-myelinating SCs, which have radially sorted axons and wrapped 1-1.5 turns around an axon, the G protein-coupled receptor (GPCR) GPR126/Adgrg6 elevates cAMP to promote expression of the transcription factor Oct6/Pou3f1 (1). Oct6 and Sox10, along with other factors, activate the master regulator of PNS myelination, Krox-20/Egr2, which is essential for expression of critical myelin genes, including Myelin basic protein (Mbp) (1).

Proliferative and migratory oligodendrocyte precursor cells (OPCs) extend and retract numerous processes during development (2). Recent work has found that OPCs can migrate along blood vessels in a Wnt-dependent manner involving the receptor-ligand pair Cxcr4-Cxcl12, which are expressed on OPCs and endothelial cells, respectively (3). Oligodendrocyte differentiation requires some shared SC factors, including Sox10 and Yin yang 1 (Yy1), in addition to the oligodendrocyte specific regulators Olig1, Olig2, Nkx2.2 (2) and Myelin regulatory factor, Myrf, which plays an analogous role to Krox-20 (4). Recent work in SCs and oligodendrocytes has identified novel roles for signaling molecules, including a suite of GPCRs, GPR17, GPR56 and GPR37 in the CNS (5-8) and GPR44 and the zinc finger Zeb2 in the PNS (9-11). While new myelin regulators remain to be uncovered, elucidating function of known molecules and pathways is key to understanding myelination in development and repair.

1.3 Mechanical regulation of myelinating glial development and differentiation

A unique signaling mechanism in SCs occurs via the basal lamina (BL), and recent evidence points to the molecular mechanisms by which this structure mechanically regulates myelination. In SCs, GPR126 can interact with axonally-derived Prion protein (PrP^c) (12) as well as two SC-derived components of the BL, collagen IV and Laminin-211 (13,14). Laminin-211 polymerization was proposed to activate GPR126 mechanically, initiating SC myelination (Fig.
1) (13), and SCs respond to mechanical properties of the BL with intracellular molecules such as Focal adhesion kinase (FAK) (15). Recently, two Hippo pathway signaling molecules, YAP and TAZ (YAP/TAZ), have been implicated as mediators of mechanotransduction during SC development. YAP/TAZ respond to mechanical or chemical stimuli and translocate to the nucleus to regulate gene transcription. In vitro culture experiments found nuclear localized YAP/TAZ during SC spreading, plating on stiffer surfaces, plating on Laminin-211, and experimentally applied stretching (Fig. 1). Analysis of mouse mutants demonstrated that YAP/TAZ signaling is required for radial sorting and myelination (16). YAP also has a role in modulating internode length during development and disease (17). In concert with TEAD transcription factors, nuclear YAP activates genes involved in the myelination program, including *Krox-20/Egr2* and *Myelin associated glycoprotein (MAG)*, *Rab11*, and *Laminin Y1*. The polarity protein Crb3 inhibits YAP nuclear translocation and knock-down of *Crb3* increases the length of SC myelin segments (17). Crb3 is therefore thought to modulate YAP activity to temper internode length. Interestingly, a dystrophic mouse model of peripheral neuropathy exhibited reduced nuclear YAP with shorter internodes, a phenotype which could be rescued by manual sciatic nerve elongation via femoral distraction to increase nuclear YAP (17). These data suggest that migration of SCs along axons and/or longitudinal nerve growth could activate YAP/TAZ signaling during development. Perhaps physical maturation of the BL and GPR126 activation is similarly linked to developmental YAP/TAZ signaling, as GPCRs are known upstream regulators of this pathway (18). Downstream of YAP/TAZ signaling, TEAD1 directly regulates *Peripheral myelin protein 22 (Pmp22)*, mis-regulation of which causes Charcot-Marie-Tooth disease (19).
While a role for YAP/TAZ signaling in oligodendrocytes has not been described, these cells are also responsive to mechanical stimuli. OPC proliferation and migration can be altered by plating on substrates of varying stiffness (20), resulting in differentiation in a density-dependent manner. Plating at high density with polystyrene beads promoted OPC differentiation, demonstrating that this process is mediated by physical space limitations, rather than by extracellular signals (21). How might external forces drive oligodendrocyte development? A recent report demonstrates that mechanical stimuli interact with the nucleus via the Linker of Nucleoskeleton and Cytoskeleton complex (LINC). One LINC complex component in particular, SYNE1, which binds the nuclear envelope and actin, was shown to link extracellular stimuli, including high density plating with beads and mechanical force using a cell-compression device, to nuclear changes (22). The switch from primarily euchromatin to heterochromatin is a hallmark of differentiation in oligodendrocytes (23) and requires SYNE1 (22). Histone modifying complexes, specifically HDAC1 and HDAC2, affect nuclear reorganization by altering chromatin configuration and are essential for oligodendrocyte and SC differentiation. Epigenetic regulation of oligodendrocytes and SCs during development and myelination is reviewed in greater detail elsewhere (1, 24).

1.4 Cytoskeletal regulation of myelination

In a feat of cellular morphogenesis, glial cells massively upregulate production of their plasma membrane and spiral it around an axon segment. These dramatic shape changes require extensive cytoskeletal rearrangements, and great inroads have been made in understanding how such rearrangements drive myelin sheath formation. Using zebrafish in vivo imaging and 3D electron microscopic reconstruction, Snaidero and colleagues demonstrated that the plasma membrane inner tongue maintains contact with the axon segment as it wraps and progressively
spreads out to form the myelin internode. Initial inner tongue movement is aided by the transport of critical material, including mRNA and protein, through nanometer wide channels, which remain in the mature myelin sheath (24). How is the inner tongue propelled around the axon? Two elegant studies suggest actin dynamics as a driving force. Nawaz et al. used zebrafish live imaging to determine that F-actin is initially localized to the leading edge, but later excluded from the developing membrane. Culture experiments demonstrated that F-actin depolymerization by drug treatment increased cell spreading, leading to a model in which the force of actin filament disassembly propels the membrane forward (Fig. 2). Interestingly, Zuchero and colleagues found that actin disassembly is driven in part by competition of MBP protein for binding to PI(4,5)P2, which then releases the actin disassembly factors gelsolin and cofilin (Fig. 2). The dynamic interplay between actin assembly during development and disassembly during myelination highlights a potential form of temporal control. Since actin assembly is necessary for OPC development (25), the timing of disassembly must be tightly regulated. What factors could influence timing? One possibility is axonal activity. *In vitro*, vesicular glutamate release from axons in response to electrical stimulation phosphorylates Fyn kinase at the oligodendrocyte membrane, leading to local translation of *Mbp* (26). Together, these discoveries implicate axons in temporally influencing myelination via actin disassembly.

A role for actin dynamics has similarly been described in the PNS. Inhibition of F-actin formation resulted in delayed SC differentiation (27) and SC-specific deletion of neural Wiskott-Aldrich syndrome protein (N-WASp), a mechanical transducer that remolds actin via Arp2/3, inhibits myelination and causes motor deficits (28, 29). Unlike oligodendrocytes, SCs must sort axons prior to myelination. Radial sorting, like myelination, requires dramatic cell shape changes that are mediated by proteins regulating the cytoskeleton, including the Rho family GTPases
Rac1 and Cdc42 (30, 31). Although these studies point to the importance of cytoskeletal rearrangements in SC development, less is known about the forces driving myelination. Interestingly, both oligodendrocytes and SCs transport Mbp along microtubules to sites of membrane elaboration (32, 33). Whether actin disassembly and local translation of Mbp in SCs have roles in driving myelination remains to be determined.

1.5 Activity-dependent control of myelination and myelin maintenance

Oligodendrocytes have intrinsic myelinating capacity and can myelinate fixed axons in addition to synthetic nanofibers and micropillars (21, 34, 35). What prevents oligodendrocytes from myelinating dendrites or other cells in the CNS? Using a candidate approach, Redmond et al. identified the transmembrane protein JAM2 as a negative regulator of oligodendrocyte myelination (Fig. 2). Overexpression of JAM2 attenuated the ability of plated oligodendrocytes to myelinate micropillars, and loss of Jam2 in a mouse model caused an increase in myelinated neuronal cell bodies, implicating repulsive cues in modulating myelination (36). Another study indicates that a component of intrinsic myelination may be hardwired in oligodendrocytes. When plated on nanofibers, spinal cord oligodendrocytes produced more myelin than cortex-derived oligodendrocytes (37). Are these regional differences due to environmental cues or other factors? One possibility is that there are specific subtypes of oligodendrocytes with distinct myelinating capacities. To this end, single-cell RNA sequencing was used to characterize cell types in the murine hippocampus and cortex. Interestingly, findings from these experiments suggested 7 distinct subtypes of oligodendrocytes, including OPCs (38). Furthermore, a recent study using the same technique to probe oligodendrocyte heterogeneity in more detail proposed 13 distinct populations of oligodendrocytes in the mouse brain (39).
While negative regulators prevent aberrant myelination in the CNS, variation in myelin distribution along single axons of the developing cortex suggests a fine-tuning of myelination capacity beyond an intrinsic program (40). Indeed, early work implicated electrical signaling as an instructive cue in oligodendrocyte development and myelination (41, 42). How might activity influence myelination? Previous work demonstrated that neurons form functional synapses on OPCs (43). Recent research suggests, however, that while oligodendrocytes are more likely to myelinate electrically active axons, this occurs independently of synapse formation, instead relying on vesicular release of glutamate and ATP (44). A critical role for vesicle transport in myelination was confirmed in vivo using zebrafish. Mensch and colleagues used tetanus toxin to inhibit vesicular release, resulting in fewer sheaths, while increasing activity led to more sheaths per oligodendrocyte (45). In a complementary study, Hines et al. found that initial oligodendrocyte axon ensheathment is activity independent, but preferential contact is maintained on axons releasing vesicles. Processes are either retracted from inactive axons or produce shorter myelin sheaths (Fig. 2) (46). However, the necessity of vesicular release is differentially regulated in the CNS. This cue is required for myelination by reticulospinal neurons but not by commissural primary ascending (CoPA) neurons (47). Why there are different regulatory mechanisms depending on neuronal subtype is an area of future investigation.

Rather than a simple static insulator deposited during development, myelin is now recognized as a player in nervous system plasticity. Myelination during development and in adulthood is modulated by an animal’s social experience (48, 49) and myelin remodeling occurs throughout life (50). Furthermore, learning new skills, such as juggling and language acquisition, results in changes to myelin (51, 52). How do myelin alterations occur and how do they affect
nervous system plasticity? One possibility is that activity stimulates formation of new oligodendrocytes. To this end, it was shown that differentiation of oligodendrocytes from precursors is necessary for mice to learn a new skill effectively (53), and that neuronal activity promotes oligodendrogenesis and concomitant behavior changes (54). What is the role of new oligodendrocytes? A recent paper examined the timing of oligodendrogenesis in response to learning and found significant formation of new oligodendrocytes in mice learning to navigate a complex wheel within the first 2.5 hours. Furthermore, mice unable to form new oligodendrocytes exhibit learning deficits as early as 2-3 hours after first encountering the wheel. This early necessity for new oligodendrocytes in the learning process indicates a level of active involvement (55). Whether this occurs through modifying circuits, providing metabolic support or an as yet undetermined mechanism is an area of future investigation.

1.6 Myelin and metabolism

In addition to promoting efficient action potential propagation, myelin is also critical for trophic and metabolic support of axons (56). To provide metabolites to axons accurately, glia must “know” the metabolic requirements of axons. Could electrical activity by axons function as a means of communication? NMDA glutamate receptors are present on oligodendrocytes (57, 58), but were thought to be dispensable for oligodendrocyte development, myelination, and injury response (59, 60). However, recent work has implicated these receptors in mediating calcium influxes in mature oligodendrocytes (61). Furthermore, NMDA receptors have been shown to link electrical activity in axons to the production of lactate by oligodendrocytes, a critical energy source for axons. By “learning” via NMDA receptor signaling which axons are fast spiking, oligodendrocytes are able to vary lactate production. Loss of NMDA receptors specifically in oligodendrocytes, while not critical during development, causes eventual
neurodegeneration from reduced metabolism (62). Lactate production and metabolic support of axons by SCs is also critical in the PNS (63, 64). The lactate transporter that is used by oligodendrocytes, MCT1, is present in SCs and mediates axonal health (65, 66). However, these studies did not address a role for electrical activity in SC regulation of axonal metabolism. Interestingly, a recent report found that ATP release by electrically active axons mediates mitochondrial signaling to promote energy production in SCs and disruption of this signaling pathway resulted in hypomyelination (67).

1.7 Conclusion and outlook

From static insulating factor to dynamic structure critical in enabling nervous system plasticity, our conceptions about myelin have changed dramatically in recent years. However, although both SCs and oligodendrocytes produce myelin, the mechanisms by which they do so are distinct (Fig. 3). Oligodendrocytes possess an intrinsic ability to myelinate that is fine-tuned by environmental cues, such as mechanical stimulation and electrical activity from axons. New studies suggest the existence of distinct subsets of oligodendrocytes, raising the possibility that such heterogeneity could contribute to differences in innate myelination and re-myelination abilities. It will be exciting to uncover the extent to which interplay between the extracellular environment and oligodendrocyte heterogeneity influences myelination during development and repair. Advances in cellular techniques, including 3D electron microscopic reconstructions and live imaging, have contributed to a better understanding of the physical process of myelination by oligodendrocytes, including a surprising role for actin dynamics. Further research into the cytoskeletal and architectural reorganization of membrane during myelination will help us better understand this feat of morphogenesis and elucidate how to promote re-myelination in disease or injury.
SCs are incapable of myelinating inert structures (37), relying instead on instructive cues. PNS myelination also appears to be less finely tuned compared to the CNS, with stricter correlations between axon diameter and myelin thickness. Whether PNS myelin undergoes dynamic changes similar to CNS myelin has not been well studied. While early work demonstrated a role for axonal activity in modulating SC development and myelination (68), this area of research has lagged behind progress made in the CNS. The mechanisms by which SCs elaborate a myelin sheath are similarly mysterious. One pertinent question is whether actin dynamics, which are vital during CNS myelination, play an analogous role in SCs. A current focus in SCs is on mechanotransduction, and advances in this area are already guiding future therapeutic developments through techniques such as optimal matrices for acellular nerve allografts (69).

In summary, the studies highlighted in this review demonstrate that myelination in the CNS and PNS is distinct while sharing some similar processes. Future work would benefit from comparing and contrasting these systems to clarify common or unique aspects of development and myelination. Therapeutic advances will be realized through continued investigation into the mechanisms and controls of myelination from genesis through maturity.

1.8 Acknowledgements

We thank members of the Monk lab for helpful discussions and acknowledge Matt McCoy for help with figures and editing the text. We apologize to our colleagues whose primary work we were unable to cite due to space limitations. A.L.H. is supported by the Philip and Sima Needleman Foundation and by F31 NS096814. Work in the Monk lab is supported by grants from the National Institutes of Health (R01 NS079445, R01 HD80601), the Muscular Dystrophy Association (MDA 293295), the Missouri Spinal Cord Injury/Disease Research Program (16-03), and K.R.M. is a Harry Weaver Neuroscience Scholar of the National Multiple Sclerosis Society.
1.9 Figures

**Figure 1:** Mechanotransduction plays a critical role in Schwann cell development and differentiation.
Immature SCs migrate and divide along growing axons. The forces associated with migration are thought to activate the mechanotransducers YAP/TAZ in SC cytoplasm, which then translocate to the nucleus where they interact with the TEAD family transcription factors to drive expression of important myelin genes (a). After SCs have formed a “1:1” relationship with axons in the pre-myelinating stage, maturation of the basal lamina and subsequent polymerization of Laminin-211 is thought to activate GPR126, which initiates a transcriptional cascade activating Oct6 and promoting myelination (b). Eventually, SCs wrap myelin around axon segments to form internodes (c).
Figure 2: Multiple factors fine tune the myelination potential of oligodendrocytes.

Oligodendrocytes preferentially myelinate electrically active axons (a) and retract processes from inactive axons (b). Furthermore, the intrinsic myelination program is moderated by negative regulators, such as JAM2, which are expressed on dendrites (c). Vesicular release from active axons initiates a cascade of events, one of which is the translation of locally transported Mbp mRNA. MBP then competes with the factors gelsolin and coflin for binding to PIP2 on the inner oligodendrocyte membrane, resulting in release of the two proteins and subsequent actin disassembly (d). During wrapping, filamentous actin is located at the leading edge of the inner tongue and is proposed to propel the membrane forward by actin disassembly (e) (image adapted from Nawaz et al. 2015).
Figure 3: Comparing and contrasting Schwann cell and oligodendrocyte development and differentiation.

Although both SCs and oligodendrocytes produce the myelin critical for nervous system function, there are important differences in the mechanisms by which they generate myelin. The similarities and differences between SCs and oligodendrocytes discussed in this review are summarized in the table above.
1.10 References


33. Trapp BD, Moench T, Pulley M, Barbosa E, Tennekoon G, Griffin J: **Spatial segregation of mRNA encoding myelin-specific proteins.** 1987, **84**:7773–7777.


Chapter 2: A forward genetic screen in zebrafish to uncover new regulators of myelinated axon development
2.1 An introduction to zebrafish screens

Forward genetic screens in model organisms have made seminal contributions to the field of developmental biology. The ability to discover new genes involved in regulating developmental processes led to the assembly of gene networks and is responsible for our current understanding of numerous developmental processes. Early work in *C. elegans* and *Drosophila* established the importance of forward genetic screens (1, 2). In order to study development in a genetically tractable vertebrate system, George Streisinger established the zebrafish as a model organism in the 1970s. The rapid generation time, transparent embryonic and larval stages, and large clutch size of zebrafish led to their quick adoption as a vertebrate model of choice, and to the establishment of forward genetic screens as an important tool (3). Classic studies demonstrated that the mutagen used in forward genetic screens in other organisms, Ethyl methanesulfonate (EMS), was less efficient than *N*-Ethyl-*N*-Nitrosurea (ENU) in introducing point mutations in the zebrafish germline (4, 5). In 1996, two groups published seminal work describing the first large-scale forward genetic screens performed in zebrafish (6, 7). These screens identified a host of new mutant zebrafish with defects in varied processes including gastrulation, notochord development, retinal development, craniofacial development, and ear development, among others (8-11).

As the cost of sequencing technology has decreased, it has become increasingly feasible to determine the genes responsible for mutant phenotypes extracted from forward genetic screens. Moreover, the sequencing of the human and zebrafish genomes has rendered it possible to perform comparative analyses between the genes identified in zebrafish screens to orthologues in humans. In the field of myelin biology, rodents were initially the model of choice. An important study identifying zebrafish orthologues of key myelin proteins, including Myelin
protein zero (P0), Proteolipid protein (Plp), and Myelin basic protein (Mbp) set the stage for zebrafish to be used as genetic models in investigating myelin development (12). Although there are some crucial differences between mammalian and zebrafish myelination, further studies have solidified numerous similarities, establishing zebrafish as a model organism at the forefront of myelin research (13, 14). To this end, several important myelin screens have been performed in zebrafish over the years, yielding critical insights into the regulation of myelin in both the central nervous system (CNS) and peripheral nervous system (PNS).

The discovery of myelin protein orthologues in zebrafish, Mbp in particular, was instrumental in facilitating myelin research. Mbp is a small, basic protein (as its name suggests), and is critical for compaction of the myelin sheath. Mbp is transported from the cell body to the periphery as mRNA, and recent studies have focused on additional roles for Mbp other than compaction, including initiation of myelination in oligodendrocytes (15-18). In zebrafish, mbp transcripts are initially distinguished beginning at 2 days post fertilization (dpf) in the CNS and the PNS and are robustly expressed by 4 dpf. The ability to visualize myelinated axons using riboprobes against mbp was particularly useful for forward genetic screens.

In 2006, two studies published the results of forward genetic myelin screens looking for disruptions to mbp mRNA that together uncovered 17 myelin mutants (19, 20). Mapping of the mutations identified important new regulators of myelination including Klf1b, Nsf, and Kbp (21-23). Of particular note was the discovery that two myelin mutants identified from the Talbot lab were the result of mutations in a G protein-coupled receptor (GPCR), Gpr126, a member of the understudied class of adhesion GPCRs (24). The necessity of cAMP signaling during Schwann cell development to facilitate the initiation of myelin production had been known for many years (25-27). However, it had been unclear what regulated the timing and production of cAMP. Monk
and colleagues demonstrated in zebrafish that signaling by Gpr126 was responsible for triggering upregulation of cAMP, which in turn initiated the transcription of a host of myelin genes necessary for myelination. Moreover, subsequent studies demonstrated that this critical role of Gpr126 was conserved in rodents and humans (28, 29). Since GPCRs are recognized as “druggable targets,” research is ongoing in zebrafish to discover small molecules or drugs that can modulate Gpr126 activity, with the potential for identifying treatments for human PNS myelin disorders (30, 31). From gene discovery to drug screens, this example highlights the efficacy of zebrafish as a tractable genetic model organism capable of yielding insights into not only development, but also human disease.

More recently, transgenes have been used in zebrafish myelin forward genetic screens. In a screen to identify regulators of oligodendrocyte development, the Appel lab used the transgene \( olig2:EGFP \) (\( tg:olig2:EGFP \)) as a marker of oligodendrocyte precursor cells (OPCs) (32). Transgenic approaches circumvent the need to fix zebrafish larvae during the \textit{in situ} hybridization process, allowing researchers to observe phenotypes in live animals. This is particularly important today when traditional recombination mapping has been replaced by next generation sequencing to determine the lesion responsible for the mutant phenotype. In the \( tg(olig2:EGFP) \) screen, several mutated genes were uncovered, including in the ubiquitin ligase \( fbxw7 \), in cytoplasmic dynein, and in the enzyme \( hmgcs1 \), which catalyzes cholesterol biosynthesis (32-34). Transgenes therefore present a viable option for forward genetic screens in zebrafish and should be used to their full advantage in the future. Importantly, the myelin screens described above were not performed to saturation and there were likely new genes involved in myelination to be uncovered. The Monk lab therefore decided to embark on a forward genetic screen to identify remaining myelin mutants that had not been found previously.
2.2 Set-up of the “LM” screen

In collaboration with the Solnica-Krezel lab, members of the Monk lab participated in a large-scale forward genetic screen in zebrafish. The purpose of the LM screen, which stands for the two transgenes used (see below), was to identify mutants in both gastrulation and myelination. Before I joined the Monk lab, the screen was initiated with ENU mutagenesis of two founding groups of males, LM1 and LM2, according to standard protocols with the addition of tricaine to the water (35). 40 males in the LM1 group were exposed to 3 mM ENU for one hour every week during a 6-week period, while 40 males in the LM2 group were exposed to a 3.5 mM dose for 4 weeks. WT males from the genetic strain SAT, which is a combination of the Sanger AB and Tubingen lines, were used. A specific locus test was utilized to determine the rate of mutagenesis in the founder males. Mutagenized F0 founder males from both LM1 and LM2 groups were outcrossed to females of homozygous albino or homozygous albino/golden backgrounds, both of which lack pigmentation. F1 progeny from these crosses were screened for pigment defects by members of the Monk and Solnica-Krezel labs at 3 dpf to determine the mutation rate in the F0 mutagenized males (35).

F0 mutagenized males were then outcrossed to WT SAT females to produce the F1 progeny, approximately half of which should have heterozygous mutations in unknown genes. F1 animals were grown to adulthood and then crossed to a double transgenic line to produce the F2 generation. The double fluorescent reporter line included tg(lhx1a:egfp), which was used by the Solnica-Krezel lab to screen for gastrulation defects, and tg(mbp:mcherry-CAAX), which was used by the Monk lab to screen for myelin mutants. I became involved with the screen after the F2 progeny had reached sexual maturity. During the summer of 2013, we set up two cohorts of intercrosses per week, using F2s that had originated from both LM1 and LM2 males. Because of
the screen set up, half of the F2 generation should be heterozygous, which allowed us to screen for recessive mutations in the F3 generation. We set up 10 pairs per family and screened between 20-40 F3 larvae per clutch under the fluorescent microscope for perturbations to myelin as visualized using \textit{tg(mbp:mcherry-CAAX)} (Figure 1).

Unfortunately, weakness of the mCherry fluorescent reporter and differences in transgene copy number resulted in difficulty screening by this transgene. To confirm mutants identified by transgene, we therefore saved larvae from screened clutches for re-screening using \textit{mbp in situ} hybridization. Eventually, it became clear that screening by transgene was not efficacious, and we switched to a purely \textit{in situ} hybridization screen. Any F2 pairs identified as producing larvae with mutant phenotypes were re-crossed and re-screened using \textit{in situ} hybridization. Furthermore, the F3 progeny from these crosses were themselves grown up and then crossed and re-screened by ISH to confirm heritability of the mutation. After passing these rigorous tests, mutants were considered to be “real” and given allele designations with the prefix \textit{stl}, which stands for “St. Louis.”

In total, Monk lab members, including myself, screened approximately 4,500 F3 clutches from 1,102 F2 families. We identified 31 mutations in total. Of these mutants, 14 were shown to be heritable while 5 did not re-screen and were subsequently discarded. We eventually stopped re-screening the F4 generation for heritability, and therefore 12 mutants are currently awaiting re-screening.
2.3 Whole genome sequencing identifies the causative lesions responsible for mutant phenotypes

The myelin mutants identified in the LM screen exhibited a variety of phenotypes (Figure 2). Although there was only one mutant identified with increased mbp expression, stl64, there were many mutants exhibiting reduced expression in either the PNS, such as stl72, stl91 and stl83, or in the CNS, such as stl90 and stl93 (Figure 2). Transitioning to the in situ hybridization screen was also effective for identifying more PNS mutants, as the lateral line had been particularly difficult to score using the transgene. The tg(lhx1a:eGFP) also allowed us to visualize axon defects, as lhx1a is expressed in a subset of neurons. This led to the identification of the stl83 mutant, which exhibited large caliber axon swellings in the CNS and the PNS (Figure 2). Mbp in situ hybridization of the stl83 mutant showed reduced expression, indicative of potential myelin defects the CNS and PNS, as well as a punctate expression of mbp in the hindbrain of the CNS (Figure 2).

The ability to identify stl83 mutants via the tg(lhx1a:eGFP) transgene meant that DNA could be collected from fresh tissue. This was advantageous for mapping purposes because DNA from fresh tissue is more amenable to sequencing than crosslinked DNA from tissue that had been fixed during the in situ hybridization process. Therefore, stl83 was the first mutant of the LM screen to be sequenced. In order to sequence the stl83 genomoe, we performed a map cross by crossing the F2 carriers that had produced mutant progeny to SJD, an isogenic strain of zebrafish with well characterized markers used for traditional recombination mapping (36). After reaching adulthood, F3 progeny from the SJD outcross were intercrossed and screened for the stl83 phenotype by transgene. The mutation was successfully passed on to the next generation.
From this intercross, I anaesthetized progeny in tricaine and screened for the mutant phenotype using the transgene \textit{tg(lhx1a:eGFP)}. 20 phenotypically WT larvae and 20 phenotypically mutant larvae were pooled separately in 1.7 ml Eppendorf tubes. 500 µL of Lysis buffer, composed of 100 µM Tris-HCL (pH 8.5), 5 mM EDTA, (pH 8.0), 0.2% SDS, 200 mM NaCL, was added to each tube, and tubes were then heated at 98°C for 10 minutes. Next, 5 µL of 20 mg/mL proteinase K was added and both tubes were incubated overnight at 55°C. The next day, samples were centrifuged at 14,000 x g for 1 minute. The supernatant was pulled off and placed in a new 1.7 mL Eppendorf tube. 600 µL of isopropanol was added to each tube, mixed by inversion, and allowed to sit for 5 minutes at room temperature. Next, samples were centrifuged at 14,000 x g for 5 minutes and supernatant was removed, leaving a pellet containing the DNA. Pellets were washed with 500 µL of 70% Ethanol (EtOH), allowed to sit for 5 minutes at room temperature, and then centrifuged at 14000 x g for 2 minutes. 70% EtOH was removed, the sample was briefly spun again, EtOH again removed, and the sample was allowed to air dry. 100 µL H2O was added to both samples and they were then incubated for 1 hour at 60°C, vortexing continuously to facilitate resuspension. Finally, the DNA concentration in the samples was obtained using a nanodrop.

1 µg in 50 µL of extracted DNA from mutant and WT samples was sent to the Genome Technology Access Center (GTAC) at Washington University. 100 bp paired end sequencing using the Hi-Seq Illumina platform was used to sequence samples. Upon receiving the sequencing results from GTAC, I worked with Ryan Gray and Thomas O’Reilly-Pol, in the labs of Lila Solnica-Krezel and Steve Johnson, respectively, to determine the gene responsible for the mutant phenotype. The pipeline established to identify the \textit{stl83} mutant was later refined and published in a research article from our lab, which can be found in Appendix A of this
dissertation (37). In brief, a Perl script was used to first split the sequencing data into 25 separate files for each of the zebrafish 25 chromosomes. There were 50 individual files, 25 from the mutant sequencing and 25 from the WT sequencing. Another Perl script was then used to compute the mutant allele frequency (MAF). By comparing the MAF between siblings and mutants, we were able to identify regions of homozygosity where the MAF approached 2. It was found that the highest peak from the stl83 sequencing was on chromosome 20 (see Chapter 3).

A subsequent SNP subtraction analysis was performed on the stl83 data using sequencing data from the Solnica-Krezel lab. This narrowed the number of candidate SNPs down to 5, including actin related protein 10 (actr10). Importantly, none of the identified SNPs was a STOP; rather, all were candidate missense mutations. I then turned to a modified traditional recombination mapping approach. I designed genotyping assays that involved PCR and digest for several of the SNPs. The SNPs themselves disrupted specific restriction enzyme sites. In this way, I was able to narrow down the potential mutant SNPs. As I was using this method of mapping, we learned of a mutant identified in another screen for axonal defects that exhibited a similar phenotype to stl83 (Figure 4).

The nl15 mutation was identified at Oregon Health and Science University (OHSU) in a forward genetic screen for regulators of axon development as assayed by transgene expression of a pan neuronal promoter, neurod, driving GFP expression tg(neurod:egfp). The nl15 mutation had been mapped by a post-doctoral fellow in the Nechiporuk lab, Katie Drerup, to the gene actin related protein 10 (actr10), which is a component of the dynactin complex that facilitates retrograde transport by the molecular motor dynein (38). After receiving actr10 nl15 fish from OHSU, I performed a complementation test by crossing actr10 nl15/+ fish to stl83 carriers. In a subset of the progeny, I observed a recapitulation of the mutant phenotype with axonal swellings
in the PNS illuminated by the \textit{tg(neurod:egfp)} transgene (Figure 4). This failure to complement confirmed that the \textit{stl83} mutation was indeed the result of a mutation in \textit{actr10}. A description of the published work done on the \textit{actr10} mutants can be found in Chapter 3 and Appendix B of this dissertation.

I used the analysis protocol to map another mutant identified in the screen, \textit{stl91}. Establishing the gene responsible for the \textit{stl83} mutation had been facilitated by the fact that I was able to collect DNA from fresh tissue. However, this was not the case for the rest of the myelin mutants identified in the LM screen. Therefore, we established a modified extraction protocol to collect DNA from larvae that had been fixed during the \textit{in situ} hybridization process. This protocol is detailed in Appendix A of this dissertation. I used this extraction protocol to prepare genomic DNA of several other mutants for sequencing, including \textit{stl91}, a strong PNS hypomyelination mutant, \textit{stl159}, which exhibited axon pathfinding defects, and \textit{stl72}, a weak PNS hypomyelination mutant (Figure 2). I used the sequencing analysis pipeline to determine that the likely candidate gene for the \textit{stl91} phenotype was a missense mutation in the gene \textit{exostosin1c} (\textit{ext1c}).

\textit{Ext1c} encodes a member of the Exostosin1 protein family that has been expanded in zebrafish. While there is only one \textit{Ext1} gene in \textit{Xenopus} and mammals, zebrafish possess \textit{ext1a}, \textit{ext1b}, and \textit{ext1c} proteins (39). Exostosin proteins are necessary for the production of heparin sulfate proteoglycans (HSPGs), which form part of the extracellular matrix (40). In humans, mutations of \textit{EXT} genes have been shown to cause benign tumors called hereditary multiple exostoses (41). Interestingly, a key difference between Schwann cells and oligodendrocytes is the presence of a basal lamina surrounding Schwann cells that plays critical roles in signaling during development and myelination (42). To this end, our lab showed that Laminin-211, part of
the Schwann cell extracellular matrix, was responsible for binding and signaling to Gpr126 (43). Moreover, HSPGs were shown to directly affect Schwann cell development via Neuregulin signaling (44). A role for ext1c in myelination of the PNS therefore would seem to fit with current work. However, when other members of the Monk lab applied a new filter called SNPFisher to the sequencing data, which was not available when I originally mapped stl91, it was determined that the mutation of interest could reside in either ext1c or another protein, angpt1 (Nick Sanchez, personal communication). SNPFisher is a publicly available database that is comprised of all known zebrafish SNPs. Complementation testing with other alleles of ext1c and angpt1 will therefore be needed in the future to confirm the gene responsible for the stl91 phenotype.

In conclusion, the LM screen was highly successful, identifying numerous myelin mutants. Two publications have already resulted from work related to the screen, and more manuscripts are currently in preparation. Although zebrafish screens have become quite common, particularly in recent years, it is clear that there is still much to be learned from a forward genetics approach to discovering new genes involved in developmental processes.
2.4 Figures

Figure 1: A forward genetic screen in zebrafish to identify regulators of myelination.
80 F0 males from the SAT background were mutagenized using ENU. F0 males were then outcrossed to SAT females to produce F1 progeny, which in theory should be heterozygous for potential mutations of interest. After growing to adulthood, F1 animals were crossed to a homozygous transgenic line with the fluorescent reporters $tg(lhx1a:eGFP)$ and $tg(mbp:mCherry-CAAX)$, which were used to screen for gastrulation defects and myelin defects, respectively. The progeny from this cross, the F2 generation, 50% of which should have the mutation of interest, were randomly intercrossed to produce the F3 generation, which were screened by transgene and in situ hybridization for alterations to mbp expression. Assuming two F2 siblings heterozygous for a mutation of interest were intercrossed, the genotypes of the F3 progeny should be as follows, according to Mendelian genetics: 25% WT, 50% heterozygous and 25% mutant for the mutation of interest. The transgene inheritance would follow a similar pattern: 25% of the larvae would have no transgene, 50% would be heterozygous for the transgenes and 25% would be homozygous for the transgenes.
Figure 2: The screen uncovered myelin mutants with a variety of phenotypes.

(A) The *tg(lhx1a:eGFP)* fluorescent reporter shows normal expression in a WT animal whereas in an *stl83* mutant (B), there are large caliber swellings in the spinal cord. (C) *Mbp* mRNA is normally expressed in the CNS (arrow) and PNS (arrowheads) of a WT zebrafish larva at 5 dpf. (D) The *stl64* mutant exhibits increased *mbp* in the CNS whereas the *stl90* mutant (E) has almost no *mbp* in the CNS (arrows). (F) *Mbp* ISH of the *stl83* mutant reveals decreased *mbp* in both the CNS (arrow) and PNS (arrowheads), as well as a punctate phenotype of *mbp* expression in the CNS. (G) The *stl93* mutant has reduced *mbp* in the CNS (arrow) but normal *mbp* in the PNS, whereas the *stl72* (H) and *stl9* (I) mutants have decreased and nearly absent *mbp* expression (respectively) in the PNS (arrowheads). (J). Finally, the *stl159* mutants likely have axon...
pathfinding defects, as demonstrated by the “wavy” *mbp* expression in the lateral line of the PNS (arrowheads).
Figure 3: Whole genome sequencing is used to identify the genes responsible for mutant phenotypes.

F2 stl83 carriers (which are genetically heterozygous for stl83) were outcrossed to the mapping strain SJD. The resulting F3 progeny, 50% of which should be heterozygous for the stl83 mutant allele, were randomly intercrossed. The F4 larvae were screened by tg(lhx1a:eGFP) for axon defects. DNA from 20 phenotypically WT and 20 phenotypically mutant animals was collected separately and sent for sequencing at the Genome Technology Access Center.
Figure 4: A complementation test confirms that actr10 is the gene responsible for the stl83 mutant phenotype.

Heterozygous nl15 animals were crossed to heterozygous stl83 animals. In the transheterozygous progeny, there was a recapitulation of the mutant axonal phenotype as visualized using the tg(neurod:egfp) fluorescent reporter.
2.5 References


32. Snyder JL, Kearns CA, Appel B: **Fbxw7 regulates Notch to control specification of neural precursors for oligodendrocyte fate.** Neural Dev. 2012, **7.**


35. Solnica-Krezel L, Schier AF, Driever W: **Efficient recovery of ENU-induced mutations from the zebrafish germline.** 1994, **136:**1401-1420.


Chapter 3: Dynein/dynactin is necessary for anterograde transport of *Mbp* mRNA in oligodendrocytes and for myelination *in vivo*
Preface:

This chapter has been reproduced and adapted in its entirety from the following published manuscript:

3.1 Significance

Oligodendrocytes in the brain insulate neuronal axons in layers of fatty myelin to facilitate fast electrical signaling. Myelin basic protein (MBP), an important myelin component, is transported as mRNA away from the cell body before being translated into protein. In zebrafish, the anterograde motor kinesin transports mbp mRNA away from the cell body. We now identify myelination defects in zebrafish caused by a mutation in the retrograde motor complex dynein/dynactin, which normally transports cargos back toward the cell body. However, this mutant displays defects in anterograde mbp mRNA transport. We confirm in mammalian oligodendrocyte cultures that drug inhibition of dynein arrests transport in both directions and decreases MBP protein levels. Thus, dynein/dynactin is paradoxically required for anterograde mbp mRNA transport.

3.2 Abstract

Oligodendrocytes in the central nervous system produce myelin, a lipid-rich, multi-lamellar sheath that surrounds axons and promotes the rapid propagation of action potentials. A critical component of myelin is myelin basic protein (MBP), expression of which requires anterograde mRNA transport followed by local translation at the developing myelin sheath. Although the anterograde motor kinesin KIF1B is involved in mbp mRNA transport in zebrafish, it is not entirely clear how mbp transport is regulated. From a forward genetic screen for myelination defects in zebrafish, we identified a mutation in actr10, which encodes the Arp11 subunit of dynactin, a critical activator of the retrograde motor dynein. Both the actr10 mutation and pharmacological dynein inhibition in zebrafish result in failure to properly distribute mbp mRNA in oligodendrocytes, indicating a paradoxical role for the retrograde dynein/dynactin.
complex in anterograde mbp mRNA transport. To address the molecular mechanism underlying this observation, we biochemically isolated reporter-tagged Mbp mRNA granules from primary cultured mammalian oligodendrocytes to show that they indeed associate with the retrograde motor complex. Next, we used live-cell imaging to show that acute pharmacological dynein inhibition quickly arrests Mbp mRNA transport in both directions. Chronic pharmacological dynein inhibition also abrogates Mbp mRNA distribution and dramatically decreases MBP protein levels. Thus, these cell culture and whole animal studies demonstrate a novel role for the retrograde dynein/dynactin motor complex in anterograde mbp mRNA transport and myelination in vivo.

3.3 Introduction

In the central nervous system (CNS), specialized glial cells called oligodendrocytes wrap axons in many layers of plasma membrane to form the myelin sheath. Oligodendrocytes originate from neuroepithelial precursors that develop into oligodendrocyte precursor cells (OPCs), which are migratory and proliferative, extending numerous processes to sample the environment (1). OPCs differentiate into post-mitotic oligodendrocytes, which activate expression of mature myelin proteins and ensheathe multiple axon segments with loose membrane spirals that are eventually compacted to form a functional myelin sheath (2). Disruption of the myelin membrane can cause debilitating human conditions, including multiple sclerosis. However, while the clinical applications of myelin research are clear, molecular mechanisms regulating basic oligodendrocyte development are not well understood.

A critical protein generated during oligodendrocyte differentiation is myelin basic protein (MBP), which is essential for proper compaction of the myelin sheath. Due to its highly basic charge and propensity to promote membrane adherence, Mbp translation is tightly regulated
during oligodendrocyte development (3). *Mbp* mRNA is trafficked to the developing sheath and translated locally (4, 5). Translation at the membrane and formation of myelin sheaths is stimulated by Fyn kinase, which is phosphorylated in response to axonal electrical activity (6–8). In addition, MBP acts as an important spatial and temporal regulator of myelination, by triggering disassembly of the actin cytoskeleton to promote initiation of myelin membrane wrapping (9, 10).

Classic experiments in cultured oligodendrocytes demonstrated that *Mbp* mRNA trafficking in the anterograde direction (away from the cell body) relies on microtubules (11). By electron microscopy, these microtubules are uniformly oriented with polymerizing plus ends directed away from the cell body (12). Two types of motors move along microtubules: a vast family of kinesin motors majority of which move toward the plus end and a single cytoplasmic dynein motor that moves toward the minus end when bound to the dynactin activator complex (13). Previously, a genetic screen in zebrafish identified a kinesin *kif1b* mutant in which anterograde *mbp* mRNA transport is disrupted, resulting in mislocalization of *mbp* mRNA in oligodendrocyte cell bodies (14). Importantly, another zebrafish genetic screen for myelination defects uncovered a dynein mutation that results in decreased *mbp* mRNA levels in both the peripheral nervous system (PNS) and CNS, highlighting the necessity of molecular motors in myelination (15, 16).

Classic transport studies have demonstrated the interdependence of oppositely directed motors. Many vesicular cargos are simultaneously associated with both dynein and kinesin motors and inhibition or loss of either motor results in transport arrest in both anterograde and retrograde directions (17). This has been observed for organelles in squid axoplasm treated with anti-dynactin antibodies (16), for synaptic vesicles in axons of *Drosophila* with mutations in
kinesin, dynein, or dynactin (17) and for vesicles and lysosomes in mammalian axons in which subunits of kinesin and dynactin have been knocked down (20, 21). Though less is known about how opposing motors regulate mRNA transport, one early study in Drosophila S2 macrophage-like cells showed that dynein knockdown leads to arrested transport in both anterograde and retrograde directions of fluorescently tagged fragile X mental retardation protein (FMRP), which is an RNA-binding protein (22). However, a role for the interdependence of kinesin and dynein motors in mRNA transport has yet to be shown in glial cells.

Here, we demonstrate in vivo in zebrafish and in mammalian oligodendrocyte cultures that dynein/dynactin is required for anterograde mbp mRNA transport. In a myelination screen in zebrafish, we discovered a mutation in a subunit of the dynein activator, dynactin. This mutation in actr10, which encodes the Arp11 protein, results in dynein/dynactin loss of function phenotypes, such as photoreceptor loss and aberrant melanosome distribution in pigment cells. Mutants have OPC proliferation defects, fewer myelinated axons by transmission electron microscopy (TEM) and reduced levels of mbp mRNA, all of which phenocopy a previously published zebrafish dynein mutant (16). Interestingly, of the oligodendrocytes that are present in the mutants, we noticed a reduction in mbp mRNA localized to processes, which is similar to the previously published kinesin kif1b zebrafish mutant and therefore may be caused by mRNA transport defects (14). To test whether dynein/dynactin directly plays a role in mbp mRNA transport, we turned to primary rodent oligodendrocyte cultures. Co-immunoprecipitations demonstrated that Mbp mRNA granules indeed associate with dynactin and dynein. Furthermore, imaging of Mbp mRNA granules in primary oligodendrocytes revealed movement in the anterograde direction, as expected; unexpectedly, the granules also move transiently backwards in the retrograde direction. To test how dynein activity affects Mbp mRNA transport, we
pharmacologically inhibited dynein using ciliobrevin in both zebrafish larvae and cell culture. In zebrafish, ciliobrevin treatment reduced mbp mRNA localization in oligodendrocyte processes; in primary oligodendrocytes, this resulted in arrested transport in both anterograde and retrograde directions and also dramatically reduced MBP protein levels. Taken together, our cellular and in vivo data demonstrate a previously unappreciated role for dynein/dynactin in anterograde Mbp mRNA transport in developing oligodendrocytes.

3.4 Results

*actr10 mutations in zebrafish cause reduced mbp mRNA levels.*

In order to investigate the development of myelinated axons, we performed a large-scale N-ethyl-N-nitrosourea (ENU)-based forward genetic screen in which we visualized axons using the transgenic reporter *tg(lhx1a:GFP)* (23), then imaged myelin using *mbp in situ* hybridization (ISH). We uncovered a mutant, designated *stl83*, with both GFP-positive axonal swellings (Fig. 1A and B) and reduced levels of *mbp* by ISH (Fig. 1C and D). Whole genome sequencing of equimolar pools of mutants and siblings and subsequent analysis using an in-house pipeline (Sanchez et al., submitted) were used to determine that the gene of interest likely resided on chromosome 20 (Fig. 1E). Further single-nucleotide polymorphism (SNP) subtraction analysis to compare this data to other whole genome sequencing data sets reduced the number of candidate genes within our mapped locus to five, including one in *actr10* (Fig. 1F). To test if the *stl83* mutation was indeed in *actr10*, we performed a complementation test using a second *actr10* mutant allele (*actr10<sup>nl15</sup>*) (24). While the putative *actr10<sup>nl15</sup>* allele results from a G-to-T transversion in exon 12 resulting in a glycine to tryptophan amino acid change, the *actr10<sup>nl15</sup>* allele is a putative null mutation in the start site of *actr10* (Fig. 1G and Fig. S1A). Complementation analysis confirmed that the *stl83* phenotype results from a mutation in *actr10,*
as transheterozygous animals have axonal swellings, which are never seen in either heterozygous or wild-type (WT) animals from either genotype (Fig. 1H and I). Although the actr10<sup>al83</sup> mutation occurs late in the genomic sequence, both mutants are lethal at the larval stage. The actr10<sup>al83</sup> mutants are grossly healthier than the actr10<sup>nl15</sup> mutants (Fig. S1D), indicating that actr10<sup>al83</sup> may be a hypomorphic allele.

**actr10 mutants have fewer myelinated axons.**

To directly observe the ultrastructure of the myelin sheath, we performed TEM of both actr10 mutant alleles and counted the number of myelinated axons in a hemi-segment of the ventral spinal cord. Of note, heterozygous actr10<sup>al83/+</sup> and actr10<sup>nl15/+</sup> animals are viable and have no discernable phenotype compared to WT siblings as assessed by tg(neurod:egfp) from the complementation cross and number of myelinated axons by TEM (Figure S1E, F) and are therefore combined with WT as “controls.” Consistent with our mbp ISH analysis, both actr10 mutant alleles have significantly fewer myelinated axons compared to control animals by TEM at 5 days post-fertilization (dpf) (Fig. 2A–J), demonstrating that dynein/dynactin dysfunction has functional consequences for myelin in the CNS.

**actr10<sup>nl15/nl15</sup> mutants have fewer OPCs.**

One explanation for reduced myelinated axon numbers in actr10 mutants could be fewer OPCs. To test this, we examined spinal cord cross-sections from double transgenic tg(olig2:dsred);tg(sox10:mgfp) (25, 26) zebrafish to quantify OPC number in putative null actr10<sup>nl15/nl15</sup> mutants. olig2 is expressed in OPCs and motor neurons at 3 dpf, sox10 is expressed in OPCs, and the tg(olig2:dsred);tg(sox10:mgfp) transgenes combined allow for visualization of OPCs with red cytoplasm and green membrane. At 3 dpf, actr10<sup>nl15/nl15</sup> mutants had fewer OPCs compared to controls (Fig. 3A–C), indicating a role for actr10 in OPC development.
In the zebrafish spinal cord, OPCs originate ventrally and then migrate dorsally on either side of the neuronal cell bodies located in the center of the spinal cord (27). A population of OPCs remains in the ventral cord to differentiate and myelinate ventral axons, while the dorsally migrated OPCs differentiate and myelinate axons in the dorsal spinal cord. Dorsal OPCs can be visualized using the \textit{tg(olig2:dsred)} transgene, which are easily distinguished by their elongated cell bodies (27). Using this elongated cellular morphology as a metric, we found that the number of DsRed-positive elongated cells in the dorsal spinal cord was reduced at 3 dpf in \textit{actr10\textsuperscript{nl15/nl15}} mutants compared to controls (Fig. 3D, E, and H). To determine whether this resulted from a delay in migration and maturation, we similarly assayed 4 dpf animals and also found a significant reduction at this later time point (Fig. 3F, G, and I). Together, these data demonstrate that \textit{actr10\textsuperscript{nl15/nl15}} mutants have reduced numbers of OPCs and fewer dorsally migrated OPCs, recapitulating early defects described in zebrafish dynein mutants (16). Importantly, however, some maturing dorsal OPCs are present in \textit{actr10\textsuperscript{nl15/nl15}} mutants, indicating that migration defects alone are not the sole cause of the myelination phenotypes observed in mutants.

**OPC proliferation is slower in \textit{actr10\textsuperscript{nl15/nl15}} mutants.**

Interestingly, depletion of Arp11 in mammalian COS7 resulted in the striking formation of multiple spindles during mitosis (28). To better understand the mechanistic role of Arp11 in OPC proliferation and cell division, we performed live imaging using the transgenic line \textit{tg(nkx2.2a:megfp)}, which also marks OPCs (1). Transgenic zebrafish were imaged for up to 12 hours starting at ~57 hours post fertilization (hpf). During this time period in oligodendrocyte development, OPCs have been specified and continue to divide and migrate from the ventral to the dorsal spinal cord. They continuously extend and retract exploratory processes to sample the environment before becoming post-mitotic, and beginning to myelinate (1). Live imaging
revealed that mutant nkx2.2a-labeled cells took longer to divide compared to cells in control sibling actr10<sup>nl15/+</sup> animals (Fig. 4, Movies S1–S4). During division, the nkx2.2a-labeled cells retract processes, and become distinctly round before dividing. In the control animals, the time from beginning of rounding until division or the end of live imaging, takes an average of 46 minutes compared to 159 minutes in mutants, suggesting that reduced OPC numbers are in part due to slower OPC proliferation.

**Dynein activity is impaired in multiple tissues in actr10<sup>nl15/nl15</sup> mutants.**

Previous studies have found that loss of actr10 disrupts the assembly of the dynactin complex in mammalian cell culture and in the filamentous fungi *Aspergillus nidulans* (28, 29). Actr10 has recently been shown to have a specific role in retrograde mitochondrial transport in peripheral axons without disrupting dynein-dynactin complex integrity (24). We hypothesized, however, that oligodendrocyte cell lineage and myelin phenotypes in actr10<sup>nl15/nl15</sup> mutants result from general disruption to dynein/dynactin function for several reasons. Firstly, mutants have defects in OPC development and myelination that are similar to those previously defined in zebrafish dynein mutants (16). Secondly, mutants phenocopy the photoreceptor loss observed in zebrafish with mutations in other subunits of dynactin, including *p150<sup>Glued</sup>* and *p50* (30–32) (Fig. S2A and B). The photoreceptor defects observed indicated that the fish might be blind. To this end, we used the Noldus behavior unit, DanioVision, to assay the response of WT, actr10<sup>nl15/+</sup>, and actr10<sup>nl15/nl15</sup> animals during light/dark cycles. Larval zebrafish, unlike their adult counterparts, avoid the dark and respond with increased movement (33, 34). The movement of larvae in a 96-well plate was tracked during an alternating light/dark cycle. Both WT (Fig. S2G) and actr10<sup>nl15/+</sup> animals (Het) (Fig. S2H) showed a significant increase in movement in the dark compared to the light. However, actr10<sup>nl15/nl15</sup> animals (Mut) showed no significant difference in
movement between the light baseline and dark response (Fig. S2I), indicating that the observed photoreceptor death likely leads to blindness.

Thirdly, actr10<sup>nl15/nl15</sup> animals phenocopy the excessive pigment observed in the dorsal head region of zebrafish dynein mutants (Fig. S2C and D)(16) and also have increased pigment along the lateral line (Fig. S2E and F). Interestingly, blindness has been shown to cause background adaptation in which zebrafish upregulate pigment production in response to constant darkness (35). Thus, the increase in basal levels of pigmentation could be connected to photoreceptor loss. In addition, pigment cells, called melanophores, can change skin color in response to environmental stimuli. This is mediated by release of the hormone epinephrine, which triggers intracellular changes in protein kinase A activity that result in increased dynein-mediated retrograde run lengths, leading to melanosome aggregation toward the melanophore cell body (16, 36, 37). Thus, to further test whether dynein activation is perturbed in actr10<sup>nl15/nl15</sup> mutant melanophores, we performed a classic melanosome aggregation assay (Fig. S2J–S). In control animals, 5 minutes of exposure to epinephrine induced noticeable melanosome aggregation toward the center of the cell (Fig. S2K) whereas actr10<sup>nl15/nl15</sup> mutants maintained relatively dispersed melanosomes (Fig. S2P). After 30 minutes, whereas melanosomes in all control animals were aggregated (Fig. S2N), mutants continued to exhibit widespread pigment distribution (Fig. S2S), indicating that activated retrograde melanosome transport is compromised in mutant melanophores.

**Actr10 function in oligodendrocytes can partially suppress myelin defects.**

To understand the function of actr10 in oligodendrocytes, a construct in which the glial sox10 promoter drives expression of actr10 tagged with monomeric RFP (sox10:mRFP-actr10) was transiently expressed in larvae from a cross between tg(mbp:gfp-caax);actr10<sup>nl15/+</sup> and
actr10<sup>nl15/+</sup> fish. sox10:mRFP-actr10 injected larvae (sox10 injected) exhibit RFP fluorescence, which is not seen in uninjected mutants (Fig. 5A–B’’). TEM analysis and quantification at 5 dpf showed partial suppression of the mutant phenotype in the dorsal spinal cord. Although sox10:mRFP-actr10-injected mutants were not rescued to control levels, sox10:mRFP-actr10-injected mutants possessed significantly more myelinated axons compared to uninjected mutant siblings, demonstrating that glial actr10 rescue can promote proper myelination in vivo.

Analysis of a stable transgenic neuronal rescue line in which mRFP-actr10 is driven by the neuronal-specific neurod promoter tg(neurod:mRFP-actr10) demonstrates that Actr10 also functions in neurons to promote oligodendrocyte myelination (Fig. S3). Given the critical functions of dynein/dynactin in all cells, it is not surprising that Actr10 functions in both neurons and oligodendrocytes to promote myelination. Of note, injection of sox10:mRFP-actr10 resulted in lower levels of actr10 as assayed by RFP fluorescence than were seen in the tg(neurod:mRFP-actr10) stable line. Thus, one reason a neuronal rescue was more effective could be due to the differences between transient injection with weak oligodendrocyte actr10 expression and a stable transgenic line with strong actr10 expression. The function of Actr10 in neurons to promote myelination forms the basis of future work; here, we focus on the cell autonomous function of Actr10 in oligodendrocytes.
*actr10*\(^{nl15/nl15}\) mutants have reduced *mbp* mRNA localization in oligodendrocyte processes.

All *actr10*\(^{nl15/nl15}\) mutants observed have reduced *mbp* mRNA localization to processes at 4 dpf (Fig. 6A–F). This phenotype cannot be explained by OPC proliferation defects alone, as the observation of dorsal elongating cells in mutants (Fig. 3) indicates that some oligodendrocytes are indeed migrating and maturing. Moreover, this phenotype is reminiscent of the kinesin *kif1b* zebrafish mutant in which disruption of anterograde transport results in accumulation of *mbp* mRNA in oligodendrocyte cell bodies (14). This led us to hypothesize that dynein/dynactin may play a role in efficient transport of *mbp* mRNA to distal oligodendrocyte processes.

**Dynein and dynactin associate with *Mbp* mRNA granules.**

To visualize *Mbp* mRNA transport, we expressed the MS2 reporter in primary rat oligodendrocyte cultures that were purified using the immunopanning technique and are free of neuronal contamination (38). MS2 is an RNA-binding protein derived from bacteriophage that binds with high affinity to specific RNA sequences that form stem loop structures (39). Using a bidirectional construct, we co-expressed two transcripts: 1) *Mbp* containing 5’UTR, CDS and 3’UTR followed by MS2-binding stem loops and 2) a GFP-tagged MS2 reporter (Fig. 6G). In oligodendrocytes differentiated in culture for four days, MS2-GFP-positive puncta associated with *Mbp* mRNA are distributed throughout the many processes that emanate outward from the cell body (Fig. 6H), while in control cells not expressing stem loops, MS2-GFP is retained in the cell body (Fig. S4A).

We can use this system to biochemically validate the association of the dynein motor and its adaptor dynactin to *Mbp* mRNA granules. Indeed, immunoprecipitations using a GFP antibody to isolate MS2-GFP-tagged *Mbp* mRNA granules also pulls out the dynactin subunit
p150\textsuperscript{Glued}, dynein intermediate chain (DIC), and Arp11 (Fig. 6f). Eluates more robustly co-immunoprecipitated p150\textsuperscript{Glued} than DIC. Interestingly, two bands are visible using the p150\textsuperscript{Glued} antibody, which indicates that the lower band is p135, a shorter splice isoform of p150\textsuperscript{Glued} that does not contain the N-terminal microtubule-binding domain (40). This was confirmed by comparing the p135 band in this anti-GFP immunoprecipitation to another immunoprecipitation using an antibody against the N-terminus of p150\textsuperscript{Glued} that preferentially isolates full-length p150\textsuperscript{Glued}, but not p135 (Fig. S4B and C). To our knowledge, this is the first description of the p135 isoform in oligodendrocytes or associated with mRNA granules.

**Acute dynein inhibition arrests Mbp mRNA transport in both anterograde and retrograde directions.**

Live-cell imaging in this system using spinning-disk confocal microscopy reveals that processive Mbp mRNA transport occurs in both anterograde and retrograde directions with average speeds of approximately 0.21 µm/s and 0.16 µm/s, respectively (Fig. 7A and B), consistent with previously measured speeds for Mbp mRNA (4). To investigate the functional contribution of dynein in Mbp mRNA transport in oligodendrocytes, we pharmacologically inhibited dynein activity. The small molecule cell-permeable dynein inhibitor ciliobrevin D works by disrupting the ATPase activity of the dynein motor and has a half-minimum inhibitory concentration (IC\textsubscript{50}) ~5–15 µM in the cilia (41). Cells acutely treated with 15 µM ciliobrevin were imaged for 60-second durations for up to an hour with no observed morphology changes or toxicity. As early as 4 minutes after ciliobrevin treatment, Mbp mRNA net speed decreases in both anterograde and retrograde directions (Fig. 7A and B; N = 20 cells from 2 biological replicates). At around 10–15 minutes after ciliobrevin treatment, Mbp mRNA transport was arrested almost completely in both directions. When speeds are binned in 7-minute increments,
this decrease in speed is significantly different when compared to untreated cells (Fig. 7C and D).

Using faster frames rates to interrogate these possibilities, we saw as late as 11 minutes after ciliobrevin treatment Mbp mRNA that is engaged in bidirectional motility characterized by many frequent back-and-forth movements and directional switches (Fig. 7A, middle panel). After ciliobrevin treatment, we also observe Mbp mRNA granules with zero net motility that are engaged in very rapid and frequent directional switches for durations of up to one minute (Fig. S4D). This may represent a tug-of-war state during which anterograde and retrograde motor forces are roughly balanced and suggests that a single Mbp mRNA granule can associate simultaneously with both anterograde kinesin motors as well as retrograde dynein motors (Fig. 7E).

To confirm our findings in vivo, we treated WT zebrafish with ciliobrevin D for 21 hours starting ~3.5 dpf. Importantly, treating at this time point permits normal OPC development so that we can specifically examine the role of dynein in mbp mRNA transport. Although there was 15% mortality overall, importantly, the ciliobrevin-treated fish that were fixed at 4.5 dpf appeared grossly normal compared to vehicle-treated fish (Fig. S5A and B). ISH for mbp mRNA revealed that ciliobrevin-treated fish had a significant reduction in mbp mRNA localized to oligodendrocyte processes compared to vehicle-treated controls (Fig. 7 F–J). Taken together, our in vitro and in vivo results show that dynein inhibition leads to reduced mbp mRNA distribution.

**Chronic dynein inhibition disrupts MBP protein translation in cultured oligodendrocytes and disrupts myelination in zebrafish.**

In order to understand how Mbp mRNA transport inhibition can lead to myelination defects, we asked whether Mbp mRNA transport is necessary for local MBP protein translation.
We hypothesized that proper translocation of *Mbp* mRNA granules precedes local translation and recruitment of ribosomes (Fig. 8A). To test this, we treated cultured rat oligodendrocytes for 21 hours with the dynein inhibitor ciliobrevin, then visualized *Mbp* mRNA localization using single molecule FISH (fluorescent ISH) and simultaneously immunostained for MBP protein. Previously, oligodendrocytes were treated acutely with 15 µM ciliobrevin for less than 1 hour for live-cell imaging. However, chronic treatment for 21 hours at this concentration resulted in some toxicity when compared to control and vehicle treated conditions (Fig. S5C). Thus, cells that had been differentiated for 3 days *in vitro* were treated for 21 hours at the non-toxic concentration of 5 µM ciliobrevin (Fig. S5C), which had been previously used for overnight dynein inhibition in neurons (4).

Ciliobrevin-treated cells had remarkably decreased *Mbp* mRNA distribution and less MBP protein (Fig. 8B and C). Whereas *Mbp* mRNA granules in control cells were dispersed throughout the oligodendrocyte processes as distally as the cell periphery, *Mbp* mRNA granules in ciliobrevin-treated cells were restricted to the cell body and to proximal processes. Consistent with live-cell imaging results from acute ciliobrevin treatment, this chronic 21-hour treatment also demonstrates that dynein inhibition disrupts distal *Mbp* mRNA localization. Quantification of MBP protein staining revealed that only 42% of ciliobrevin-treated cells contained MBP protein, compared to 63% and 62% of control and vehicle-treated cells, respectively. These MBP-containing cells can be further binned as low expressers that have punctate MBP protein localization and high expressers that display pancake-like widespread MBP protein distribution. Binning demonstrates that while 29% and 27% of control and vehicle-treated cells are high expressers, only 8.5% of ciliobrevin-treated cells are high expressers. These data indicate that dynein inhibition leads to inefficient MBP translation.
3.5 Discussion

Myelination by oligodendrocytes is critical for proper function of the nervous system. In a forward genetic screen in zebrafish, we identified the dynactin subunit Actr10 as a new regulator of myelination. actr10 mutants are lethal and display signs of impeded dynein loss of function in some tissues, including blindness due to photoreceptor loss and aberrant pigment cells with defects in melanosome motility. In the nervous system, mutants have fewer myelinated axons by TEM and fewer OPCs in part due to proliferation defects, phenocopying a previously described dynein zebrafish mutant (16). In addition, mutants exhibit less mbp mRNA in oligodendrocyte processes, similar to previous observations made in kinesin kif1b zebrafish mutants (14). Thus, we propose that proper function of the retrograde motor dynein and its adaptor dynactin is paradoxically required for anterograde transport of mbp mRNA. Indeed, we demonstrate in purified rodent oligodendrocyte cultures that acute pharmacological inhibition of dynein activity arrests Mbp mRNA transport in both anterograde and retrograde directions. Moreover, ciliobrevin treatment also decreases MBP protein levels, suggesting that mRNA transport is necessary for local translation of MBP. We confirmed these results in vivo in ciliobrevin-treated zebrafish, in which mbp mRNA distribution is perturbed in oligodendrocytes.

The combination of our in vivo and cellular data suggests that dynein/dynactin activity is necessary for anterograde Mbp mRNA transport. In zebrafish, both Actr10/Arp11 loss of function and chronic dynein inhibition prevent proper anterograde distribution of mbp mRNA in oligodendrocytes. In cultured oligodendrocytes, acute dynein inhibition disrupts Mbp mRNA transport in both anterograde and retrograde directions. Our transport results are consistent with ciliobrevin treatment of cultured dorsal root ganglion neurons, which display arrest of mitochondrial and lysosomal axonal transport in both anterograde and retrograde directions (42,
43). Furthermore, our observations are consistent with earlier experiments in *Drosophila* macrophage-like S2 cells that demonstrate dynein knockdown leads to arrested transport in both anterograde and retrograde directions of fluorescently tagged RNA-binding protein FMRP (22).

Our results also demonstrate that mRNA granules are highly heterogeneous, both in motility and in composition. We observe *Mbp* mRNA granules that move quickly with average net speeds ~0.21 and 0.16 µm/s in anterograde and retrograde directions, respectively; we also observe *Mbp* mRNA granules that have very little net motility and move bidirectionally with frequent directional switches. Each bidirectional mRNA granule likely associates with multiple opposing motors that are engaged in a “tug-of-war” in which opposing motor forces are nearly balanced. Since one kinesin has 7 pN of unitary force while one dynein only has 1 pN of unitary force, this suggests that a bidirectional mRNA granule likely associates with at least 1 kinesin and 6–8 dyneins. Thus, our data support a model whereby each *Mbp* mRNA granule simultaneously associates with kinesin and dynein/dynactin and the full assembly of opposing motors may be required to sustain transport (Fig. 7E). Though it is unclear why both kinesins and dyneins are simultaneously associated with individual cargos, one possibility is that the ability to transiently step back and forth may allow more flexibility in circumnavigating roadblocks or switching microtubules (44). This may be particularly important in the crowded cytoskeletal environment of the oligodendrocyte, in which microtubules penetrate many concentric layers of the dense myelin sheath (45).

Moreover, association of different dynactin isoforms with mRNA granules may play a role in regulating *Mbp* mRNA transport as well. Co-immunoprecipitations revealed that *Mbp* mRNA granules not only associate with p150Glued, but also its shorter isoform p135, which is unable to bind to microtubules (40). Though a human neuronal cell line was used for the original
characterization of p135, we now show that p135 is also present in primary oligodendrocytes. Though it is still unclear what functional role p135 may play in transport regulation, a classic in vivo labeling study in the optic nerve demonstrated that p150Glued-associated cargos travelled much faster than p135-associated cargos, arriving past the optic chiasm at 2 days and 4 days after initial labeling, respectively (46).

These data highlight that there is much more to learn about the composition of vertebrate mRNA granules, including how many motors are associated and what adaptor proteins link these motors to mRNAs. In neuronal axons, the RNA-binding protein La is transported in the anterograde direction in its native form and in the retrograde direction in its sumoylated form; sumoylation is required for dynein association to La and may act as a mechanism to return mRNA-free La protein to the nucleus by decreasing its ability to bind to mRNA (47). Furthermore, transport of mRNA in neurons can be stimulated in response to electrical activity (48, 49) and in oligodendrocytes, activation of Fyn kinase has been shown to trigger MBP translation (7, 8, 50). Further research on regulation at the adaptor level may yield clues on how environmental stimuli can trigger Mbp mRNA transport in oligodendrocytes.

Together, our results suggest that actr10 mutants display phenotypes similar to dynein/dynactin loss of function in photoreceptors, melanophores, and oligodendrocytes. Interestingly, recently identified human patients with mutations in dynein and dynactin primarily present with sensory and motor neuron symptoms and are diagnosed with Charcot-Marie-Tooth distal neuropathy or spinal muscular atrophy (51–60). Thus, in both humans and zebrafish, the nervous system is exquisitely sensitive to mutations in dynein and dynactin. Though a link between myelination and dynein/dynactin mutations has not been established in humans, our
work in zebrafish and mammalian oligodendrocytes demonstrates that dynein/dynactin function in Mbp mRNA transport is critical for proper myelination in vivo.

3.6 Materials and methods

Zebrasfish lines and rearing conditions.

All zebrafish work was done in compliance with Washington University institutional animal protocols, and zebrafish were housed in the Washington University zebrafish facility (http://zebrafish.wustl.edu). Embryos were raised in incubators at 28.5°C in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄). ENU mutagenesis was performed according to standard protocols (61) and larvae were screened at 5 dpf for myelin abnormalities by two transgenes, tg(lhx1a:gfp) and tg(mbp:mCherry), as well as by in situ hybridization for mbp mRNA levels. The following transgenic and mutant zebrafish lines were used: SAT, SJD (62), actr10⁠¹⁰₈₃, actr10⁠¹⁰₁₅ (24), tg(lhx1a:gfp)(23), tg(mbp:mCherry)(a kind gift from Dave Lyons, University of Edinburgh), tg(neurod:egfp) (K.D. and A.V.N.), tg(olig2:dsred) (25), tg(sox10:mgfp) (26), tg(nkx2.2a:megpf) (1) and tg(mbp:gfp-caax) (63).

Whole genome sequencing and mapping.

The F2 generation of stl83 heterozygotes was outcrossed to SJD and raised. These animals were intercrossed and F4 progeny were screened for axonal swellings. DNA was extracted from phenotypically WT and mutant larvae at 5 dpf and sent to the Genome Technology Access Center (GTAC) at Washington University for whole genome sequencing. An in-house analysis pipeline (Sanchez et al. submitted) was used to determine that the mutant-to-WT allele ratio was highest on chromosome 20. A SNP subtraction analysis using other whole genome sequencing datasets narrowed the number of possible mutations down to five. The gene was confirmed by a complementation test using the actr10¹⁰₁₅ allele (24).
Genotyping.

A derived cleaved amplified polymorphic sequences (dCAPS) assay was developed to genotype the actr10<sup>at83</sup> allele (http://helix.wustl.edu/dcaps/dcaps.html). An A-to-C mismatch is introduced in the forward primer (forward primer: 5’-GTCAGAGAACTT GCTGATA<sub>TC</sub>TC-3’, reverse primer: 5’-AGCAGTTCA<sub>G</sub>GGCAGCTTTA-3’). This causes an AvaI restriction digest site in the WT haplotype and digestion generates two visible WT products of 22 and 224 base pairs (bps), and a mutant product of 244 bps. To genotype the actr10<sup>nl15</sup> allele, polymerase chain reaction (PCR) was used to amplify the first exon (forward primer: 5’-ACCCAGCCGTTCTCTCTAATG-3’, reverse primer: 5’-CCGCTCCTAATCAA TCACC-3’). Mutagenesis introduced a T-to-G mutation in the start site. This inserts an HaeIII site and digestion results in two WT fragments of 126 and 50 bps, and three mutant fragments of 112, 50 and 14 bps.

In situ hybridization (ISH).

In brief, embryos were treated with 0.003% phenylthiourea (PTU) at 24 hpf to prevent pigmentation. Embryos were reared in egg water with PTU until the desired age and fixed in 4% paraformaldehyde overnight at 4°C in 1.5 ml Eppendorf tubes with ~20 embryos per tube. A standard ISH protocol was then used (64) and all phenotypes were scored blindly.

Transmission electron microscopy.

Transmission electron microscopy (TEM) was done according to standard protocols (6). More detail can be found in Supplemental Information.

Marker analysis using transgenes.

Two transgenes, tg(olig2:dsred) and tg(sox10:mgfp), were crossed into an actr10<sup>nl15/+</sup> background and grown to adulthood. Animals were intercrossed to produce double transgene
labeling, screened for double fluorescence and fixed at 3 dpf for OPC cross-section analysis. Single transgene \textit{tg(olig2:dsred)} animals were used to count the number of dorsal, elongated \textit{olig2}-labeled cells at 3 and 4 dpf. For details, see SI Materials and Methods.

\textbf{Time-lapse imaging.}

\textit{tg(nkx2.2a:megfp);actr10^nl15/+} and \textit{actr10^nl15/+} animals were crossed and live imaging was performed for up to 12 hours beginning at 57 hpf on a Zeiss LSM 880 confocal microscope. For details, see SI Materials and Methods.

\textbf{Melanosome aggregation.}

Control and \textit{actr10^nl15/nl15} mutants were separated at 3 dpf by pigment phenotype. Larvae were treated with 0.5 mg/ml epinephrine (Sigma-Aldrich) dissolved in egg water. One representative animal from control and mutant groups was selected at random, treated with tricaine, mounted on an agarose mold, imaged and returned to the epinephrine treatment. This was repeated every approximately every 5 minutes, from 5–15 minutes, and again at 30 minutes. After 30 minutes, larvae were collected and genotyped.

\textbf{Noldus DanioVision assay.}

The Noldus DanioVision system was used to determine whether mutants were blind. Larvae were placed individually in a 96-well plate and the following protocol was used: light on (baseline) 0–4 minutes, light off (dark response) 4–8 minutes, light on (recovery) 8–12 minutes. The average distance moved from the center point during the baseline and dark response was calculated and analyzed for statistical significance. Two technical replicates were performed.

\textbf{Primary rat oligodendrocyte culture and electroporation.}

Oligodendrocyte precursor cells were purified from enzymatically dissociated P6–P8 Sprague-Dawley (Charles River) rat brain cortices by immunopanning and differentiated in serum-free
defined medium containing T3, as previously described (38). Cells were electroporated using Amaxa Nucleofector (Lonza, Program O-17 for rat oligodendrocytes) with a bicistronic construct (pBI, Clontech) simultaneously expressing MBP 5’UTR, CDS, 3’UTR, and MS2-binding stem loops (24x) as well as MS2-GFP(39, 65).

**Live-cell imaging and ciliobrevin D treatment.**

Live-cell imaging was performed at the Stanford Cell Sciences Imaging Facility on a Nikon spinning disk confocal (TiE inverted microscope body equipped with Perfect Focus mechanism, Yokogawa spinning disk) with an Andor EMCCD camera inside an environmental chamber (37°C, CO₂). Cells were treated with 15 µM ciliobrevin D (Calbiochem) and images were acquired at 1–3 frames per second for 60 seconds for a different cell at each time point to minimize photobleaching. Kymographs were generated from these movies using the Multiple Kymographs plug-in (FIJI, NIH) with a line width of 3 pixels. Net speed was calculated from the net duration and net distance of each motile event and represented as the average net speed for each cell. N = 2 biological replicates (2 OPC preps from 2 different animals), 10 imaging plates, 20 cells. WT zebrafish at 3.5 dpf were separated into 6-well plates with 20 larvae per well. Larvae were treated with either 2.5 µM ciliobrevin D (Calbiochem) or DMSO vehicle in egg water for 21 hours. Larvae were fixed in 4% paraformaldehyde for ISH after 21 hours and scored blindly for localization of mbp mRNA in oligodendrocyte processes.

**Co-immunoprecipitations.**

1–4 million primary oligodendrocytes per condition were lysed in HEM buffer (50mM HEPES, 1mM EDTA, 1mM MgCl₂) containing 25mM NaCl, 0.5% Triton X-100, and protease inhibitors (Sigma, P8340). Cell lysates were incubated with Protein-G Dynabeads (Invitrogen) and co-immunoprecipitations were performed following manufacturer’s instructions using a monoclonal
antibody against GFP (Clontech, JL-8). Western blots were probed with antibodies against DIC (Chemicon), p150\textsuperscript{Glued} (BD Transduction, 610474) and Actr10 (Proteintech, rabbit polyclonal, 20101-1-AP).

**Single Molecule FISH.**

Single Molecule FISH probes were designed against *Mbp* mRNA CDS (Stellaris, LGC Biosearch Technologies). Cells were stained according to manufacturer’s protocol. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized in 70% ethanol for 1 hour at 4°C, hybridized with smFISH probes and primary antibody against MBP (Abcam, ab7349) for 4–16 hours at 37°C, washed for 30 minutes at 37°C and then stained with secondary antibody.

**Statistical analysis.**

GraphPad Prism 7 and RStudio were used for statistical analysis. Unpaired t-tests with Welch’s correction were used for comparing all experiments with two variables. A one-way ANOVA was used to calculate the glial and neuronal TEM rescue experiments. Fisher’s exact test in R was used for calculating the ciliobrevin D experiments in zebrafish.

### 3.7 SI Materials and methods

**Transmission electron microscopy.**

Zebrafish larvae (5 dpf) were fixed in modified Karnovsky’s solution (2% glutaraldehyde, 4% paraformaldehyde and 0.1 M sodium cacodylate buffer, pH 7.4) using a Pelco BioWave Pro with Steady Temp Plus water recirculation system. To ensure accurate quantification, samples were cut between body segments 5–7. Preparation of tissue for analysis was performed as described (50). ~70-nm thin sections were cut using an ultramicrotome and mounted on copper mesh grids. Sections were stained using uranyl acetate and Sato’s lead stain. A Jeol JEM-1400 (Jeol USA) electron microscope was used to view samples and images were obtained using an AMT V601
digital camera. Images were analyzed using FIJI software (NIH) and all quantification was performed blinded to genotype.

**Spinal cord OPC quantifications**

tg(sox10:mgfp);actr10^{a115/+} animals were crossed to tg(olig2:dsred);actr10^{a115/+} animals. At 3 dpf, double transgenic larvae were identified on a Zeiss fluorescent stereoscope by screening for dual-color fluorescence. Larvae were fixed for 30 minutes at room temperature using 4% EM-grade paraformaldehyde diluted in 1x PBS. Larvae were then transferred to 30% sucrose for overnight immersion at 4°C. The next day, larvae were transected between segments 5–7. Heads were saved for genotyping and bodies were immersed and frozen in Tissue-Tek OCT compound using dry ice. Samples were sectioned at 10 µm on a cryostat and mounted on charged slides. 7 sections per animal were imaged at 20X on an inverted Zeiss LSM 880 confocal microscope and OPC numbers were counted and averaged. All work was performed blinded to genotype.

**Quantification of dorsal elongated OPCs.**

tg(olig2:dsred);actr10^{a115/+} animals were crossed to actr10^{a115/+} animals. At 3 and 4 dpf, larvae were screened for DsRed on a Zeiss fluorescent stereoscope. Larvae were then immobilized in tricaine and mounted on an agarose mold. The numbers of dorsally migrated olig2-labeled cells on either side of the spinal cord along the entire length of the animal were counted using a Zeiss fluorescent stereoscope. Quantifications were notated and animals were saved for DNA extraction.

**Time-lapse imaging.**

tg(nkx2.2a:megfp);actr10^{a115/+} animals were crossed to actr10^{a115/+} animals. Larvae were screened for fluorescence at 2 dpf. At ~55 hpf, mEGFP-positive embryos were immersed in 0.8% low melting point agarose and mounted on 22x22 mm² glass coverslips. Vacuum grease
was applied to a glass slide and egg water with 0.2% tricaine was used to fill the space. The coverslip containing mounted embryos was placed on top of the water to immerse embryos. At ~57 hpf, larvae were imaged on an inverted Zeiss LSM 880 confocal microscope at 20X for up to 12 hours and Z-stacks were collected every 10 minutes. Blinded analyses were performed using FIJI software (NIH). Cell division times were calculated blinded to genotype from TIFF images of each time point.

**Rescue experiments.**

The transgenic tg(neurod:mrfp-actr10) rescue line was generated by concurrent injection of 5kbneurod:mRFP-actr10 (12–25 pg) and transposase RNA (50–100 pg) into 1-cell stage embryos from an actr10<sup>nl15/+</sup> intercross. Larvae with green hearts and mRFP fluorescence were grown to adulthood and genotyped for actr10<sup>nl15/+</sup>. These animals were then crossed to actr10<sup>nl15/+</sup> animals and progeny were scored for germline transmission of 5kbneurod:mRFP-actr10. Animals were again grown, genotyped for actr10<sup>nl15/+</sup> and crossed to actr10<sup>nl15/+</sup> for experiments. To generate the sox10:mRFP-actr10 construct, primers were designed to amplify the sox10 promoter (66) and to add Fse1 and Asc1 restriction enzyme sites to the 5’ and 3’ ends, respectively (forward primer: 5’GACTACGGCCGGCCGGATCCCCTTATCAGAGTCAACATTCATGGATAATGAAAAAGGC-3’, reverse primer: 5’TGGCAAGGCGCGCCACTAGTCGGTCCACTCGTTCTGCGGCCA-3’). The sox10 promoter fragment (insert) was amplified using a 2X Phusion Master Mix (Thermo Scientific) and PCR purified using the QIAquick PCR purification kit (Qiagen). The insert and 5kbneurod:mRFP-actr10 plasmid (vector) were digested with Fse1 and Asc1 and the vector was subsequently treated with Antarctic phosphatase. Both vector and insert were visualized using gel electrophoresis and the correct bands were excised and gel purified. Vector and insert were ligated overnight using T4 ligase (New England
Biolabs). The next day, ligated product was transformed and grown overnight on antibiotic plates. Subsequently, colonies were grown in liquid culture overnight and mini preps were made the next day. 25 pg of mini prep and 100 pg of transposase RNA were simultaneously injected into 1-cell zebrafish embryos from \(tg(mbp:egfp-caax);actr10^{nl15/+}\) fish crossed to \(actr10^{nl15/+}\) fish. Injected larvae were screened for green hearts and mRFP fluorescence as early as 24 hpf. \(tg(mbp:egfp-caax)\) uninjected \(actr10^{nl15/nl15}\) mutants and mRFP-positive injected \(actr10^{nl15/nl15}\) mutants were imaged on the 2-photon LSM 880 at 4 dpf. In a separate experiment, \(tg(mbp:egfp-caax)\) uninjected \(actr10^{nl15/nl15}\) mutants and mRFP-positive injected \(actr10^{nl15/nl15}\) mutants were saved for TEM analysis at 5 dpf.

### 3.8 Author contributions

A.L.H., M.-m.F., and K.R.M. designed the project, and K.R.M. A.L.H, B.L.H, and S.D.A performed the forward genetic screen. R.S.G, T.O.P., and S.L.J. designed the original whole genome sequencing analysis pipeline and R.S.G. assisted in mapping the \(stl83\) mutation. C.M.D. and A.V.N. provided the \(actr10^{nl15}\) allele prior to publication and the \(5kbneurod:mRFP-actr10\) plasmid. A.L.H. performed zebrafish experiments, and M.-m.F performed all primary oligodendrocyte experiments with input from B.A.B. A.L.H., M.-m.F., and K.R.M. wrote the manuscript, which was edited by all authors.

### 3.9 Acknowledgements

We thank members of the Solnica-Krezel lab, C. Johnson, Z. Spence, and S. Canter for assistance with the screen and fish maintenance. We thank members of the Monk lab and Matt McCoy for helpful discussions. We thank D. Lyons and M. Voigt for transgenic fish lines and P. Gontarz for assistance with statistical analyses. A.L.H. is supported by the Philip and Sima
Needleman Foundation and by F31 NS096814, and M.m.-F. is supported by T32 HD7249, F32 NS090721, and a National Multiple Sclerosis Society (NMSS) postdoctoral fellowship. This work was also supported by F31 NS094004 (B.L.H.), F31 NS087801 (S.D.A.), R01 GM056988 (S.L.J.), the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (B.A.B.), a Myra Reinhard Foundation grant (B.A.B.), R01 HD80601 (K.R.M.), and K.R.M. is a Harry Weaver Neuroscience Scholar of the NMSS.
3.10 Figures

Figure 1: A forward genetic screen uncovers actr10 mutants.

(A) tg(lhx1a:gfp) marks axons in the tail of a control zebrafish larva at 4 dpf. (B) stl83 mutants exhibit axonal swellings in the CNS (arrows). (C) mbp ISH in a control animal at 5 dpf shows robust mbp mRNA levels. (D) An stl83 mutant animal exhibits reduced mbp mRNA in the hindbrain (arrow) and spinal cord (arrowheads). (E) Analysis of whole genome sequencing data revealed a higher mutant-to-WT allele ratio on chromosome 20, and SNP subtraction analysis (F) narrowed the number of candidate genes to 5. (G) Diagram of actr10 genomic structure shows the location of the nl15 mutation in exon 1 and the stl83 mutation in exon 12. (H) In a tg(neurod:egfp) background, the lateral line is normal in the tail of control animals at 2 dpf. (I) A complementation cross demonstrated that transheterozygous stl83/nl15 animals have axonal swellings in the PNS (actr10<sup>stl83/nl15</sup>; N = 22), which are never seen in either heterozygous or wildtype (WT) animals from either genotype (actr10<sup>+/+</sup>; N = 47, actr10<sup>stl83/+</sup>; N = 13, actr10<sup>nl15/+</sup>; N = 9) (arrows). (A, B, H, I), lateral views, dorsal up; (C and D), dorsal views, anterior up.
Figure 2: *actr10* mutants have fewer myelinated axons in the ventral spinal cord.

(A) Myelinated axons in a control larva in a hemi-segment of the ventral spinal cord at 5 dpf. (B) Pseudocoloring and higher magnification enhance visualization of control myelinated axons. (C and D) Myelinated axons are similarly shown in a quadrant of the ventral spinal cord in an
actr10^{nl15/nl15} mutant animal. (E and F) Myelinated axons in a control larva in a quadrant of the ventral spinal cord at 5 dpf. (G and H) Myelinated axons are similarly shown in a quadrant of the ventral spinal cord in an actr10^{stl83/stl83} mutant animal at 5 dpf. (I and J) Quantification revealed a significant difference in the number of myelinated axons in a ventral quadrant between control animals (N = 6) and actr10^{nl15/nl15} mutants (N = 4, p < 0.0006) (I) and between control animals (N = 8) and actr10^{stl83/stl83} mutants (N = 5, p < .0001) (J) at 5 dpf. Unpaired t-tests with Welch’s correction were used for statistical analysis.
Figure 3: actr10<sup>nl15/nl15</sup> mutants have fewer OPCs.

(A and B) Double transgenic tg(sox10:mgfp);tg(olig2:dsred) larvae were used to identify OPCs in spinal cord cross-sections at 3 dpf. Arrowheads mark OPCs, which are labeled by cytoplasmic DsRed and membrane-tagged GFP. (C) Mutants (N = 7) have significantly fewer OPCs in the spinal cord compared to sibling controls (N = 16, p < 0.0002). (D–G) The tg(olig2:dsred) line was used to count the number of olig2-labeled elongated cells in control animals (D, D’, arrows) compared to actr10<sup>nl15/nl15</sup> mutants (E, E’, arrows) at 3 dpf and at 4 dpf (pictured). (F) Quantification revealed a significant difference between the number of olig2-labeled dorsal cells in controls (N = 48) compared to actr10<sup>nl15/nl15</sup> mutants (N = 16, p < 0.0067) at 3 dpf. (G) Similarly, there was also a significant difference between controls (N = 28) and actr10<sup>nl15/nl15</sup> mutants (N = 16, p < 0.0001) at 4 dpf. Unpaired t-tests with Welch’s correction were used for statistical analysis.
Figure 4: Cell division is delayed in *actr10nl15nl15* mutants.

(A–E) Time-lapse imaging from ~57 hpf using the *tg(nkx2.2a:megf4)* line to mark OPCs allows for visualization of individual *nkx2.2a*-labeled cells that retract processes and become round in preparation for division (arrowhead, A). In *actr10nl15/+* heterozygous control animals, the cell remains in this state for only two panels of 10 minutes (B and C) before becoming two cells (arrowheads, D) that divide rapidly (E). (F–R) In contrast, a cell from an *actr10nl15nl15* mutant animal that has already retracted its processes (arrowhead, F) takes over 3 hours to divide (G–R). (S) Quantification revealed a significant difference in either time to cell division or the end of imaging between heterozygous control animals (N = 11 cells from 5 animals) and mutants (N = 7 cells from 6 animals, p < 0.0194, unpaired t-test with Welch’s correction). See also Movies S1–S4.
Figure 5: Transient expression of actr10 in oligodendrocytes partially suppresses myelination defects in actr10<sup>nl15/nl15</sup> mutants.

(A–B”) Confocal images of mbp-labeled oligodendrocytes in uninjected (A) and sox10:mRFP-actr10 (“sox10 injected”) actr10<sup>nl15/nl15</sup> mutants (B). While uninjected actr10<sup>nl15/nl15</sup> mutants do not exhibit RFP fluorescence (A’, A”), injection of sox10:mRFP-actr10 results in monomeric RFP fluorescence in actr10<sup>nl15/nl15</sup> mutants (B’, B”). (C–E) TEM images show dorsal spinal cords of uninjected WT and actr10<sup>nl15/+</sup> controls (C), uninjected actr10<sup>nl15/nl15</sup> mutants (D), and sox10:mRFP-actr10-injected
actr10^{nl15/nl15} mutants (E). Myelinated axons are pseudocolored in purple. (F) Quantification shows that sox10-injected actr10^{nl15/nl15} mutants (N=6) have significantly greater numbers of myelinated axons in the dorsal spinal cord compared to uninjected actr10^{nl15/nl15} mutant siblings (N=5, p < 0.0377), although sox10:mRFP-actr10 injection does not restore myelination to WT control levels (N=5, p < 0.0001), indicative of partial rescue. One-way ANOVA with Tukey’s multiple comparisons test used for statistical analyses.
Figure 6: Dynein and dynactin are associated with Mbp mRNA granules.

(A) ISH shows robust levels of mbp mRNA in a representative control larva at 4 dpf and higher magnification (B) shows mbp processes in the control (arrowheads) (N = 47/47). In contrast, an actr10^{nl15/nl15} mutant (C) has a punctate mbp phenotype and higher magnification of the same image (D) shows mbp positive cell bodies (arrows) but no processes (N = 18/18). (E) A lateral view of the brain and spinal cord of a WT animal shows mbp mRNA in processes (arrowheads) while actr10^{nl15/nl15} mutant animals (F) have cell bodies (arrows) but reduced mbp mRNA bearing processes. (G) A bidirectional construct expressing MS2-GFP, a RNA-binding reporter, as well as MBP 5'UTR, CDS, and 3'UTR tagged with MS2 stem loops was electroporated into
purified rat oligodendrocytes. MS2-GFP binds to stem loops to allow visualization of Mbp mRNA motility. (H) Primary rat oligodendrocyte expressing MS2-GFP labeled Mbp mRNA imaged using spinning-disk confocal microscopy shows distribution of Mbp mRNA throughout the oligodendrocyte processes. (I) Lysates from primary rat oligodendrocytes expressing the MS2-GFP labeled Mbp mRNA construct were immunoprecipitated using an anti-GFP antibody and probed with p150Glued, DIC (dynein intermediate chain), and Actr10/Arp11 antibodies (N = 4 independent experiments).
Figure 7: Acute dynein inhibition arrests both anterograde and retrograde Mbp mRNA transport in cultured oligodendrocytes and perturbs mbp localization in zebrafish.

(A) In kymographs representing 60 seconds of live-cell imaging, MS2-GFP labeled Mbp mRNA can be seen moving in the retrograde direction in untreated cells (top panel). Following acute ciliobrevin D (15 µM) addition, Mbp mRNA displays bidirectional motility characterized by frequent back-and-forth movement and many directional switches (middle panel). Finally, around 15 minutes after ciliobrevin D (15 µM) addition, most cells display arrested motility (bottom panel). (B) A scatter plot represents the average anterograde or retrograde net speeds for individual oligodendrocytes. (C and D) Anterograde or retrograde net speeds were averaged for each cell and binned across 27-minute time periods following ciliobrevin treatment. At 8–15 minutes following ciliobrevin treatment, speeds significantly decreased compared to earlier time points. One-way ANOVA with post-hoc Tukey's test were performed. Anterograde speeds: No CilioD vs. 1–7 min: p = 0.109, No CilioD vs. 8–15 min: p = 0.0011, 1–7 min vs. 8–15 min: p = 0.077. Retrograde speeds: No CilioD vs. 1–7 min: p = 0.666, No CilioD vs. 8–15 min: p = 0.106, 1–7 min vs. 8-15 min: p = 0.027. (E) A model shows that Mbp mRNA granules can move processively in the anterograde and retrograde directions as well as bidirectionally and that each Mbp mRNA granule can simultaneously bind to both kinesin and the dynein/dynactin complex. (F–I) mbp ISH of zebrafish larvae treated with ciliobrevin D for 21 hours. Larvae were scored as having strong (F), normal (G), reduced (H) or absent (I) mbp localization in oligodendrocyte processes (arrowheads, processes; arrows, cell bodies). (J) Quantification of phenotypic distribution shows that there was a significant difference in scores for DMSO (N = 71) and ciliobrevin D-treated larvae (N = 68), with the latter exhibiting more larvae with reduced or absent mbp localization in oligodendrocyte processes (p < 4.5E-14, Fisher’s exact test) (scale bar = 50 µM).
Figure 8: Chronic dynein inhibition disrupts MBP protein translation in cultured oligodendrocytes and disrupts myelination in zebrafish.

(A) A model shows that transport of Mbp mRNA granules by microtubule-based motors precedes local translation and recruitment of ribosomes. (B) Quantification of the percentage of DIV3 oligodendrocytes treated for 21 hours with 5 µM ciliobrevin that express MBP protein demonstrate that a lower percentage of ciliobrevin-treated cells (42%) are positive for MBP protein compared to control cells (63%, p < 0.001) and DMSO-treated cells (62%, p < 0.001). Of these MBP-containing cells, only 8.5% of ciliobrevin-treated cells highly expressed MBP protein compared to 29% (p <0.001) and 27% (p < 0.001) in control and DMSO-treated cells. (C) Cells treated with 5 µM ciliobrevin for 21 hours were co-stained with smFISH probes against Mbp mRNA and with a monoclonal antibody against MBP protein. MBP protein images (middle panels) show examples of high expressers (arrows) in control cells and low expressers.
(arrowheads) in ciliobrevin-treated cells (N = 2 biological replicates using primary cultures from 2 different animals, 4 cover slips, 23–32 fields of view per group).
3.11 Supplementary figures

**Figure S1 (related to Fig. 1 and Fig. 2):** *nl15* and *stl83* are alleles of *actr10*.

(A) The *nl15* allele is the result of a mutation in the start site (T to G), while the *stl83* allele results from a G-to-T transition. (B) The Actr10/Arp11 amino acid sequence is highly conserved across species, and the glycine altered in the *stl83* allele is completely conserved from zebrafish to human (black rectangle). (C) A dCAPs genotyping assay for *stl83* inserts an AvaI restriction site in WT, leading to a 224 bp major band in WT used for genotyping (arrowhead) and a minor fragment of 22 bps. Mutants have a 244 bp fragment (asterisk). The *nl15* mutation introduces an HaeIII site in mutants resulting in a major band at 112 bp (asterisk) and shorter bands at 50bp and 14bp, while WT has a major band at 126 bps (arrowhead) and a minor band of 50 bp. (D) Neither *stl83* nor *nl15* mutants develop a swim bladder. However, *stl83* mutants appear generally healthier than *nl15* mutants at 6 dpf. (E) There is no significant difference in number of myelinated axons in the ventral spinal cord between sibling *actr10*+/− (*N* = 3) and *actr10*H183+/− (*N* = 4) animals (p<.6120). (F) Similarly, there is no difference in number of myelinated axons
between sibling $actr10^{+/+}$ (N = 3) and $actr10^{+/15}$ (N = 3) animals. Unpaired t-test with Welch’s correction used for statistical analyses.
Figure S2 (related to Fig. 6): *actr10^{nl15/nl15}* mutants have similar phenotypes to dynactin and dynein mutants.
(A, B) Photoreceptor layer analysis by DAPI staining at 3 dpf. Controls (N = 4) have a normal photoreceptor layer (A, arrowheads), while actr10nl15nl15 mutants (N = 3) have a disrupted photoreceptor layer (B, arrowhead). (C, D) Head pigment analysis at 6 dpf. WT controls have normal pigmentation (C), while actr10nl15nl15 mutants have expanded pigment (D, arrows). (E, F) Lateral line pigment analysis at 5 dpf. WT control animals have intermittent pigment along the lateral line (E), while actr10nl15nl15 mutants have increased pigment (F, arrows). (G-I) The Noldus DanioVision behavioral system was used to track larval movement at 6 dpf. WT (G, N = 23, p<.0002) and heterozygous (H, N = 36, p<.0001) controls showed a significant increase in movement in response to dark, while actr10nl15nl15 mutants did not (I, N = 26, p<.9823). An unpaired t-test with Welch’s correction was used for statistical analysis. (J-S) Epinephrine treatment to assay retrograde melanosome transport. Before epinephrine treatment at 3 dpf, control animals (N = 19) exhibit similar pigment patterns. After 5 minutes of epinephrine exposure (K), melanosomes have begun to move back toward the melanophore cell body via retrograde transport. After 10 (L) 15 (M) and 30 minutes (N) all control animals have small pigment patches representative of aggregated melanosomes. Before epinephrine treatment, actr10nl15nl15 mutants (N = 11) can be distinguished phenotypically from control animals by excessive pigment (O). After 5 (P), 10 (Q) 15 (R) and 30 (S) minutes of epinephrine treatment, melanosomes in mutants are still more dispersed relative to those in control animals. Note that representative animals were selected at random and images may be different animals.
Figure S3 (related to Fig. 5): Stable expression of *actr10* in neurons suppresses myelination defects in *actr10*\(^{nl15/nl15}\) mutants.

(A–D) Fluorescent micrographs showing *tg(mbp:gfp-caax)* and *tg(neurod:mRFP-actr10)* expression in zebrafish of the indicated genotypes harboring the indicated transgenes at 5 dpf. (A) The ventral and dorsal spinal cords are normal in WT control animal lacking the *neurod:mRFP-actr10* transgene. (B) Large caliber swellings (arrows) are present in an *actr10*\(^{nl15/nl15}\) mutant lacking the *neurod:mRFP-actr10* transgene. (C, D) Swellings are not observed in a WT control (C) and are ameliorated in *actr10*\(^{nl15/nl15}\) mutants harboring the *neurod:mRFP-actr10* transgene (N = 15/17). (E–G) TEM images of the dorsal spinal cord show...
myelinated axons (pseudocolored purple) in WT control animals (E) (N = 4), actr10<sup>n115/n115</sup> mutants lacking the stable neurod:mRFP-actr10 transgene (F) (N = 3), and actr10<sup>n115/n115</sup> mutants harboring the stable neurod:mRFP-actr10 transgene (G) (N = 4). (H) Quantification shows that stable neuronal expression of actr10 suppresses actr10<sup>n115/n115</sup> mutant myelination defects to WT levels (p<.9936, N.S), while there is a significant difference between control and actr10<sup>n115/n115</sup> mutants (p<.0184) and actr10<sup>n115/n115</sup> mutants and actr10<sup>n115/n115</sup> mutants with the neurod:mRFP-actr10 transgene (p<.0224).
Figure S4 (related to Fig. 6 and Fig. 7): Co-immunoprecipitation pulls out p135 in addition to p150\textsuperscript{Glued}.

(A) Image of a cell expressing a control construct for MS2-GFP, which contains a nuclear localization sequence (NLS). In the absence of any stem loop containing constructs, such as MBP, MS2-GFP localizes to the nucleus. (B) Identity of the p135 band from an anti-GFP immunoprecipitation against MS2-GFP-associated MBP mRNA granules was confirmed by comparing it to the eluted band from an immunoprecipitation using an antibody targeted against the N-terminus of p150\textsuperscript{Glued}, which contains its microtubule-binding domain. (C) Ribbon diagram of DCTN1 isoform 1 (p150\textsuperscript{Glued}) shows a CAP-Gly microtubule-binding domain, which is lacking in DCTN1 isoform 2 (p135). (D) Kymograph analysis shows rapid back and forth movement of Mbp mRNA granules.
Figure S5 (related to Fig. 7): Effects of ciliobrevin D treatment on zebrafish and cultured oligodendrocytes.

(A–B) DMSO and ciliobrevin D treated fish at 4.5 dpf. Compared to fish treated with DMSO for 21 hours (A), ciliobrevin D treated fish look relatively normal. (C) Treatment of oligodendrocytes differentiated for 3 days in vitro with different concentrations of the dynein inhibitor ciliobrevin for 21 hours. Cell death was assayed by morphology using DIC microscopy; living cells contained continuous, non-bulbous branches and adhered to coverslips. Concentrations of 1 µM and 5 µM did not significantly alter the percentage of living cells while 10 µM and 15 µM significantly decreased the percentage of living cells (n = 4 cover slips per group; * p = 0.023, ** p = 0.0001, one-way ANOVA).
3.12 References


Chapter 4: Investigating the role of ACTR10 in human disease
Preface:

The contents of this chapter may be adapted and reproduced at a later date in a published manuscript.
4.1 Abstract

Precision medicine, targeting therapy to the underlying molecular cause of disease, is increasingly used to maximize positive outcomes in patients. To this end, better technology and lower costs have led to a massive increase in the use of sequencing to understand the etiology of human diseases. This approach has resulted in numerous studies associating new genes with diseases. However, validation of many of the genes found in these studies has lagged behind the influx of human sequencing data. The next phase in precision medicine research will be confirming sequencing data and establishing mechanisms for studying patient specific diseases in the lab. Zebrafish, already well established as an important organism for developmental biology, are increasingly being used in precision medicine research.

In a previous paper, we described uncovering a mutation in the gene \textit{actin related protein 10} (\textit{actr10}), through a forward genetic screen in zebrafish. \textit{actr10} mutants presented with myelin defects in the central nervous system (CNS) and we showed that this defect was due in part to reduced transport of \textit{mbp} mRNA in oligodendrocytes, the myelinating glia of the CNS. \textit{actr10} mutants also exhibit axonal swellings in the peripheral and central nervous systems with neurofilament accumulation, reminiscent of many neurological disorders. In collaboration with two neurologists from Washington University, we searched patient sequencing databases to determine if any patients with neurological disorders of unknown origin had mutations in \textit{ACTR10}. Indeed, five families presenting with Amyotrophic Lateral Sclerosis (ALS), Charcot-Marie-Tooth disease Type 2 (CMT2), and distal myopathy were identified as having missense mutations in \textit{ACTR10}. Here, we focus on the \textit{ACTR10} single nucleotide polymorphism (SNP) identified in the CMT2 family. We generated zebrafish mutants with the patient specific \textit{ACTR10} SNP in order to use zebrafish to model this patient specific form of CMT.
4.2 Introduction

The use of whole genome sequencing (WGS), whole exome sequencing (WES), and genome wide association studies (GWAS) to both find new genes and to elucidate gene networks in human diseases has increased dramatically in recent years. Furthermore, the ability to sequence an individual’s genome at low cost has made precision medicine a reality for some diseases. However, the rapid pace of next generation sequencing has vastly outpaced researchers’ ability to validate and study many of the genes identified. A critical next step in advancing precision medicine research will be to establish pipelines for taking the many genes identified from these studies back to the bench for validation and further study. To this end, recent advances in molecular techniques in zebrafish have made them an attractive organism for precision medicine research.

Zebrafish are a well-established model organism used widely in many different areas of research. Large clutch size, rapid embryogenesis, transparency of the early embryo, and a relatively short generation time are a few of the attributes that have made zebrafish so ubiquitous, particularly in fields such as developmental biology. Taking advantage of these traits in zebrafish, large-scale forward genetic screens led to the discovery of important genes regulating development and are still used today (1, 2) (see Chapter 2 of this dissertation). However, technology in zebrafish to disrupt specific genes via reverse genetics lagged behind what was possible in other models, such as mouse. Approaches such as TILLING relied on finding mutations in zebrafish populations that had been mutagenized, rather than targeting specific genes (3). Furthermore, antisense morpholinos have increasingly been shown to have off-target effects and more severe phenotypes than mutants in the targeted genes, confounding research (4).
Recent advances have brought zebrafish to the forefront of reverse genetics in model organisms. TALENs and CRISPR-Cas9 technology have made it feasible not only to disrupt genes efficiently, but also to insert nucleotides or specific sequences into the zebrafish genome (5). Using gene editing technology, it is now possible to introduce a single nucleotide polymorphism (SNP) into a gene of interest, which can be inherited by progeny. Zebrafish are therefore becoming increasingly suited to modeling patient specific diseases. Furthermore, the ability to perform transgenesis, xenografts, behavioral studies and live imaging have also made zebrafish incredibly useful for precision medicine research. Moreover, zebrafish can absorb small molecules and drugs through their skin, facilitating the use of drug screens (6). To this end, a recent study grafted patient specific tumors onto zebrafish, which were then treated with a drug, and found that not all the tumors responded in the same way (7). Zebrafish are therefore an important model organism that can be used to confirm sequencing data and study patient specific diseases in the lab.

Zebrafish hold promise in particular for studying neurological diseases which may have multigenic or unknown etiology. One example of this is the complex disease Charcot-Marie-Tooth (CMT) disease. Although there are currently 80 genes identified as causative for CMT, it is estimated that there remain many genes still be discovered (8). To this end, a recent paper described the discovery of three new CMT genes using whole exome sequencing of 40 CMT patients followed by tests for functional significance using morpholinos in zebrafish (9). CMT is a heterogenous group of progressive peripheral neuropathies that afflict one in 2,500 people, impacting both sensory and motor nerves (10). CMT neuropathy is broadly characterized by muscle weakness and atrophy in limbs beginning in the extremities (11, 12). Patients initially exhibit trouble walking, resulting in tripping and falling (11, 12). Patients may have difficulty
keeping their foot horizontal, called foot drop, or exhibit the classic neuropathic foot deformity “pes cavus,” which is characterized by a high arch (11, 12). In some instances, severe sensory loss can necessitate amputation of the afflicted foot. Although CMT is not fatal, its debilitating effect on patient quality of life, prevalence in the general population, and current dearth of treatment options make it a key candidate for precision medicine research.

CMT neuropathy is divided broadly into two categories: CMT disease type 1, which is a myelinopathy originating in the myelinating Schwann cells of the PNS, and CMT disease type 2, which is an axonopathy originating in the axons of the PNS (13). Classical characterization of CMT involved testing patient nerve conduction velocities (NCVs) (14). Patients exhibiting low NCVs, below 38 m/s, are characterized as having CMT disease type 1, while patients with a normal NCV, above 38 m/s, are placed in the type 2 category (14). Since these early distinctions between myelinopathy and axonopathy, the classification of CMT disease has grown to include other characterizations, such as how the neuropathy is inherited. CMT 1 and CMT 2 can both be inherited in an autosomal dominant or autosomal recessive fashion (15). Other categories include CMT4, which is demyelinating and characterized by autosomal recessive inheritance, CMT X or CMT5, which is X-linked, and DI-CMT, which is inherited in a dominant intermediate manner (15). Currently, the number of CMT categories has expanded into nine different subgroups (16). Moreover, in addition to the classic Mendelian inheritance patterns, there are frequently instances of de novo mutations that cause CMT which, prior to the advent of low cost sequencing, would have gone undetected in afflicted patients (8).

As the specific genes responsible for CMT have been identified, categorization has expanded to include this information. For example, the demyelinating autosomal dominant class of CMT disease type 1 is now subdivided into CMT1A-F. One of the most well-known genes
involved in CMT1 encodes a critical component of the myelin sheath. PMP22 is a small, integral protein critical for compaction of the myelin sheath of Schwann cells and was the first protein identified as causative for CMT (17–20). Patients with a duplication in \textit{PMP22} are classified as having CMT1A. Other mutations in \textit{PMP22} are responsible for CMT1E, autosomal recessive CMT1, and hereditary neuropathy with pressure palsy (HNPP) (21, 22). Other genes affected in CMT1 include neurofilament light (\textit{NEFL}), the transcription factor \textit{EGR2}, and \textit{Myelin protein zero (MPZ)} (15). \textit{PMP22}, however, appears to be the gene primarily affected in demyelinating CMT disease. A recent study involving 17,000 patients found that 78.6% of those sequenced had mutations in \textit{PMP22} (23). To this end, many drug and treatment studies have targeted CMT1A (16).

In CMT2, the most common mutations are in the genes \textit{MFN2} and \textit{GDAP1}, both of which affect mitochondrial localization and transport (24–26). However, unlike in CMT1A, these two genes only account for 25% of the total mutations involved in CMT2 (16). Therefore, it is critical to continue to identify new genes that cause CMT2. An important component of CMT axonopathies is disruption to cytoskeletal integrity and intracellular trafficking (27). As is the case with many neurological disorders, the length of axons in the nervous system renders them acutely sensitive to transport defects (28). Thus, a primary phenotypic hallmark of CMT2 is disrupted transport (27). Indeed, mutations in genes involved in transport have been shown to be responsible for various forms of CMT2. Mutations in \textit{KIFb\beta}, an anterograde molecular motor that transports cargo from the cell body to the periphery along microtubules, were shown to be causative for CMT2A1 in a Japanese family (29). As in CMT1, mutations in \textit{NEFL} are also causative for a form of CMT2, CMT2E. In a mouse model of CMT2E, axons swollen many times their normal diameter were shown by electron microscopy to be filled with disorganized...
neurofilaments (30). Neurofilaments are specialized neuronal intermediate filaments that are critical for cytoskeletal integrity and transport. In addition to NEFL being directly mutated in CMT disease, abnormal neurofilament accumulation and/or disorganization is a hallmark of many neurodegenerative diseases, including CMT, ALS, Parkinson’s disease, and Alzheimer’s disease, among others (31).

A recently characterized form of CMT2, CMT2O, was identified via exome sequencing of a family in which 4 generations were affected by the disease, and shown to be the result of a mutation in cytoplasmic dynein (32). A subsequent study of a separate afflicted family identified the same lesion in dynein as causative for the neuropathy (33). A mouse model of the dynein mutation similarly recapitulated the patient phenotypes (34). Unlike the large kinesin family of anterograde molecular motors, cytoplasmic dynein is the primary retrograde motor involved in transport in eukaryotes (35). Dynein thus requires multiple adaptors to promote facile transport of many different types of cargo along microtubules. One such adaptor complex is dynactin. Dynactin is composed of 12 different proteins, several of which have been implicated in neurodegenerative diseases (36). A family afflicted with motor neuron disease was found to have a point mutation in p150Glued, the microtubule binding component of dynactin that regulates microtubule stability (37–39). Follow up work in a mouse model recapitulated the degenerative phenotypes and demonstrated that lack of binding by p150Glued to microtubules and the protein EB1 played a role in the etiology (40). Another dynactin subunit, dynamitin or p50, when overexpressed in a mouse model results in general dynactin and dynein dysfunction leading to a progressive motor neuron degeneration phenotype (41). Components of the retrograde motor complex, including dynein, dynactin subunits and other adaptor proteins, are therefore important
to study for their role in neurodegenerative diseases. However, relatively little is known about the other proteins in the dynactin complex.

In a large scale forward genetic screen in zebrafish, we identified a missense mutation in the gene actin related protein 10 (actr10), which encodes a component of the pointed end complex of dynactin (42). The actr10<sup>stl83</sup> mutant identified in the screen is the result of a missense mutation in exon 12 out of 13 total exons. Using another allele of actr10, actr10<sup>nl15</sup>, which is a presumptive null, we previously demonstrated that actr10 mutants had reduced myelination in the CNS in part due to trafficking defects of myelin basic protein (mbp) mRNA (43). In collaboration with another group, we also demonstrated that actr10 has a role in mitochondrial trafficking in axons of the PNS which, when disrupted in the mutants, results in axonal swellings (44)(Appendix B of this dissertation).

Here, we show that actr10 mutants have defects in PNS development and myelin. Zebrafish homozygous for either stl83 or nl15 alleles of actr10 also exhibit reduced numbers of total axons in the PNS at 3 and 5 dpf. We observed axonal swelling and abnormal neurofilament accumulations in actr10 mutants, reminiscent of neurodegenerative diseases. These observations led us to wonder whether ACTR10 could have a role in human disease. To investigate this possibility, we worked with Drs. Matt Harms and Conrad Weihl to search sequencing databases for patients with neurological disorders that also had mutations in ACTR10. Five patient groups in total were identified: three with ALS, one with distal myopathy and one with CMT2. All mutations were missense mutations and corresponded to completely conserved amino acids between zebrafish and humans. We are currently working to test whether the patient mutation in ACTR10 may be contributing to the CMT2 and ALS diagnosis by knocking down Actr10 via
shRNA in mouse dorsal root ganglion (DRG) culture and assaying for degeneration and regeneration.

Since the zebrafish actr10\textsuperscript{stl83} allele had such a severe phenotype, we hypothesized that the human mutations in ACTR10 might also cause a similar phenotype when recapitulated in the zebrafish. We found that the CMT2 allele was not present in the human population as a background mutation, and therefore decided to leverage the power of zebrafish to model the CMT2 mutation. Using TALENs, we generated a line of zebrafish with the CMT2 patient ACTR10 mutation. To determine if the mutant fish had a phenotype, we performed a complementation test between actr10\textsuperscript{CMT/+} F1 fish and actr10\textsuperscript{nl15/+} fish in a tg(neurod:egfp) background to screen for lateral line swellings. To our surprise, no phenotype was observed in the transheterozygous animals, unlike the complementation test performed previously between actr10\textsuperscript{stl83/+} and actr10\textsuperscript{nl15/+} fish, which yielded a strong phenotype in transheterozygous animals by 2 dpf. We discuss the potential reasons for the lack of an early phenotype in the Discussion section of this Chapter. While it is still possible that there may be an age dependent phenotype, and ongoing work will test this hypothesis, the potential lack of a phenotype demonstrates that there can be pitfalls in attempting to use model organisms to study human diseases. In this particular case, the complex nature of CMT2 may have precluded the ability to model the disease with a single mutation; rather, there may be multiple mutations in the human patient that are acquired over an individual’s lifetime, which eventually result in the disease diagnosis. Nonetheless, this work demonstrates the feasibility of reproducing human specific mutations in zebrafish, which was unthinkable just 10 years ago. While future studies in disease modeling must take into account the complexity and heterogeneity of neurodegenerative diseases, new advances in zebrafish techniques bode well for this work.
4.3 Materials and methods

Zebrasfish maintenance and husbandry

All zebrafish used in these studies were kept in the Washington University zebrafish facility (zebrafish.wustl.edu). Zebrafish experiments were executed according to Washington University IRB standards and described in Monk lab animal protocols approved by Washington University (animal protocol no. 20160174; Institutional Biological & Chemical (IBC) Safety Committee protocol no. 10-643). Larvae were reared in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$) at 28.5°C until 5 dpf, when they were transferred to the recirculating water system to be grown to adulthood. The mutant strains actr10$^{nl15}$ and actr10$^{stl83}$ were obtained from OHSU and from a forward genetic screen, respectively (43). Other lines used in this work include tg(neurod:egfp)(45) and tg(mbp:gfp-caax) (46). The actr10 CMT mutant strain was generated at Washington University using TALEN technology.

Genotyping of actr10 mutants

Genotyping for actr10$^{nl15}$ and actr10$^{stl83}$ was performed as described previously (43). To genotype actr10 CMT mutants, PCR was used to amplify the actr10 sequence of interest (forward primer: TGCAGGAGCGATATTTGGAG; reverse primer: CTCTTCATGAGCGGTGGAG). The C-T CMT human SNP abrogated a BseR1 restriction enzyme site normally present in the WT sequence. Digestion of the amplified PCR product at 37°C for two hours with the BseR1 enzyme results in an uncut mutant band of 165 bps, while the WT allele results in two bands of 107 and 58 bps.

Protein alignment

Actr10 cDNA sequences for Homo sapiens and Danio rerio were acquired using ensemble (https://useast.ensembl.org/index.html). Versions GRCh38.p10 for human and GRCz10 for
zebrafish were utilized. Sequences were added to the sequence editor ApE and the translation tool was used to generate the protein sequence. Sequences for human and zebrafish were aligned using the EMBOSS needle pairwise sequence alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

**In situ hybridization**

*In situ* hybridization was performed according to standard protocols (47). Briefly, embryos were raised egg water and then treated with 0.003% phenylthiourea (PTU) beginning at 24 hpf to prevent pigment from forming. Larvae were grown in incubators until 5 dpf, when they were fixed in 4% paraformaldehyde (PFA) in 1xPBS overnight at 4 °C. An *in situ* hybridization was then performed using a riboprobe against *mbp* mRNA (48). Larvae from these experiments were scored blindly and subsequently genotyped.

**Transmission electron microscopy**

For all TEM experiments, zebrafish larvae from *actr10* nl15/+ and *actr10* stl83/+ heterozygous intercrosses were grown in egg water until 3 or 5 dpf. All samples were cut between segments 5-7 and larval heads were saved for genotyping by PCR and digest, while bodies were fixed in a modified Karnovsky’s solution composed of 2% glutaraldehyde, 4% PFA, and 0.1 M sodium cacodylate buffer, pH 7.4. Fixation was aided by temperature shifts in a Pelco BioWave Pro with Steady Temp Plus water recirculation system and proceeded according to standard protocols (49). The larvae used for analysis of number of myelinated and unmyelinated axons were mounted in epon with the cut tissue nearest the edge, while the *actr10* nl15 samples being used for analysis of axonal swellings in the CNS and neuromasts were mounted with the tail nearest the edge, to allow for sectioning through posterior swellings. Sections were cut on an ultramicrotome using a diamond knife. The zebrafish being used to examine the swellings were
then stained using Tol-Blue to ensure that the swellings were indeed present in the \textit{actr10}^{nl15/nl15} mutants. Thin \textasciitilde 70 nm sections were cut using an ultra diamond knife and placed onto copper mesh grids. Staining with uranyl acetate and Sato’s lead stain were performed according to standard protocols (50). Electron micrograph images were obtained using a Jeol JEM-1400 (Jeol USA) electron microscope and captured by an AMT V601 digital camera.

**Generation of \textit{actr10} CMT mutants**

The \textit{actr10} CMT zebrafish mutants were produced using TALEN technology. TALEN arms were designed using the TALEN targeter tool (https://tale-nt.cac.cornell.edu/) and generated using the GoldyTALEN kit (51). The left repeat variable di-residue sequence (RVD) was as follows: NG NN HD NG NN NG HD NG NN NI. The right RVD was as follows: NG NI NN NI NG NN NN NI NN NG. Additionally, the following oligonucleotide containing the CMT SNP (in brackets), was ordered through IDT:

CATGAGCGGTGGAGGGGTTTTTCCAGCCTCATAAGATGGGATCGGCTGAGGA[A]GAGCATGACAGCACCAGTCCGGATGCAGCGCCTATGGTTGTAGTAAT. The reverse complement was used because it has been shown to insert more efficaciously after disruption by TALEN arms. 25-50 pg/nL total of mRNA of both TALEN arms were co-injected with 50 pg/nL of the oligonucleotide in a 10 µL solution containing milliQ water and 1 µL phenol red. At 24 hpf, DNA from injected and uninjected control embryos was collected and the functionality of the TALEN mutagenesis was tested using the PCR and digest assay described above.

**Deep sequencing**

TALEN injected F0 animals were grown to adulthood in the Washington University zebrafish facility. After they were sexually mature, sperm was collected via squeezing from F0 males. DNA was extracted according to standard protocols. Up to 17 sperm samples were pooled into
PCR tubes and sent for deep sequencing at the Washington University Genome Engineering and iPSC Center (GEIC). Sequencing analysis revealed the SNP of interest in two out of five samples sent for sequencing. Sperm from the pooled animals was then collected from individual fish and sent again for sequencing to identify the specific F0 carrier. Sequencing identified three F0 carriers. F0 carrier males were outcrossed to WT AB females and F1 animals were grown until adulthood. F1 animals were fin clipped and genotyped for the CMT mutant allele. Mutant bands of 165 bps were excised from the gel and DNA was collected via gel extraction using the QIAquick gel extraction kit. DNA was sent for Sanger sequencing to Genewiz (https://www.genewiz.com/en) using the forward and reverse primers used in the genotyping assay. F1 fish with the CMT C-T SNP were identified from sequencing.

**Regeneration assay in mouse dorsal root ganglia (DRG)**

Dorsal root ganglia were dissected from embryonic 13.5 day old mice. Neurons were plated in spots in 24-well plates. At DIV2, neurons were transduced with FCIV-Bclxl-IRE5-GFP [multiplicity of infection (MOI) 10] to ensure survival of embryonic DRGs. At DIV 4, cells were transduced with three different shRNAs (MOI 10) targeting Actr10. shRNAs were ordered from Sigma-Aldrich as MISSION bacterial clones. Efficacy of Actr10 knock-down was determined by qPCR following shRNA addition to DRG culture. At DIV9, DRG spots were axotomized along a single plane using a microtome blade pressed into 8 mm chunks. Cells were allowed to regenerate for 48 hours before being fixed in 4% PFA and stained for SCG10, which labels regenerating axons. Length was measured at multiple points across the injury to determine regenerative growth. The regeneration experiment was performed in technical triplicate, N=4. Human constructs used for rescue were ordered from Genecopoeia.
4.4 Results

**actr10<sup>nl15/nl15</sup>** and **actr10<sup>stl83/stl83</sup>** mutants have reduced mbp expression in the lateral line

In a previously published study, we showed that **actr10<sup>nl15/nl15</sup>** and **actr10<sup>stl83/stl83</sup>** mutants had reduced expression of **mbp** in the hindbrain of zebrafish in addition to a punctate phenotype (43). Here, we show that both **actr10** mutants also exhibit reduced **mbp** expression in the lateral line of the PNS (Figure 1). Zebrafish larvae from an **actr10<sup>nl15/+</sup>** and **actr10<sup>stl83/+</sup>** intercross were scored as having either “WT” or “mutant” **mbp** expression following *in situ* hybridization (ISH) at 5 dpf. All **actr10<sup>nl15/+</sup>** and **actr10<sup>+/+</sup>** animals were scored as having WT **mbp** expression (N=67/67) while all **actr10<sup>nl15/nl15</sup>** mutants had mutant expression (N=27/27) (Figure 1A-B). Similarly, all **actr10<sup>stl83/+</sup>** and **actr10<sup>+/+</sup>** zebrafish had WT expression (N=30/30) while all **actr10<sup>stl83/stl83</sup>** mutants were scored as having mutant **mbp** expression (N=21/21) (Figure 1C-D).

**actr10** has previously been shown to be involved in mitochondrial trafficking, which, when disrupted in **actr10<sup>nl15/nl15</sup>** mutants, results in swellings in peripheral axons of the lateral line (Appendix B). These experiments show that there are additional myelin defects not only in the **actr10<sup>nl15/nl15</sup>** mutant, which is presumed to be a null, but also in the **actr10<sup>stl83/stl83</sup>** mutants.

**actr10<sup>nl15/nl15</sup>** and **actr10<sup>stl83/stl83</sup>** mutants have reduced numbers of myelinated axons at 3 and 5 dpf compared to WT animals

While we use **mbp** expression as a marker of myelinating glial cell development, it does not necessarily indicate that the structure of myelin is altered. To examine the ultrastructure of the myelin sheath, we used transmission electron microscopy (TEM) to visualize myelinated axons in the PNS. At 3 dpf, there were significantly fewer myelinated axons in **actr10<sup>nl15/nl15</sup>** mutants compared to **actr10<sup>+/+</sup>** larvae (Figure 2A-C). TEM of the **actr10<sup>stl8</sup>** allele showed similar
results. At 3 dpf, there was a significant reduction in number of myelinated axons in $actr10^{nl15/nl15}$ mutants compared to $actr10^{+/+}$ animals (Figure 2E-G).

The reduction in myelinated axons persisted at 5 dpf, indicating that this defect was not a developmental delay. TEM at 5 dpf revealed significantly fewer axons in $actr10^{nl15/nl15}$ mutants compared to $actr10^{+/+}$ animals (Figure 2I-K). There was also a significant reduction in number of myelinated axons in $actr10^{nl15/nl15}$ mutants compared to $actr10^{+/+}$ animals (Figure 2M-O).

$actr10^{nl15/nl15}$ and $actr10^{stl83/stl83}$ mutants have fewer axons at both 3 and 5 dpf.

To understand whether the myelin defects shown by TEM were the result of axon defects, we also quantified total axon number in the lateral line. There was a significant decrease in the total number of axons in $actr10^{nl15/nl15}$ mutants at 3 dpf compared to $actr10^{+/+}$ animals (Figure 2D). Similarly, $actr10^{stl83/stl83}$ mutants had fewer axons compared to $actr10^{+/+}$ animals (Figure 2H).

We next counted the total number of axons at 5 dpf. There were significantly fewer axons in $actr10^{nl15/nl15}$ mutants compared to $actr10^{+/+}$ animals (Figure 2L). Moreover, $actr10^{nl15/nl15}$ mutants exhibited abnormalities, including large swellings with neurofilaments and cargo (Figure 2J, arrowheads). In the $actr10^{stl83}$ background, we also saw a decrease in total number of axons in mutants. There was a significant reduction in total number of axons in $actr10^{stl83/stl83}$ mutants compared to $actr10^{+/+}$ animals (Figure 2P). There were also axonal abnormalities in the $actr10^{stl83/stl83}$ mutants at this stage (Figure 2N, arrowhead). These data indicate that in addition to the myelin defects, there are axonal defects at both 3 dpf and 5 dpf in $actr10^{nl15/nl15}$ and $actr10^{stl83/stl83}$ mutants.

$actr10^{nl15/nl15}$ and $actr10^{stl83/stl83}$ mutants exhibit neurofilament-filled axonal swellings in PNS and CNS axons.
The \textit{actr10}^{nl15} mutant was first identified in a forward genetic screen to uncover axon defects in the lateral line using the transgene \textit{tg(neurod:egfp)}(44). At 3 dpf, swellings in the lateral line are visible in \textit{actr10}^{nl15/nl15} mutants, which are not present in control animals (Figure 3). Neither \textit{actr10}^{nl15/+} nor \textit{actr10}^{+/+} animals ever exhibited swellings, and were therefore combined as controls (Figure 3). Previously, we showed that these large swellings were the result of stalled mitochondria in axon terminals of neuromasts, which are sensory structures along the lateral line which aid fish in sensing water movements (44). Upon further inspection, we found that in addition to the mitochondria, there is an excess of neurofilaments in the swellings not observed in control animals (Figure 3).

We also previously showed that there were additional large caliber swellings in the CNS, which we hypothesized were in the Mauthner axon (43). By using a construct in which \textit{actr10} tagged with mRFP was driven by the \textit{neurod} promoter, we were able to rescue axon defects in both the CNS and in the PNS (43). At 5 dpf, the large caliber swellings can be visualized in both \textit{actr10}^{nl15/nl15} and \textit{actr10}^{stl83/stl83} mutants using the \textit{tg(mbp:egfp-caax)} line (Figure 4). Swellings are never observed in WT or heterozygous animals of either genotype (Figure 4). We wondered whether these swellings were similarly filled with neurofilaments. Therefore, we examined \textit{actr10}^{nl15} animals using TEM. All \textit{actr10}^{+/+} animals examined had normal Mauthner axons (Figure 4I-K). In contrast, all mutant animals imaged had abnormal neurofilament accumulation in the Mauthner axon (Figure 4L-N). Furthermore, all mutants exhibited cargo accumulation that appeared to be degenerating (Figure 4M). We also observed axons other than the Mauthner with neurofilament accumulation (arrows, Figure 4M). In some instances, the Mauthner axon was swollen to many times its normal size (Figure 4O-Q).
Neurofilament accumulation is a hallmark of many neurological disorders, including ALS, Parkinson’s disease, and Charcot Marie Tooth disease. Furthermore, a mouse model of CMT type 2E was shown to exhibit giant axons filled with neurofilaments, very similar to the actr10\textsuperscript{nl15/nl15} mutant. We therefore wondered whether ACTR10 mutations might play a role in human neurological disorders.

**Human patients with missense mutations in ACTR10 have neurological disorders**

Working with Dr. Matt Harms, who has since moved to Columbia University, and Dr. Conrad Weihl from Washington University School of Medicine, Department of Neurology, we obtained sequencing data from patients with neurological disorders that also had mutations in ACTR10. Sequencing identified ACTR10 mutations in three patients with ALS, one patient with distal myopathy, and one patient with CMT2 (Figure 5A). While one ALS patient had a C9orf72 mutation that was causative for the disease, the cause of disease for the other patients was unknown. All ACTR10 mutations were missense and all patients were heterozygous for the mutant allele (Figure 5A). This fits with the zebrafish data that both actr10 alleles are lethal when homozygous (43). Moreover, a recent study of humans with homozygous mutations in Iceland did not find any humans that had homozygous mutations in ACTR10, indicating that this gene may be necessary for human survival (52). All of the human ACTR10 missense alleles identified via sequencing were in completely conserved amino acids between humans and zebrafish (Figure 5B) and were also relatively close to the actr10\textsuperscript{alt83} allele, which has a severe phenotype (Figure 5B).

Using the Exome Aggregation Consortium (ExAC) browser, it was found that the patients with CMT2 and distal myopathy had ACTR10 mutations that have not been identified in the general population, meaning that they are not common background mutations. As a result of
this finding, and from our investigation of actr10\textsuperscript{nl15} and actr10\textsuperscript{nl83} phenotypes, we decided to pursue the CMT2 mutant allele to understand whether this could have a role in the patient disease. In order to study the patient specific ACTR10 mutation, we decided to generate zebrafish mutants with the patient disease.

**An actr10\textsuperscript{CMT/+} complementation cross with actr10\textsuperscript{nl15/+} fish does not result in a mutant phenotype**

To generate a zebrafish strain with the patient specific mutation, we used TALEN technology. The human SNP is the result of a C-T nucleotide change that causes a proline to serine amino acid change at amino acid 393 (Figure 5B). We injected TALEN mRNA targeting the actr10 locus near the codon encoding the amino acid 393 affected by the missense mutation in human, as well as an oligonucleotide containing the patient specific nucleotide into 1-cell stage zebrafish larvae. The human SNP is the result of a C-T nucleotide change that causes a proline to serine amino acid change at amino acid 393 (Figure 5B). In the zebrafish, we were able to generate this nucleotide change in F0 embryos (Figure 5C). We designed a PCR-based genotyping assay in which the mutant nucleotide alters a restriction enzyme binding site, resulting in the mutant allele having a larger band (Figure 5D).

We grew up the F0 fish with the CMT mutation to adulthood and then outcrossed several founders to another strain of zebrafish to produce the F1 generation. After the F1 fish reached adulthood, we found that many F1 animals were indeed heterozygous for the CMT humanized allele (actr10\textsuperscript{CMT/+}). We then performed a complementation test with the actr10\textsuperscript{nl15/+} animals harboring the tg(neurod:eGFP) transgene to determine if the actr10\textsuperscript{CMT} mutation causes a phenotype. Previously, the complementation cross between actr10\textsuperscript{nl15/+} and actr10\textsuperscript{nl83/+} produced a strong swelling phenotype in the lateral line by 2 dpf, as visualized by tg(neurod:eGFP) (43).
However, the complementation cross with the \( actr10^{CMT/+} \) allele did not produce larvae with a lateral line phenotype by 5 dpf, as 12 larvae, which had been scored as WT, were transheterozygous animals (Figure 6).

### 4.5 Discussion

Here, we demonstrated that two \( actr10 \) mutant alleles in zebrafish resulted in reduced myelination in the PNS, as well as reduced numbers of axons and a neurodegenerative phenotype, including neurofilament accumulation in axonal swellings. These phenotypes led us to wonder whether there might be human conditions caused by \( ACTR10 \) mutations. Working with two neurologists, we identified five different groups of human patients with the diseases ALS, distal myopathy, and CMT2, that had missense mutations in \( ACTR10 \). All of the mutations were in completely conserved amino acids between zebrafish and human. Although the mutations were far in the protein sequence, they were in a relatively similar position to the \( actr10^{stl83} \) mutation, which was also a missense mutation resulting in a single amino acid change. Since the \( actr10^{stl83} \) had such a severe phenotype, we hypothesized that the other human \( ACTR10 \) mutations might produce a similar phenotype in zebrafish. Furthermore, using the ExAC browser, we found that no humans have been identified with the CMT2 mutation, meaning that it is likely not a common background SNP. Therefore, we decided to generate a “humanized” zebrafish line containing the patient specific SNP.

In order to identify whether the \( actr10^{CMT} \) line had a phenotype, our first step was to perform a complementation test, which had been done previously between \( actr10^{nl15} \) and \( actr10^{stl83} \) to demonstrate that the \( stl83 \) phenotype was indeed the result of an \( actr10 \) mutation. However, when we performed the complementation cross between \( actr10^{nl15/+} \) and \( actr10^{CMT/+} \) animals, there was no observable phenotype in the transheterozygous larvae by \( tg(neurod:egfp) \).
at 3 dpf. In the original complementation cross, the swelling phenotype was obvious by 2 dpf. The larvae from the complementation cross were grown until 5 dpf to observe if there might be subtler defects. However, at 5 dpf, all larvae were scored as having a WT phenotype. The first possibility for this lack of phenotype is that there may be an age-dependent phenotype that is not present at early stages. Another possibility is that the phenotype is subtle and cannot be observed via transgene analysis, but may require other methods, such as TEM.

However, if experiments are performed to test the two possibilities listed above, and there is still no phenotype, there could be several reasons to explain this observation. The first is that the actr10<sup>stl83</sup> mutation may be in a unique and important domain necessary for the Actr10 protein to be produced properly, while the actr10<sup>CMT</sup> mutation may be in a less important domain. Furthermore, the actr10<sup>stl83</sup> mutation causes a glycine to tryptophan amino acid change, a drastic change which might affect the folding of Actr10 and prevent it from being processed properly.

The lack of phenotype in the larval zebrafish does not preclude the possibility that the missense mutation in the human patient may still have a role in CMT2 disease. Although the mutation occurs in a completely conserved amino acid, the protein sequence between human and zebrafish ACTR10 is ~75% conserved and there are amino acid differences between the two species. Therefore, the CMT mutation could be more important in humans than it is in zebrafish. This brings up an important point which will have to be addressed in order for human disease research in zebrafish to proceed: zebrafish and human genes can have divergent protein sequences, and in many cases, zebrafish can possess multiple copies of a human gene.

Another possibility is that the human disease phenotype is the result of multiple mutations in different genes over the course of the patient’s life. Many neurological disorders in
particular are considered multigenic and are unlikely to be the result of one mutation. To this end, the human CMT2 patient was heterozygous for the *ACTR10* mutation, while the *actr10* zebrafish mutants only exhibit defects when the animals are homozygous for the CMT mutation. In zebrafish, complete loss of *actr10* is lethal, as it likely is in humans, and the reason *actr10* homozygous zebrafish mutants are able to live until ~day 7 is likely due to maternal deposition of WT *actr10* mRNA in the egg. It was unlikely that there would be a dominant CMT phenotype in the zebrafish, since neither *actr10*<sup>nl15/+</sup> nor *actr10*<sup>stl83/+</sup> animals exhibited any defects. Therefore, if the human *ACTR10* mutation does not directly cause the disease phenotype, the mutation could function in conjunction with other mutations. Addressing the multigenic nature of many human diseases will be another hurdle to overcome in order to effectively create animal models of patient specific diseases.
4.6 Figures

Figure 1: \textit{actr10}^{nl15/nl15} and \textit{actr10}^{sfl83/sfl83} mutants have reduced \textit{mbp} expression in the PNS.

(A) A lateral view of a control zebrafish larva at 5 dpf shows normal \textit{mbp} expression along the lateral line (arrowhead) (N=67/67) in contrast to an \textit{actr10}^{nl15/nl15} mutant (B), which exhibits reduced \textit{mbp} expression (arrowhead) (N=27/27). (C) Similarly, in the \textit{actr10}^{sfl83} background, a WT control zebrafish shows normal levels of \textit{mbp} (arrowhead) (N=30/30) while an \textit{actr10}^{sfl83/sfl83} mutant (D) has reduced peripheral \textit{mbp} expression (arrowhead) (N=21/21).
Figure 2: *actr10*<sup>n15/n15</sup> and *actr10*<sup>stl83/stl83</sup> mutants have axonal and myelin defects.

TEM images of the lateral line of *actr10*<sup>+/+</sup> (WT, A), and *actr10*<sup>n15/n15</sup> (MUT, B) zebrafish larvae at 3 dpf show myelinated axons (pseudocolored) and unmyelinated axons (asterisks). (C) Quantification shows a significant difference in the number of myelinated axons between WT (N=4) and MUT (N=7, p<0.00534) (D) There is also a significant difference in the number of total axons (p<0.00486). TEM images of *actr10*<sup>+/+</sup> (WT, E), and *actr10*<sup>stl83/stl83</sup> (MUT, F) zebrafish larvae are similarly pseudocolored to show myelinated axons and naked axons (asterisks). (G) There is a significant reduction in myelinated axons between WT (N=6) and
MUT (N=6, p<1.68457e-7)). (H) There were also significantly fewer axons in MUT compared to WT (p<0.04974). TEM images at 5 dpf of actr10+/+ (WT, I), and actr10nl15/nl15 (MUT, J) show myelinated axons pseudocolored in blue. (J) The actr10nl15/nl15 mutant shows neurofilament accumulation and abnormal degeneration and swelling at 5 dpf (arrowheads, N=4/6) (K) Quantification of number of myelinated axons shows a significant decrease in MUT (N=6) compared to WT (N=4, p<8.71309e-7). (L) At 5 dpf, there is also a significant decrease in number of total axons between MUT and WT (p<4.51358e-5). TEM images of actr10+/+ (WT, M), and actr10nl83/stl83 (MUT, N) zebrafish larvae at 5 dpf show myelinated axons (pseudocolored) and unmyelinated axons (asterisks). (N) actr10nl83/stl83 mutants exhibit degenerated or abnormal axons (arrowhead, N=4/6). (O) Myelin defects persist at 5 dpf, with a significant reduction between WT (N=3) and MUT (N=6, p<0.03255). (P) At 5dpf, there are significantly fewer axons in MUT compared to WT (p<5.46213e-6). *p<0.05. **p<0.01, ***p<0.001, ****p<0.0001. A two-sided student’s t-test in R was used for quantification.
Figure 3: actr10<sup>p115/n115</sup> mutants exhibit swellings in the lateral line and neurofilament accumulation in neuromasts.

Both control, which includes both actr10<sup>+/+</sup> and actr10<sup>p115/+</sup> animals (A), and actr10<sup>p115/n115</sup> mutants (B) appear grossly normal by brightfield microscopy at 3 dpf. (C) However, using fluorescent microscopy to observe tg(neurod:egfp) expression demonstrates that while the lateral line in control animals is normal, actr10<sup>p115/n115</sup> mutants (D) have visible swellings (arrows, WT: N=14/14, actr10<sup>p115/+</sup>:N=34/34, actr10<sup>p115/n115</sup>:N=25/25). (E) TEM of an actr10<sup>+/+</sup> animal shows a normal neuromast (N=3/3). (F) A higher magnification shows the absence of swellings. (G) An actr10<sup>p115/n115</sup> mutant, however, shows a large caliber swelling (box, N=4/4). (H) A higher magnification image shows that the swelling is filled with neurofilaments (arrowheads).
Figure 4: actr10 mutants exhibit swelling in the large caliber Mauthner axon and neurofilament accumulation.
(A-D) Brightfield images of control animals and actr10 mutants demonstrate that the WT controls (A, C) are grossly healthier at 5 dpf compared to actr10 mutants (B, D). (E) A lateral view of the zebrafish spinal cord in a control animal shows normal *tg(mbp:egfp-caax)* expression (WT: N=4/4, *actr10*<sup>nl15/nl15</sup>: N=7/7), whereas in an *actr10*<sup>nl15/nl15</sup> mutant (F) there are large caliber swellings (arrowheads, *actr10*<sup>nl15/nl15</sup>: N=7/7). Similarly, a control animal from the *actr10*<sup>sl83</sup> background shows normal expression (G, WT: N=2/2, *actr10*<sup>sl83/+</sup>: N=9/9) while an *actr10*<sup>sl83/sl83</sup> mutant (H) exhibits similar swellings (arrowheads, *actr10*<sup>sl83/sl83</sup>: N=10/10). (I) An electron micrograph of a quadrant of the ventral spinal cord from an *actr10*<sup>+/+</sup> (WT) zebrafish taken shows neuronal cell bodies (asterisks) and the large caliber myelinated Mauthner axon (arrow). (J) A higher magnification image shows myelinated axons (pseudocolored blue) as well as the myelinated large caliber Mauthner axon (arrow). (K) All WT animals had normal Mauthner axons without neurofilament accumulation (N=4/4) (L). An EM image from an *actr10*<sup>nl15/nl15</sup> mutant animal shows no myelinated axons and a swollen Mauthner axon (arrow). (M) A higher magnification image reveals that the swollen Mauthner, in addition to other axons, is filled with neurofilaments (arrows). Additionally, there are swellings that appear to be filled with mitochondria and other cargo (arrowhead). (N) Neurofilaments are visualized in the swollen Mauthner. All mutants examined had neurofilament accumulations in axons (5/5). (O) Another *actr10*<sup>nl15/nl15</sup> mutant shows an extreme swelling of the Mauthner axon. This correlates with the large caliber swellings observed in *actr10*<sup>nl15/nl15</sup> and *actr10*<sup>sl83/sl83</sup> mutants. (P-Q) Higher magnification images show the accumulated neurofilaments in the swollen Mauthner axon (arrow).
Figure 5: Generation of a humanized zebrafish strain with a patient specific actr10 mutation.
(A) Table shows sequencing information from 5 patient families diagnosed with ALS, distal myopathy and CMT2 that also have missense mutations in ACTR10. (B) A protein sequence comparison between human and zebrafish shows the location of the human ACTR10 mutations as well as the location of the stil83 mutation. All mutations are in completely conserved amino acids. (C) A sequence comparison between WT zebrafish DNA and the CMT sequence shows the C-T nucleotide change that results in the proline to serine amino acid change. (D) A gel image of the CMT genotyping assay shows the 165 bp mutant band, which is not cut by the restriction enzyme BseR1. The WT sequence is cut by the enzyme, resulting in two fragments of lengths 107 and 58 bps.
Figure 6: Transheterozygous actr10<sup>nl15/+</sup>; actr10<sup>CMT/+</sup> zebrafish do not have an axonal swelling phenotype at 5 dpf.

(A) A brightfield image shows a zebrafish larva at 5 dpf. (B) All zebrafish scored from the complementation cross had a WT phenotype by <i>tg(neurod:egfp)</i>, including 12 transheterozygous animals.
Figure 7: Actr10 knockdown in mouse DRG culture results in a regeneration defect.

(A) Three different shRNA constructs demonstrated efficient knockdown of Actr10 by qPCR.
(B) Axon regeneration (arrow) in DRG spot culture (arrowhead) after axotomy was normal when
a control shRNA was used. (C) Knocking down Actr10 with shRNA decreased regeneration. (D) The regeneration phenotype was not rescued with addition of a WT ACTR10 construct. (E) Quantification shows decreased regeneration after shRNA Actr10 knock down, which is not rescued by WT, ALS or CMT constructs.
4.7 References


Chapter 5: Conclusions and future directions
5.1 Conclusions and future directions

Starting with a large-scale forward genetic screen in zebrafish, I identified a mutant exhibiting myelin defects in the central and peripheral nervous systems (Chapter 2). I used a whole genome sequencing approach to determine that the gene responsible for the mutant phenotype was in *actin related protein 10 (actr10)* (Chapter 2, Chapter 3, Appendix A). I found through studying the zebrafish *actr10* mutant, and also using oligodendrocyte cell culture, that dynein and dynactin are required for the anterograde movement of *mbp* mRNA granules (Chapter 3). In addition to this role in oligodendrocytes, a role for *actr10* specifically in mitochondrial retrograde transport was identified in peripheral nervous system axons (Appendix B). I demonstrated that *actr10* zebrafish mutants exhibit neurodegenerative phenotypes and I worked with neurologists at Washington University to identify patients with neurodegenerative diseases who also had mutations in *ACTR10*. One patient in particular, who had been diagnosed with Charcot-Marie-Tooth disease type 2, had an *ACTR10* mutation that was not identified in the ExAC browser, indicating it was not a common human background mutation. I therefore decided to generate a zebrafish line with the CMT2 patient *ACTR10* SNP. This was done successfully using TALEN technology. However, I did not observe a neuronal mutant phenotype when I did a complementation cross between *actr10*°°$$^{nl15/}$ and *actr10*°°$$^{CMT/}$ heterozygotes.

In order to determine whether the CMT allele produces a phenotype, the next step will be to outcross F1 *actr10*°°$$^{CMT/}$ animals to produce the F2 generation, which will then be intercrossed to look for a phenotype in homozygous mutants. Another future direction to fully explore the *actr10*°°$$^{CMT}$ allele will be to determine if there are age-dependent phenotypes. Since the patient with CMT2 did not present with the disease until middle age, this is an important experiment that could explain the lack of an early defect. To test whether age could play a role in the zebrafish
phenotype, $actr10^{CMT/+}$ F2 animals will be intercrossed to produce WT, heterozygous, and homozygous $actr10^{CMT/CMT}$ mutants. All viable genotypes will be grown to adulthood (~1-year-old). TEM on zebrafish maxillary barbels will be done to determine if there are myelin or axonal defects at this later stage.

Finally, we are currently collaborating with the lab of Dr. Valeria Cavalli to test whether loss of Actr10 function in mouse dorsal root ganglia (DRG) culture results in regeneration and/or degeneration phenotypes. Thus far, we have knocked down Actr10 in mouse using shRNA (Figure 7A). A regeneration assay in which axons from DRG spot culture are cut found that there was a regeneration defect in the Actr10 knockdown condition (Figure 7B, C). When we attempted to rescue the phenotype with a human ACTR10 construct containing the ALS and CMT2 mutations, there was no rescue (Figure 7D, E). However, there was also no rescue of the phenotype when we added a WT ACTR10 construct (Figure 7E). This preliminary data is therefore inconclusive due to the lack of rescue by WT ACTR10. Future directions will be to determine why the WT ACTR10 construct did not rescue and to perform the assay again to conclude if the ALS and CMT2 ACTR10 human constructs do not rescue. Additional future directions will be to test the degenerative phenotype after knocking down Actr10 in mouse DRG culture and to examine the $actr10^{CMT/CMT}$ mutant zebrafish in the context of peripheral nerve injury. These experiments in mouse will contribute to a more complete modeling of the human diseases.

Although it is too early to irrefutably determine whether the "humanized" zebrafish model was successful, this project has demonstrated some of the constraints with human disease modeling in animal models, and in zebrafish in particular. Future work must address issues such as the multigenicity of human diseases, the differences between zebrafish and human proteins,
and the potential for age related phenotypes not obvious at early larval stages, when the majority of zebrafish research is performed. However, the current rapid pace of innovation makes it quite likely that modeling of patient specific diseases in zebrafish will be a reality in the not too distant future. In conclusion, I used a variety of genetic tools in zebrafish to study neural development. Using a forward genetics approach, I identified new factors involved in myelination, including a mutation in actr10 that results in defects in transport of an mRNA critical for myelination by oligodendrocytes. By using a reverse genetics approach, I generated a zebrafish strain with a human ACTR10 mutation to model a neurological disorder. This dissertation demonstrates the breadth of research possible using zebrafish to investigate the mechanisms of key developmental processes.
Appendix A: Whole genome sequencing-based mapping and candidate identification of mutations from fixed zebrafish tissue
Preface:

This chapter has been reproduced and adapted in its entirety from the following published manuscript:

A.1 Abstract

As forward genetic screens in zebrafish become more common, the number of mutants that cannot be identified by gross morphology or through transgenic approaches, such as many nervous system defects, has also increased. Screening for these difficult to visualize phenotypes demands techniques such as whole-mount in situ hybridization (WISH) or antibody staining, which require tissue fixation. To date, fixed tissue has not been amenable for generating libraries for whole genome sequencing (WGS). Here, we describe a method for utilizing genomic DNA from fixed tissue and a bioinformatics suite for WGS-based mapping of zebrafish mutants. We tested our protocol using two known zebrafish mutant alleles, gpr126<sup>st49</sup> and egr2b<sup>fh227</sup>, both of which cause myelin defects. As further proof of concept we mapped a novel mutation, stl64, identified in a zebrafish WISH screen for myelination defects. We linked stl64 to chromosome 1 and identified a candidate nonsense mutation in the F-box and WD repeat domain containing 7 (fbxw7) gene. Importantly, stl64 mutants phenocopy previously described fbxw7<sup>vu56</sup> mutants, and knock-down of fbxw7 in wild-type animals produced similar defects, demonstrating that stl64 disrupts fbxw7. Together, these data show that our mapping protocol can map and identify causative lesions in mutant screens which require tissue fixation for phenotypic analysis.

A.2 Introduction

Zebrafish (Danio rerio) have emerged as an ideal model organism for both genetic and chemical screens due to their vertebrate anatomy and physiology, large clutch sizes, fully sequenced genome, transparency during development, and ease of maintenance. Forward genetic screens in zebrafish have uncovered many new mutant alleles that disrupt key regulators of a wide variety of developmental and cellular biological processes (Driever et al. 1996; Haffter et
al. 1996). However, the rate at which new mutants are generated has not been matched by the rate of linking a mutant phenotype to a specific causative lesion, which has created a backlog of unmapped mutants. Traditional PCR-based mapping methods are extremely time consuming, taking months or even years (Talbot & Schier 1998; Geisler et al. 2007; Zhou & Zon 2011). With the relatively recent advent of next-generation sequencing approaches such as whole genome sequencing (WGS) and RNA-sequencing, a phenotype can be linked with a genomic region or a specific mutation in a matter of days or weeks (Bowen et al. 2012; Leshchiner et al. 2012; Obholzer et al. 2012; Voz et al. 2012; Miller et al. 2013).

However, while mapping with WGS can allow rapid identification of causative mutations, this technology to date has only been applied to screens wherein phenotypes can be easily identified by gross anatomical observation or by fluorescent transgenes, which allow genomic DNA (gDNA) extraction from fresh tissue. To our knowledge, WGS-based mapping protocols have not been applied to screening strategies that require tissue fixation such as whole-mount in situ hybridization (WISH) or antibody staining. In these strategies, the phenotypes can only be identified after the samples have been fixed and have undergone a variety of other chemical manipulations, which compromise not only the ability to extract sufficient amounts of gDNA, but also the quality of gDNA obtained.

One good example of a biological process that is difficult to screen without tissue fixation is myelination. Myelin is a multilamellar, lipid rich membrane that is iteratively wrapped around neuronal axon segments. It is produced by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Myelin is a jawed vertebrate innovation (Zalc et al. 2008) and as such, zebrafish are the most tractable model organism to screen for genes involved in the formation of myelin and the development of the myelinating glia
(Kazakova et al. 2006; Pogoda et al. 2006). However, while disruptions to myelinating glia result in debilitating symptoms in a wide variety of neurological disorders in humans, disruptions to genes specifically affecting myelination do not typically result in gross morphological abnormalities during early zebrafish development. Further, although there are transgenic lines that label myelinating glia, changes in myelination can be difficult to assess by simple transgenic screening, especially in the PNS. Therefore, one common approach to screen for myelination defects in both the CNS and PNS has been to assess the expression of *myelin basic protein (mbp)* by WISH or antibody staining (Kazakova et al. 2006; Pogoda et al. 2006).

Here we describe methods for extracting gDNA from zebrafish larvae after WISH and present a WGS and bulked segregate analysis (BSA) based approach to link mutant phenotypes identified in forward genetic screens to a genomic region and identify possible causative mutations. We validated our approach using known mutations and report a novel mutation uncovered in a forward genetic screen for regulators of myelination.

### A.3 Results

The specialized glia that produce myelin provide vital trophic support to ensheathed axons, while the myelin sheath itself protects axons and allows for saltatory conduction of action potentials (Nave 2010). The first forward genetic screens in zebrafish for disruptions in myelinating glial cell development utilized WISH to assess changes in the levels or patterns of *mbp* expression in the CNS and PNS (Pogoda et al. 2006; Kazakova et al. 2006). Over the years, as the causative mutations for these mutant phenotypes were identified, the study of these genes has contributed tremendously to the understanding of the development of myelinating glia. However, there are still many aspects of myelinating glial cell development and myelination that remain mysterious.
Therefore, to identify novel regulators of oligodendrocyte and Schwann cell development, we conducted a large-scale three-generation forward genetic screen in zebrafish. The genomes of 80 adult male zebrafish were randomly mutagenized with N-ethyl-N-nitrosourea (ENU) to produce the founder generation. We then drove these mutations to homozygosity and assessed the third generation (F3) progeny for changes in mbp expression patterns in the CNS and PNS by WISH using a riboprobe for mbp (Brösamle & Halpern 2002). The extensive tissue fixation and chemical manipulation of these samples precluded known WGS-based methods for mapping putative causative mutations for any phenotypes recovered. Therefore, to take advantage of the power and speed of WGS in mapping mutations, we developed a WGS-BSA pipeline using gDNA isolated from fixed tissue.

The WGS-BSA pipeline accurately maps the st49 allele to gpr126 using gDNA from fresh tissue

To establish our pipeline, we first sequenced a known mutation that results in phenotypes easily observed by gross morphology so that we could test the ability of our WGS analysis scheme to correctly identify a causative lesion. The st49 mutant allele was uncovered in a forward genetic screen in zebrafish for mutants with developmentally disrupted mbp expression (Pogoda et al. 2006). The causative mutation was later identified through traditional PCR-based linkage mapping and Sanger sequencing of genes in the linked area as a T to A transition resulting in a nonsense mutation in the gene adgrg6/gpr126 (Monk et al. 2009). Importantly, in addition to decreased mbp expression in the PNS observed by WISH, at 5 days post-fertilization (dpf), gpr126st49/st49 animals display a swollen ear phenotype (Fig 1A-B) that readily distinguishes mutant animals from their wild-type and heterozygous siblings in living larvae (Monk et al. 2009; Geng et al. 2013). We took advantage of this obvious morphological defect to
collect a pool of phenotypically mutant larvae with swollen ears and a pool of siblings that displayed phenotypically normal ears (N=30 larvae per pool).

We then extracted gDNA from each pool and submitted both samples to the Genome Technology Access Center (GTAC) at Washington University for WGS on a single lane of the Illumina HiSeq3000. Sequencing reads for both pools were aligned to the Zv9 genome build of the zebrafish genome using Novoalign (Howe et al. 2013) (Table 1), and variants (SNPs, insertions, and deletions) were called using SAMtools (Li et al. 2009; Li 2011) and annotated using snpEFF (Cingolani et al. 2012). Our sequence analysis pipeline consists of three different custom Perl scripts that can be run locally on any workstation and easily tailored to specific circumstances. Henceforth, these scripts will be referred to as “ChromSplit,” “Allele Ratio Calculator (ARC),” and “SNPfilter.”

For both sibling and mutant datasets, we used the ChromSplit script to split each variant in the BED file based on genomic position into separate files for each of the 25 zebrafish chromosomes. Mitochondrial DNA and variants currently assigned to genomic scaffolds were excluded. The second script, ARC, then used the 50 individual chromosome files – 25 sibling and 25 mutant – to group variants into non-overlapping bins based on chromosomal position and calculate the mutant allele frequency (MAF) in each bin. As a starting point, we set the bin size to 200 kilobases (kb). In total, 6,735 bins of a 200 kb size were generated containing at least one SNP with the variant allele being seen in some frequency in both the sibling and mutant pools. Finally, using ARC we compared the MAF for each bin between siblings and mutants to calculate the ratio of homozygosity between these two groups. Any bins with fewer than 10 SNPs were subsequently removed due to the outsized ability of any outliers to affect the
calculated ratios. In this instance, 6,531 bins had 10 or more SNPs with variant alleles seen in both the mutant and sibling \textit{st49} pools data.

To determine the specific chromosome linked to the mutant phenotype, we graphed the mutant/sibling allele ratios for all bins by genomic position and looked for regions of high homozygosity as seen by sequential bins with an allele ratio of approximately 2. For a variant that is homozygous in the mutant pool and with perfect Mendelian ratios in the sibling pool, the theoretical expected allele ratio/mapping score is 3 at single bp resolution. We expect our mapping scores to approach this limit but rarely, if ever, reach it because we utilize a non-overlapping bin size of 200 kb to calculate the mapping score. The overall allele ratio/mapping score is reduced in the individual bins because we are using a large bin filled, in some cases, with hundreds of SNPs from highly heterogenous genetic backgrounds several generations removed from isogenic mapping strains. Additionally, we do not control for the genotypic ratio in the sibling pool, thus in practice we observe allele ratios closer to 2 for bins with high rates of homozygosity. When graphing the mutant/sibling allele ratios of all the bins across the entire genome for \textit{st49} mutants, there was a clear peak at chromosome 20, indicating linkage to the swollen ear phenotype (Fig 1C). When viewing only chromosome 20, three distinct regions displayed the highest levels of SNP ratio imbalances between the mutant and sibling pools, indicating linkage of these regions to the mutant phenotype used to sort the pools. These three distinct regions were centered around chromosomal positions 12 MB, 25 MB, and 39 MB (Fig 1D).

Finally, we used SNPfilter to eliminate variants in protein-coding sequences that had been previously annotated by SNPFisher (Butler et al. 2015), a database of SNPs observed in non-mutagenized zebrafish. This process eliminated SNPs that were present in WT populations
of zebrafish, and therefore were unlikely to cause our phenotype of interest. All SNPs annotated by SNPeff as protein coding that resulted in non-synonymous amino acid changes and that were verified to be homozygous in the mutants but not siblings using the integrative genomics viewer (IGV) (Thorvalsdóttir et al. 2013; Robinson et al. 2011) were considered putative causative lesions. The st49 mutant pool had 71,791 SNPs annotated by SNPeff (Table 2) as coding SNPs. After filtering out known SNPs, 35,730 SNPs remained. Only 29 SNPs fall into any of the three intervals linked to the st49 mutant phenotype (Fig 1E-G). Of all the SNPs that pass all filtering, the predicted most deleterious SNP was a T to A nonsense mutation in gpr126, which is the causal mutation for the gpr126st49 allele (Monk et al. 2009).

The WGS-BSA pipeline accurately maps the fh227 allele to egr2b using gDNA from fixed tissue

Our WGS analysis pipeline accurately predicted the st49 allele to be a nonsense mutation in gpr126, but the gDNA used for WGS was from fresh tissue. To determine if we could use the same process to map mutants from our WISH-based forward genetic screen, we tested our pipeline using a mutation known to disrupt mbp expression in the PNS by WISH. The fh227 allele was discovered through targeting induced local lesions in genomes (TILLING) (Moens et al. 2008) for mutations in egr2b (krox20) and is a C to A point mutation that leads to a premature termination codon (PTC) (Monk et al. 2009). egr2bfh227 mutants display severely decreased mbp expression in the posterior lateral line nerve (pLLN) of zebrafish (Monk et al. 2009). Zebrafish genotyped as egr2bfh227/+ were intercrossed and WISH was performed on the resulting progeny at 5 dpf using an mbp riboprobe. Individuals were pooled based on the mbp expression phenotype – either phenotypically wild-type (Fig 2A; N=33) or reduced mbp expression (Fig 2B; N=33).
To extract gDNA from fixed WISH larvae with high yields, we modified the animal tissue (column-based) protocol included with the Qiagen DNeasy Blood & Tissue kit. Two of these modifications were incubating 10 minutes at 56°C in ATL buffer and again when samples are in AL buffer. Per the manufacturer, the ATL and AL buffers occasionally generate precipitates that can be prevented by brief warming at 56°C. Therefore, we added short incubations as precautions given the precious nature of the samples. To increase gDNA yield, we made two additional modifications – the Proteinase K digestion was increased from 30 minutes to 3 hours, and the elution incubation was lengthened to at least 20 minutes. We found that using the recommended digestion and elution times resulted in at least 10-fold lower yield. Thus, since many non-column-based gDNA extraction protocols for a variety of animal tissues involve Proteinase K digestion steps of 12 hours or more, we increased the digestion time to 3 hours and elution time to >20 minutes and saw a dramatic improvement in gDNA yield (from ~2-10ng/ul to ~75-150ng/ul). Digestion times greater than 3 hours did not continue to notably increase yield.

All samples were submitted for WGS using the same specifications as described above to map *st49*. We also subjected the *fh227* WGS data to the same analysis paradigm as outlined for *st49*. In total, 6,783 bins of a 200 kb size were generated for the *fh227* pools, 6,725 of which contained 10 or more SNPs. We then graphed the mutant/sibling allele ratio by genomic position and found a clear peak of homozygosity on chromosome 12, indicating linkage to the *fh227* phenotype (Fig 2C). When viewing only chromosome 12, the most divergent region between mutant and sibling pools, and thus most linked to the *fh227* phenotype, is a single distinct genomic region centered around the 10 MB mark (Fig 2D).

The *fh227* mutant pool had 167,060 SNPs annotated by SNPeff as coding SNPs (Table 2). After filtering out known SNPs, 81,832 SNPs remained. Notably, only 21 SNPs fell into the
interval linked to the *fh227* mutant phenotype (Fig 2E). Of all SNPs that passed all filtering, the predicted most deleterious SNP was a nonsense mutation in *egr2b*, which is the known causal mutation for the *fh227* allele (Monk et al. 2009).

**The WGS-BSA pipeline accurately maps the novel *stl64* mutation to *fbxw7***

We have demonstrated that we can utilize gDNA from fixed tissue and successfully perform WGS with that DNA. We have also shown that we can use BSA to link a phenotype to a specific genomic region, and through SNP filtering, we can identify the specific causative mutation. To demonstrate that we can successfully map a novel allele, we ran one of the first mutants from our screen though the WGS-BSA pipeline. This mutant allele, provisionally designated *stl64*, displayed striking overexpression of *mbp* in the CNS when compared to siblings at 5 dpf (Fig 3A-B).

To determine the causative lesion in the *stl64* mutants, we crossed *stl64* carriers and used WISH with an *mbp* riboprobe to identify mutants. As before, we pooled the larvae into two groups based on their *mbp* expression phenotype – normal *mbp* levels (Fig 3A; N = 23) and enhanced *mbp* expression (Fig 3B; N=26). gDNA from both samples was submitted for WGS on the Illumina HiSeq2500 as described above for the *st49* and *fh227* alleles. Sequencing reads for both pools were aligned using Novoalign to the same Zv9 genome build of the zebrafish genome as was used to map *st49* and *fh227* (Table 1). SNPs were again called and annotated using SAMtools and snpEFF. In total, 6,790 bins of a 200 kb size were generated for the *stl64* pools, of which 6,746 bins contained 10 or more SNPs. The mutant/sibling allele ratios for *stl64* were highest on chromosome 1 (Fig 3C). Closer inspection of chromosome 1 showed that the ratios of SNPs in the mutant and sibling pools were most different, and thus most highly linked, at a single distinct region centered around chromosomal position 23 MB (Fig 3D).
The stl64 mutant pool had ~155,550 SNPs annotated by SNPeff as coding SNPs (Table 2), 84,553 of which remained after filtering out known SNPs. Of those 84,553 SNPs, only 3 SNPs fell into the interval linked to the stl64 mutant phenotype, were protein coding, and were verified in IGV as homozygous in the mutant pool but not in the sibling pool (Fig 3E). Among these 3 SNPs, the predicted most deleterious SNP was a C to A transition resulting in a PTC in fbxw7.

Previously, zebrafish fbxw7^{vu56} mutants were shown to possess an increased number of oligodendrocytes and to display hypermyelination in the CNS (Snyder et al. 2012; Kearns et al. 2015). To determine if the overexpression of mbp in the CNS of stl64 mutants was similarly due to increased oligodendrocyte numbers, we performed WISH using a riboprobe against nkx2.2a, which marks the oligodendrocyte lineage. At 65 hours post-fertilization (hpf), stl64 mutants displayed more nkx2.2a positive cells in the spinal cord than their WT siblings (Fig 4A-B). Further, ultrastructural analyses by transmission electron microscopy (TEM) revealed increased numbers of myelinated axons as well as thicker myelin in the spinal cords of stl64 mutants at 8 dpf (Fig 4 C-J). To further test if fbxw7 regulates myelination we employed an established antisense oligonucleotide morpholino (MO) (Snyder et al. 2012) to reduce fbxw7 levels in WT embryos. We then assessed mbp expression by WISH, and found that larvae injected with low doses of the fbxw7-MO displayed increased mbp expression (Figure 4K-L). Together, our WGS mapping using fixed-tissues, the phenotypic similarities of stl64 and fbxw7^{vu56} mutants, and our fbxw7-MO analyses strongly support that the stl64 mutation represents a new allele of the fbxw7 gene.
A.4 Discussion

Here, we have described a WGS-BSA pipeline for identifying the causative mutations for phenotypes discovered in zebrafish forward genetic screening strategies requiring fixed tissue. We tested this protocol by mapping two alleles, \textit{gpr126}$^{st49}$ and \textit{egr2b}$^{fh227}$, known to disrupt myelination. Furthermore, we successfully mapped a novel allele, \textit{stl64}, generated in a WISH-based forward genetic screen, to a nonsense mutation in the gene \textit{fbxw7}.

In validating the WGS-BSA pipeline, the importance of genetic variation in the lines sequenced became clear with the sequencing of the \textit{gpr126}$^{st49}$ pools. There were three linkage peaks for the \textit{gpr126}$^{st49}$ pools compared to only a single linkage peak in the \textit{egr2b}$^{fh227}$ and \textit{fbxw7}$^{stl64}$ pools. We believe this is due to the crossing history of each line. The \textit{egr2b}$^{fh227}$ line was maintained on an AB* (ZFIN ID: ZDB-GENO-960809-7) background with intermittent outcrossing to the wild-caught, IND line (ZFIN ID: ZDB-GENO-980210-28). Similarly, the screen that generated the \textit{fbxw7}$^{stl64}$ allele started on the SAT background (ZFIN ID: ZDB-GENO-100413-1) before being outcrossed to SJD (ZFIN ID: ZDB-GENO-990308-9) (Johnson et al. 1996) and other fish with mixed backgrounds (pigment mutants and transgenes) before sequencing. The outcrossing of both the \textit{egr2b}$^{fh227}$ and \textit{fbxw7}$^{stl64}$ lines drove down the level of homozygosity across their respective genomes and allowed the difference in ratio between the sibling and mutants pools to be more easily observed. In contrast, the \textit{gpr126}$^{st49}$ line in our lab has been maintained exclusively on the AB* background without intermittent outcrossing. This difference in crossing history has driven up the level of homozygosity and led to three distinct regions of the genome being linked to the \textit{st49} phenotype. This large linkage peak underscores the importance of performing at least one outcross before collecting mutants for pooled sequencing.
It was also apparent from our analyses that coverage of the genome when using WGS was especially important for filtering of putatively causative SNPs. For *st49*, in the middle of the third linked region on chromosome 20 (spanning 37.4 Mb to 41.6 Mb), 8 bins had fewer than 10 SNPs fall into them and were subsequently filtered out. This differs from both the *fh227* and *stl64* sequencing, which had hundreds of SNPs across the entire region. The loss of the 8 bins in the middle of the region likely suppressed the expected signal from the true *gpr126*<sup>st49</sup> mutation, contributing to the generally broad linkage observed. Additionally, when verifying the homozygosity of the *gpr126*<sup>st49</sup> allele in the mutant sequencing, a single read was all that covered the mutation in the *st49* sibling pool, further underscoring the importance of coverage depth in calling variants. Based on the sequencing described here, a target of 100 million mapped reads is the minimal target recommended for future experiments.

We have shown that linking a genomic region to a phenotype using our WGS-BSA approach is relatively robust and is able to overcome excessive inbreeding and relatively low coverage; however, determining if a specific mutation from the linked region is causative has several important caveats. The filtering methods described here are specific to defining coding variants introduced by mutagenesis and are not applicable to all scenarios. For example, in the case of an allele that was simply an endogenous, rare, recessive mutation driven to homozygosity in the process of the screen, the SNPFisher database of naturally occurring variants would be inappropriate to use as a filter. Similarly, if the responsible mutation is in a regulatory region, limiting SNPs to only those designated as coding by SNPeff is similarly inappropriate. In both these cases, SNPs previously observed as homozygous should still be removed, but the SNPFisher database should not be included.
In the case where an allele’s causative mutation is a nonsynonymous mutation introduced through mutagenesis, as opposed to the three nonsense mutations described here, we propose a schema to prioritize the most likely causative mutation. Highest priority should be given to mutations affecting splice sites or out of frame insertions and deletions (INDELS). Below that, priority should be given to nonsynonymous SNPs that disrupt protein domains or cause shifts in polarity or hydrophobicity between amino acid side chains. The mutation with the highest probability to be causative should be confirmed by creation of a second allele and complementation testing.

Despite the caveats regarding coverage, outcrossing, and SNP filtering, our demonstrated ability to extract gDNA from fixed tissue in order to map and characterize mutations is important. Using WGS with BSA over traditional PCR-based mapping methods can dramatically shrink the time required to map some mutants. Including fixed tissue as a DNA source for this procedure opens up this approach for a cadre of mutants not easily identified by gross morphological changes. As a result, many new genes may be discovered from existing mutants and new screens can be envisioned involving these types of screens using fixed zebrafish tissues. We note that our protocol to obtain high-quality gDNA from fixed tissue can be paired with any number of useful pipelines already described to define causative lesions by WGS (e.g., Henke et al. 2013).

In conclusion, as WGS has increased the speed by which mutants discovered in forward genetic screens can be linked to a causative region and mapped to a specific lesion, the inability to use WGS to map alleles that need to be phenotyped using fixed tissue has held back the mapping of an entire class of alleles. To address this hurdle, we created a WGS-BSA pipeline for sequencing and analysis of gDNA extracted from pools of fixed or fresh tissue. This pipeline
compares areas of the genome between mutant and sibling pools to calculate the ratios of variant to reference alleles for that specific region. Furthermore, filtering out natural variants and variants previously observed as homozygous from the list of predicted protein coding variants in the linked regions allows for an accurate attribution of causality to a specific mutation. Using this WGS-based mapping pipeline, we correctly identified the previously described mutations, \textit{gpr126}^{st449} \textit{and egr2b}^{fh227}, when gDNA was extracted from pools of either fresh or fixed tissue, respectively. To test the ability of the WGS-BSA pipeline to map a novel mutant, we used this pipeline to identify the causative lesion in an unidentified CNS hypermyelination mutant, designated \textit{stl64}, from our WISH-based forward genetic screen. We found that \textit{stl64} was most strongly linked to a 3 Mb region of chromosome 1, with the most deleterious mutation being a C to A transition leading to a premature termination codon in the gene \textit{fbxw7}. Importantly, \textit{stl64} mutants phenocopy the CNS myelination defects observed in a previously described mutation in the same gene, \textit{fbxw7}^{vu56}. Finally, loss of \textit{fbxw7} in WT zebrafish larvae resulted in similar overexpression of \textit{mbp}, further supporting the conclusion that \textit{stl64} disrupts the gene \textit{fbxw7}.

These experiments demonstrate that our new pipeline can successfully identify causative lesions for mutants that can only be analyzed using fixed tissue preparations. We believe that this pipeline can be easily applied to extract gDNA from pooled fixed or fresh tissue for WGS with BSA to determine the protein coding causative mutation in zebrafish screen mutants.
A.5 Figures and tables

Figure 1: The st49 allele is accurately mapped to chromosome 20 and gpr126 using gDNA extracted from fresh tissue.
Dorsal views of phenotypically wild-type (A) and gpr126<sup>st49/st49</sup> (B) zebrafish show the characteristically enlarged ears (arrowheads) of mutants at 5 days post-fertilization (dpf). When the ratio of variant to reference alleles in the mutant pool is compared to the sibling pool and graphed across the whole genome for gpr126<sup>st49</sup>, there is a clear spike on chromosome 20 for the gpr126<sup>st49</sup> mutation (Box) (C). This spike indicates linkage of the region to the trait used to sort the mutant and sibling pools. gpr126<sup>st49</sup> is linked to three separate regions on chromosome 20 (Arrows) (D). Linkage map of the gpr126<sup>st49</sup> allele showing the 3 different regions of chromosome 20 linked to the expanded ear phenotype that was used to sort the mutant and sibling pools. Between all 3 linked regions there are 29 different protein coding, homozygous, non-synonymous SNPs (E-G). The single introduced stop is the mutation responsible for the gpr126<sup>st49</sup> mutant phenotype.
Figure 2: The fh227 allele is accurately mapped to chromosome 12 and egr2b using gDNA extracted from fixed tissue.

Dorsal view of mbp expression in 5 dpf zebrafish using WISH and the mbp riboprobe shows phenotypically normal expression (purple) of mbp along the pLLNs (arrowheads) (A) compared to severely reduced mbp expression along the pLLN (arrowheads) in egr2b<sup>fh227/fh227</sup> mutants (B). When the ratio of variant to reference alleles in the mutant pool is compared to the sibling pool and graphed across the whole genome for egr2b<sup>fh227/fh227</sup>, a clear spike on chromosome 12 is observed (Box) (C). This spike indicates genotypic linkage to the trait used to sort the mutant and sibling pools. When looking at chromosome 12, egr2b<sup>fh227</sup> is linked to a single region centered around 10 Mb (Arrow) (D). Linkage map of the egr2b<sup>fh227</sup> allele showing the 21 different homozygous, non-synonymous, protein coding SNPs in the single chromosome 12 region linked to the
decreased $mbp$ expression in the PNS that was used to sort the mutant and sibling pools (E). The single introduced stop is the mutation responsible for the $egr2^{b227}$ mutant phenotype.
Figure 3: The *stl64* phenotype is linked to chromosome 1 and is likely caused by a nonsense mutation in *fbxw7*.

Dorsal views of 5 dpf zebrafish showing *mbp* expression by WISH using the *mbp* riboprobe. Normal expression in the CNS (arrowhead) by phenotypically wild-type siblings (A) compared to the dramatic overexpression of *mbp* in the *stl64* mutants (B) shows increased *mbp* expression in the *stl64* mutants. When the ratio of alleles in the mutant pool compared to the sibling pool is graphed across the whole genome for the *stl64* allele, a clear spike on chromosome 1 (Box) is
observed for *stl64* (C). This spike indicates genomic linkage to the trait used to sort the mutant and sibling pools. When viewing chromosome 1, *stl64* is linked to a single region of the chromosome centered around 24 Mb (Arrow) (D). Linkage map of the *stl64* allele showing the three protein coding, homozygous, non-synonymous SNP linked to the increased *mbp* expression in the CNS that was used to sort the mutant and sibling pools. The most deleterious SNP is the introduced stop codon in *fbxw7* (E).
Figure 4: The \textit{fbxw7}^{stl64} allele phenocopies the \textit{fbxw7}^{vu56} allele.

\textit{stl64} mutants display more \textit{nklx2.2a}+ cells in the dorsal spinal cord (SpC) relative to their WT siblings at 65 hours post-fertilization (hpf) (A,B). TEM analysis of the ventral (C-F) and dorsal (G-J) SpC at 8 dpf shows that \textit{fbxw7}^{stl64} mutants have more myelinated axons in the dorsal SpC (I) and thicker myelin in both regions (F, J). Transient morpholino (MO) knockdown of \textit{fbxw7} in
WT embryos results in \textit{mbp} overexpression at 65 hpf compared to control injected siblings (K, L). Error bars are S.D. ** = p < 0.01, *** = p < 0.001, NS = not significant.
Table 1: WGS Coverage of the *gpr126, egr2b* and *stl64* Pools.

Coverage statistics for each pool sequenced. Mapped reads are counted as mapped when an individual read is mapped to the genome without regard for its pairs. Aligned pairs are counted as only those reads where both reads of the pair were able to be aligned. All alignment calculations are in regard to the ability of Novoalign to map a read accurately. Coverage calculations are based off the actual depth of coverage across the Zv9 build of the zebrafish genome.

<table>
<thead>
<tr>
<th></th>
<th>Total Mapped Reads</th>
<th>Total Aligned Read Pairs</th>
<th>% Genome ≥ 5x</th>
<th>% Genome ≥ 10x</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gpr126</em>&lt;sup&gt;st49&lt;/sup&gt; Siblings</td>
<td>63,359,360</td>
<td>27,209,088</td>
<td>67.34%</td>
<td>49.39%</td>
</tr>
<tr>
<td><em>gpr126</em>&lt;sup&gt;st49&lt;/sup&gt; Mutants</td>
<td>58,637,164</td>
<td>24,936,351</td>
<td>66.59%</td>
<td>49.37%</td>
</tr>
<tr>
<td><em>egr2b</em>&lt;sup&gt;fh227&lt;/sup&gt; Siblings</td>
<td>95,221,594</td>
<td>44,326,398</td>
<td>74.86%</td>
<td>46.83%</td>
</tr>
<tr>
<td><em>egr2b</em>&lt;sup&gt;fh227&lt;/sup&gt; Mutants</td>
<td>93,143,924</td>
<td>43,264,449</td>
<td>74.86%</td>
<td>47.31%</td>
</tr>
<tr>
<td><em>stl64</em> Siblings</td>
<td>99,225,270</td>
<td>41,977,069</td>
<td>65.45%</td>
<td>20.94%</td>
</tr>
<tr>
<td><em>stl64</em> Mutants</td>
<td>116,915,836</td>
<td>49,043,710</td>
<td>69.69%</td>
<td>30.12%</td>
</tr>
</tbody>
</table>
Table 2: Number of Exonic SNPs and INDELS in the *gpr126*, *egr2b* and *stl64* Pools.

Number of mutations, by SNPs and INDELS, to the reference genome (Zv9) for all pools sequenced.

<table>
<thead>
<tr>
<th></th>
<th>Number of Exome SNPS</th>
<th>Number of Exome INDELS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>gpr126&lt;sup&gt;st49&lt;/sup&gt; Siblings</strong></td>
<td>74,146</td>
<td>1,843</td>
</tr>
<tr>
<td><strong>gpr126&lt;sup&gt;st49&lt;/sup&gt; Mutants</strong></td>
<td>66,055</td>
<td>1,798</td>
</tr>
<tr>
<td><strong>egr2b&lt;sup&gt;fh227&lt;/sup&gt; Siblings</strong></td>
<td>153,942</td>
<td>2,862</td>
</tr>
<tr>
<td><strong>egr2b&lt;sup&gt;fh227&lt;/sup&gt; Mutants</strong></td>
<td>155,256</td>
<td>2,884</td>
</tr>
<tr>
<td><strong>stl64 Siblings</strong></td>
<td>126,026</td>
<td>2,644</td>
</tr>
<tr>
<td><strong>stl64 Mutants</strong></td>
<td>160,112</td>
<td>3,275</td>
</tr>
</tbody>
</table>
A.6 Methods

Zebrarfish Husbandry and Genotyping

All Danio rerio stocks were maintained in the Washington University Zebrafish Consortium facility (http://zebrafish.wustl.edu/husbandry.htm). Embryos were collected from paired matings and raised in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) at 28.5°C. At 5 dpf, larvae were transitioned to a rotifer-based diet for 10-14 days before incorporating flake food and flowing water. Traditional morphological markers were used to stage embryos (Kimmel et al. 1995). When necessary for WISH, development of pigment was prevented by adding 0.003% phenylthiourea (PTU) to egg water around 24 hpf and maintained until fixation at 5 dpf. All animal experiments were performed in compliance with Washington University’s institutional animal protocols.

Forward Genetic Screen

A standard three-generation forward genetic screen was performed in zebrafish using the chemical mutagen, N-ethyl-N-nitrosourea (ENU) (Mullins et al. 1994; Solnica-Krezel et al. 1994). Briefly, adult WT (SAT strain) males were mutagenized using 3-3.5 mM ENU over the course of 4-6 weeks, allowed to recover for 1 month, and then crossed to WT (SAT) females, resulting in the F1 generation (Draper et al. 2004). Each F1 individual was then outcrossed to a double transgenic line [tg(lhx1a:egfp) (Swanhart et al. 2010); tg(mbp:mcherry-CAAX) (kind gift from Dave Lyons, University of Edinburgh), on a mixed-strain WT background], which marked a subset of neurons (lhx1a) and all myelinating glia (mbp). This cross produced F2 families, which were raised together and then intercrossed to drive putative mutations to homozygosity in the F3 progeny. Clutches of F3 larvae that showed altered mbp promoter expression by transgene were selected for verification using WISH to directly assess mbp expression. When initial
transgenic screening proved to be inconsistent, all subsequent screening was done exclusively by WISH using the *mbp* riboprobe.

**Whole Mount *in situ* hybridization (WISH)**

We used standard protocols (Thisse & Thisse 2008) to perform WISH on zebrafish larva. After 24 hpf, embryos were raised in egg water with 0.003% PTU to prevent the development of pigment. At the desired developmental stages, pools of 25-40 larvae were anesthetized and then fixed in 4% paraformaldehyde (PFA) for 2 hours at room temperature or 4°C overnight with gentle agitation. After fixation, samples were then dehydrated in 100% methanol overnight at -20°C. Following dehydration, embryos were washed in 0.2% PBS-Tween (PBSTw), permeabilized with proteinase K (20 mg/µl diluted 1:1000 in 0.2% PBSTw), and incubated with digoxygenin-labeled riboprobe overnight at 65°C in hybridization buffer (50% formamide). Following probe treatment, embryos were washed and then blocked for at least 1 hour at room temperature in a solution of 2% blocking reagent made in maleic acid buffer with 0.2% triton (MABTr) supplemented with 10% normal sheep serum. Samples were then incubated overnight at 4°C in primary antibody (Anti-Dig, Fab fragments (1:2000); Roche, Pleasanton, CA) diluted in blocking solution, with gentle agitation. Following primary antibody treatment, embryos were repeatedly washed in MABTr, and developed by alkaline phosphatase reaction. Embryos were then post-fixed in 4% PFA, passaged through increasing concentrations of glycerol, and stored long-term in 70% glycerol at 4°C, protected from light. The *mbp* (Brösamle & Halpern 2002) and *nkx2.2a* (Barth & Wilson 1995) riboprobes have been previously described. All scoring was performed blinded to genotype.
Genotyping $egr2b^{fh227}$ and $fbxw^{7d64}$

DNA for genotyping was extracted by adding 50 µl of fish lysis buffer (10 mM pH 8 Tris, 1 mM EDTA 0.3% tween and 0.3% glycerol) to tissue. After cooling from a 10 minute digestion at 98°C, 10 µl of 10 mg/ml proteinase K was added to each sample and all samples were digested for 12 hours at 55°C. The proteinase K was inactivated by incubating at 98°C before storing the DNA for later use. This process tended to provide low amounts of fragmented gDNA that was sufficient for genotyping but not sufficient for WGS.

The $egr2b^{fh227}$ allele was genotyped by using the following primers 5’-GAGGACTTTCGCTCTTTTTG-3’ and 5’-TCGGACGAACTTACCAGACAC-3’, which amplified a 228 base pair (bp) region including the $egr2b^{fh227}$ mutation. These primers were redesigned from those described in Monk et al. (2009) to better amplify the smaller size fragments of gDNA that typically were extracted from fixed tissue. A 40 cycle PCR with 60°C annealing temperature and 45 second extension time was performed on all samples. To assay the $egr2b^{fh227}$ mutation status, disrupts of an NsiI restriction site was used as described previously (Monk et al. 2009). PCR amplicons were digested with the NsiI enzyme (New England Biosciences, Ipswich, MA) for 2 hours at 37°C, and then heat inactivated for 20 minutes at 65°C. The sizes of digested products were visualized using a 3% agarose gel with ethidium bromide. Samples with two bands at 161 and 67 bps were scored as $egr2b^{+/+}$ (wildtype), a single uncut band at 228 bp was scored as $egr2b^{fh227/fh227}$ (mutant), individuals with all three size bands were called $egr2b^{fh227/+}$ (heterozygous).

The $fbxw^{7stl64}$ mutation was genotyped using the following primers: 5’-CTCTCCAGTGAGCCAGGTT-3’ and 5’-GCTTCAGGGTCCTACCAAGC-3’, which amplify a 147 bp region surrounding the $stl64$ lesion. PCR was performed for 40 cycles with an
annealing temperature of 55° C and an extension time of 45 seconds. The \textit{stl64} mutation disrupts a Hpy188I restriction site, so this disruption was used to genotype individual fish. PCR amplicons were digested with the Hpy188I enzyme (New England Biosciences) for at least 1-2 hours at 37° C, and then heat inactivated at 65° C for 20 minutes. Products sizes were visualized on a 2-2.5% agarose gel with ethidium bromide. A thick band around 75 bp was scored as \textit{fbxw7}^{+/+} (wildtype), a single uncut band at 147 bp was scored as \textit{fbxw7}^{stl64/stl64} (mutant), and individuals with both size products were called \textit{fbxw7}^{stl64/+} (heterozygous).

**Genomic DNA Extractions from Fresh Tissue**

Pools of anaesthetized fish in egg water were collected in a 1.5 ml tube, and as much liquid as possible was removed from each tube. 500 µl of fish lysis buffer was added, and the tubes were heated at 98° C for 10 minutes before cooling. Next, 5 µl of 20 mg/ml proteinase k was added, and all tubes were incubated overnight at 55° C with gentle agitation. After digestion, the samples were spun in a centrifuge at 17,900 x g for 1 minute. The resulting supernatant was moved to a new tube and 500 µl of 100% isopropanol was added. The samples and isopropanol were incubated overnight at -20° C then spun for 15 minutes at 17900 x g at room temperature. After centrifuging, the supernatant was removed and 500 µl of 70% ethanol was added. This mixture was then incubated at room temperature for at least 5 minutes before as much liquid as possible was removed with a pipette. Finally, the sample was air dried and the resulting pellet was suspended in TE for later use.

**Genomic DNA Extractions from Fixed Tissue**

For \textit{egr2b}^{fh227} mutants, after \textit{mbp} WISH was completed, zebrafish larvae were passaged into 70% glycerol and scored for \textit{mbp} expression. Mutant animals were separated from their phenotypically wild-type siblings, and phenotypically wild-type and mutant larvae were pooled.
separately. These pools were then backed out of glycerol by replacing the 70% glycerol with 50% glycerol for 5 minutes and then replacing the 50% glycerol with 30% glycerol for another 5 minutes. The 30% glycerol was then replaced with PBSTw. On the other hand, stl64 mutants were not passaged into glycerol before scoring and pooling, and were instead pooled directly into PBSTw. For both egr2b\textsuperscript{fh227} and stl64 mutants, all samples were washed at least three times in PBSTw. Finally, as much PBSTw as possible was removed, and gDNA was extracted using the DNeasy Blood & Tissue (Qiagen cat# 69506) kit with four key modifications to the manufacturer’s protocol.

These four modifications substantially increased our gDNA yield; specifically, we: (1) incubated for 10 minutes at 56° C when the samples are in the ATL buffer; (2) incubated a minimum of 3 hours at 200 rpm during the proteinase k digestion; (3) incubated for 10 minutes at 56° C when the samples were in AL buffer; (4) incubated at least 20 minutes (can go longer; \textit{e.g.}, >90 minutes) after the buffer AE is added before centrifuging for 2 minutes. Additionally, we found that it is of paramount importance to use a fresh kit, ideally less than three months old, to process samples. Using older kits resulted in significantly decreased or in failed extraction.

**Whole Genome Sequencing (WGS)**

All gDNA was extracted and submitted to the Genome Technology Access Center (GTAC) at Washington University for WGS. Quantification of concentration and integrity of all genomic DNA, was determined by using Qubit (ThermoFisher) and Tapestation (Agilent), respectively. All samples submitted had a minimum of 1 µg of gDNA. Each sample was barcoded, pooled, and paired-end sequencing was done in a single lane of a HiSeq2500 or 3000 (Illumina). After demultiplexing, all reads were aligned using Novoalign (Novocraft). From the alignments, SNPs
and INDELS were called using SAMtools (Li et al. 2009; Li 2011), and any effects to protein coding genes were predicted using snpEFF (Cingolani et al. 2012).

**Bulled Segregate Analysis**

There are three custom Perl scripts referred to as “ChromSplit,” “Allele Ratio Calculator (ARC),” and “SNPfilter” that link mapping and filtering of causal variants. ChromSplit takes the bam file generated in the process of aligning the reads to the genome and splits them into two files per chromosome. The first contains all SNPs called and the second contains all INDELS observed. SNPs and INDELS observed in the mitochondrial genome or on unattached chromosomal scaffolds are discarded prior to linkage mapping. ARC tiles bins of custom size across the different chromosomes and fills each bin with the ratio of mutant to reference allele for all SNPs observed in both the mutant and sibling pools. When all SNPs are sorted into their appropriate bins, the ratio of mutant to reference allele is calculated for the whole bin and that ratio is compared between the mutant and sibling pools. The mutant to sibling pool ratio for each bin is generated by ARC.

With the output of ARC, all bins with less than 10 SNPs are thrown out because of the outsized effect that a single SNP can have. To determine a linked chromosome, the ratios of the mutant and sibling bins are graphed and the highest peak that approaches a ratio of two indicates the linked chromosome. To identify the region of a single chromosome is most linked to the phenotype used to sort pools, all bins from that chromosome were sorted by their ratios. The 20 bins with the highest ratios are selected, and any area where those selected bins are clustered is the region of the chromosome most closely linked to the phenotype.
SNP Filtering and Prioritization

The SNPfilter script takes the output from the SNPeff program and sorts out all SNPs and INDELS previously observed in unmutagenized strains as well as SNPs and INDELS previously observed as homozygous that are not causative. The database of variants from unmutagenized lines used here was the SNPFisher database (Butler et al. 2015). SNPfilter was written to use the SNPFisher database as a base while adding the homozygous SNPs observed in the SNPeff files being filtered to the database of known SNPs for future filtering. In this way, as SNPfilter is run on more samples, its ability to filter out non-causative SNPs and INDELS increases. Any SNPs not removed by SNPfilter that are homozygous and non-synonymous are candidates for the phenotype used to sort the mutant and sibling pools.

Transmission electron microscopy (TEM)

TEM was performed between body segments 5-7 of 8.5 dpf zebrafish larvae using established protocols employing microwave assistance (Czopka & Lyons 2011). In brief, individual larval trunks were fixed in modified Karnovsky’s fixative (4% PFA, 2% glutaraldehyde, 0.1M sodium cacodylate, pH 7.4) via microwave assistance and then overnight at 4° C. After genotyping and pooling samples with the same genotypes, samples were washed with 0.1M sodium cacodylate and post-fixed for 1 hour in 2% osmium tetroxide in 0.1M sodium cacodylate and 0.1M imidazole. Samples were then washed with ultrapure water and stained with saturated uranyl acetate. Larvae were dehydrated using increasing concentrations of ethanol then 100% acetone, and finally were infiltrated overnight with a 1:1 acetone:EPON mix at room temperature using gentle agitation of the tubes. The next day, samples were transferred to 100% EPON, while allowing any residual acetone to fully evaporate. Finally, individual larvae were embedded in 100% EPON and baked at least 48 hours at 65° C.
When the EPON was solid, excess material was trimmed off each block and thin (70 nm) sections were mounted on copper mesh grids (Electron Microscopy Sciences, Hatfield, PA). Grids were stained again with saturated uranyl acetate for an hour, rinsed with ultrapure water, and then stained with Sato’s lead stain for 6 minutes. Grids were imaged using a Jeol (JEM-1400) electron microscope and images were collected using an AMT V601 digital camera. All images were analyzed using the FIJI module of Image J and Adobe Photoshop.

**Morpholino Injections**

A morpholino targeting *fbxw7* has been previously described and validated (Snyder et al. 2012). For injections, the morpholino was diluted in ultrapure water supplemented with phenol-red dye (10%) to obtain a final concentration of 2.5 µg/µl. Embryos were injected at or before the 1-cell stage with 2.5 ng of morpholino in a total volume of 2 nl. To control for potential adverse effects of the injections, control siblings were also injected with an equal volume of phenol-red dye diluted 1:10 in ultrapure water. Finally, we scored morpholino and control-injected animals by *mbp* WISH. All scoring was performed blind to treatment.

**Data Access**

.bed files produced during sequencing the *stl64* mutation, the three Perl scripts, and a guide to using these scripts are freely available (https://zenodo.org/record/843605).

**A.7 Acknowledgments**

The authors would like to thank past and present members of the Monk Lab for valuable discussions, with special thanks to Charleen Johnson, Ian Hakkinen, and Zachary Spence, who contributed many hours to the screen and maintaining the *egr2b*6227 and *gpr126*649 alleles. We would also like to thank the Washington University Zebrafish Facility, particularly Stephen Canter and John Englehard, for providing excellent zebrafish care and assistance with the genetic
screen. We thank Dave Lyons for his kind gift of the \textit{tg(mbp:mcherry-CAAX)} zebrafish line. We thank the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine for help with genomic analysis. The Center is partially supported by NCI Cancer Center Support Grant #P30 CA91842 to the Siteman Cancer Center and by ICTS/CTSA Grant# UL1 TR000448 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. This publication is solely the responsibility of the authors and does not necessarily represent the official view of NCRR or NIH. This work was supported by: NIH/NINDS to BLH (F31 NS094004), NIH/NINDS to SDA (F31 NS087801), NIH/NIGMS to SLJ (R01 GM056988) and NIH/NICHD to KRM (R01 HD080601), the Edward J. Mallinckrodt Jr. Foundation (KRM), and KRM is a Harry Weaver Neuroscience Scholar of the National Multiple Sclerosis Society.

\textbf{A.8 Author contributions}

NES performed all work to map the \textit{egr2b}^{fh227} and \textit{gpr126}^{at449} alleles. BLH, SDA, ALH, and KRM conducted the screen that identified the \textit{fbxw7}^{stl64} allele. BLH, SDA and ALH optimized the extraction protocol for gDNA from fixed tissue. BLH performed gDNA extraction and sequence analysis for \textit{fbxw7}^{stl64} with additional filtering by NES. Phenotypic characterization of \textit{fbxw7}^{stl64} mutants and \textit{fbxw7} MO experiments were conducted by BLH, with the help of MH. TOP and RSG wrote the initial scripts with oversight from SLJ, and modifications were implemented by NES, BLH, and SDA with oversight from KRM. NES generated the Perl script for SNP filtering. The manuscript was written by NES, BLH, and KRM and edited by all authors.
A.9 Disclosure

Nothing to disclose
A.10 References


Appendix B: Regulation of mitochondria-dynactin interaction and mitochondrial retrograde transport in axons
Preface:

This chapter has been reproduced and adapted in its entirety from the following published manuscript:

B.1 Abstract

Mitochondrial transport in axons is critical for neural circuit health and function. While several proteins have been found that modulate bidirectional mitochondrial motility, factors that regulate unidirectional mitochondrial transport have been harder to identify. In a genetic screen, we found a zebrafish strain in which mitochondria fail to attach to the dynein retrograde motor. This strain carries a loss-of-function mutation in actr10, a member of the dynein-associated complex dynactin. The abnormal axon morphology and mitochondrial retrograde transport defects observed in actr10 mutants are distinct from dynein and dynactin mutant axonal phenotypes. In addition, Actr10 lacking the dynactin binding domain maintains its ability to bind mitochondria, arguing for a role for Actr10 in dynactin-mitochondria interaction. Finally, genetic interaction studies implicated Drp1 as a partner in Actr10-dependent mitochondrial retrograde transport. Together, this work identifies Actr10 as a factor necessary for dynactin-mitochondria interaction, enhancing our understanding of how mitochondria properly localize in axons.

B.2 Introduction

Mitochondrial transport in axons is critical for the formation and function of the nervous system. This organelle generates the ATP necessary for energy demanding functions in all cells, but neurons are especially reliant on mitochondria to maintain their electrically polarized state. After depolarization, ATP-dependent ion pumps are employed to repolarize the cell and prepare it for another action potential. It is estimated that at rest alone, neurons use 4.7 billion molecules of ATP per second (Zhu et al., 2012). Because of this large ATP requirement, mitochondria need to be properly localized to regions of high ion influx, such as at synapses (reviewed in Schwarz, 2013). In addition to their critical role in cellular metabolism, mitochondria also regulate local
calcium ion levels (Werth and Thayer, 1994). Calcium efflux from intracellular stores mediates synaptic activity. During inactivity, this ion needs to be contained in intracellular compartments as high cytoplasmic calcium levels correlate with axonal degeneration (Avery et al., 2012; Vargas et al., 2015; Yang et al., 2013). The proper localization of mitochondria to sites of high ATP consumption and calcium ion flux requires active transport.

In addition to maintaining axon health, mitochondrial motility is also necessary to maintain mitochondrial health and function. Mitochondria undergo fission-fusion dynamics that facilitate both the replenishment of proteins in this organelle and the maintenance of mitochondrial DNA quantity and integrity (reviewed in Scheibye-Knudsen et al., 2015). Interrupted mitochondrial fission or fusion has been linked to loss of mitochondrial DNA, loss of oxidative potential, and mitophagy. Axonal transport of mitochondria is tightly linked to mitochondrial dynamics as mitochondrial fusion requires the coalescence of mitochondria and fission requires the active separation of dividing organelles. The relationship between mitochondrial dynamics and transport is also apparent at the molecular level: Mitofusin, an essential protein for mitochondrial fusion, participates in the anterograde transport of this organelle as well (Misko et al., 2010). Furthermore, manipulation of the dynamin-like protein Drp1, necessary for mitochondrial fission, impacts the localization of mitochondria (Smirnova et al., 2001; Varadi et al., 2004). The mechanistic bases for these relationships are still largely unclear.

Elegant work in *Drosophila, C. elegans*, and cultured neurons has begun to elucidate the mechanisms of mitochondrial axonal transport. The primary anterograde mitochondrial motor, Kinesin-1, attaches to mitochondria via the proteins Miro (RhoT1/2; Guo et al., 2005) and Milton (TRAK1/2; Glater et al., 2006; Stowers et al., 2002). Miro contains calcium sensitive EF
hands that, when exposed to high levels of this ion, change their confirmation, resulting in the uncoupling of the Kinesin-1 motor from microtubules (Saotome et al., 2008; Wang and Schwarz, 2009). This mechanism, in conjunction with mitochondrial tethering factors (Kang et al., 2008), allows mitochondrial congregation at sites of high synaptic activity. The Miro/Milton transport machinery is not specific for anterograde transport, however, as loss of either protein impacts anterograde and retrograde mitochondrial movement (Guo et al., 2005; Saotome et al., 2008). Therefore, how mitochondria are specifically moved in a unidirectional manner by either the Kinesin or Dynein motor protein complex is not well understood.

Retrograde mitochondrial transport is known to depend on the cytoplasmic dynein complex (Pilling et al., 2006; Schnapp and Reese, 1989). The core dynein motor (reviewed in Holzbaur and Vallee, 1994) is oftentimes associated with dynactin, itself a multi-protein complex, during retrograde axonal transport. Dynactin, specifically its p150 subunit, has been shown to facilitate dynein processivity and also serves as an anchor for dynein at microtubule plus ends, facilitating cargo loading (Lloyd et al., 2012; Moughamian and Holzbaur, 2012; Schroer, 2004). The dynactin accessory complex is attached to dynein through interaction of p150 with the tails of the dynein intermediate chains (Vaughan and Vallee, 1995). In addition to p150, dynactin contains two actin-related proteins, Arp1 and Actr10 (also known as Arp11; Eckley et al., 1999; Eckley and Schroer, 2003). Together with p25, p62 and p27, Actr10 is a part of the dynactin pointed end complex which is predicted to be in an ideal location for cargo binding (Yeh et al., 2012). Structural work suggests that one key function for Actr10 is capping the actin-like Arp1 filament, to regulate filament length and facilitate attachment of other pointed end proteins (Urnavicius et al., 2015); however, this does not preclude an additional role for Actr10 in cargo attachment to the dynein complex, a function which has not been explored.
In a forward genetic screen for mediators of retrograde axonal transport, we identified a strain with a loss-of-function mutation in Actr10. \( actr10^{nl15} \) mutants (hereafter referred to as \( actr10 \)) display axon terminal swellings in the central and peripheral nervous systems indicative of retrograde transport abnormalities. Analysis of cargo localization and movement in the \( actr10 \) mutant revealed clustering of mitochondria, but not other cargos analyzed, at microtubule plus ends due to failed retrograde mitochondrial movement. This phenotype was vastly different from loss of either Dynein heavy chain or p150, potentially indicating a unique function for Actr10 in mitochondrial retrograde movement. Furthermore, we demonstrated that abnormal mitochondrial movement in \( actr10 \) mutants is due to failed attachment of mitochondria to the dynein-dynactin complex in the absence of Actr10. Importantly, Actr10 engineered to lack the dynactin binding domain maintains mitochondrial interaction, hinting at a specific role for Actr10 in mediating dynactin-mitochondria interaction. Finally, we provide biochemical and genetic evidence that Drp1, a Dynamin-related protein previously implicated in microtubule minus end-directed mitochondrial movement (Smirnova et al., 1998), partners with Actr10 in mitochondrial retrograde transport. Together our data support a model in which Actr10 functions to scaffold mitochondria to the dynein-dynactin complex for retrograde transport in axons.

B.3 Results

Mitochondria accumulate in \( actr10 \) mutant axon terminals due to failed retrograde transport

We used the zebrafish posterior lateral line (pLL) system to identify novel mediators of retrograde cargo transport in axons (Drerup and Nechiporuk, 2013). pLL axons develop early (axon extension is complete by 2 days post-fertilization (dpf) and synapse formation occurs by 4 dpf), are superficially localized and are largely planar, making them an ideal system for \textit{in vivo}
observations and manipulations (Figure 1A; Ghysen and Dambly-Chaudière, 2004; Metcalfe, 1985; Metcalfe et al., 1985). Using an ENU-based forward genetic screen, we isolated a larval-lethal, recessive mutant strain with large swellings in pLL axon terminals (Figure 1B). 

RNAmapper analysis (Miller et al., 2013) identified the causal mutation as a single nucleotide change (T to G) in the start codon of actr10. We confirmed actr10 as the affected gene using RNA-mediated rescue of axon terminal swellings and by performing TALEN- (Transcription Activator Like Effector Nuclease) mediated disruption of the first exon of actr10, which phenocopied the mutant axon terminal swellings (Figure 1C–F).

Axonal swellings, like those observed in actr10 mutants, can arise due to a number of intracellular abnormalities, including disruptions in retrograde cargo transport (Drerup and Nechiporuk, 2013; Martin et al., 1999). To determine if a particular cargo was accumulating in actr10 mutant axon terminals, indicative of impaired retrograde movement, we performed immunolabeling on mutants and wildtype siblings at 4 dpf with antibodies against various axonal cargos. This revealed an accumulation of Cytochrome c, a mitochondrial protein, in actr10 axon terminals (Figure 2A,B,E). To confirm mitochondrial accumulation in mutant axon terminals, mitochondria were labeled in neurons with TagRFP by zygotic injection of a plasmid containing a mitochondrial targeting sequence tagged with this fluorescent protein (5kbneurod:mito-TagRFP). actr10 mutants displayed mitochondrial accumulation in pLL axon terminals (Figure 2C,D). Other known pLL axon retrograde cargos, including those labeled by Lamp1 (Lysosome associated membrane protein1; late endosome/lysosome marker) and phosphorylated c-Jun N-Terminal Kinase (pJNK) did not accumulate (Figure 2F–K; Drerup and Nechiporuk, 2013). Expression of wildtype Actr10 using zygotic injection of in vitro synthesized mRNA rescued
mitochondrial accumulation in *actr10* mutants (Figure 2L–O), confirming that Actr10 is necessary for proper mitochondrial positioning.

As our immunolabeling experiments were not exhaustive for every possible cargo in axons, we undertook transmission electron microscopy (TEM) analyses of pLL axon terminals to determine if mitochondria are the predominant cargo mislocalized in *actr10* mutants. This experiment demonstrated mitochondria are highly enriched in the axon terminal swellings of *actr10* mutants compared to other intracellular structures (Figure 3A,B; n = 3 wildtypes, n = 4 mutants). Together, our immunolabeling and TEM analyses showing mitochondrial accumulation in axon terminals could indicate interrupted retrograde mitochondrial transport in *actr10* mutants.

To determine if mitochondrial retrograde transport was disrupted in *actr10* mutants, mitochondria in single pLL axons were tagged with TagRFP using 5kbneurod:mito-TagRFP plasmid injection and organelle movement was visualized by confocal microscopy (Figure 4A,B). Kymograph analysis demonstrated a 37% and 39% reduction in the distance moved by mitochondria in the anterograde and retrograde directions respectively in *actr10* mutants but no change in mitochondrial transport velocity (Figure 4C–F). Despite the decrease in anterograde transport distance, the frequency of anterograde mitochondrial transport was unchanged in *actr10* mutants. We also analyzed reversal frequency to determine if there was a defect in directional persistence of mitochondrial transport in *actr10* mutants but found no difference in the proportion of mitochondria that reversed direction during our imaging sessions (wildtype: 0.011 ± 0.008; *actr10* mutants: 0.000 ± 0.009; ANOVA; p=0.4003). In contrast, both the frequency of retrograde mitochondrial transport and the proportion of mitochondria moving in the retrograde direction were dramatically decreased in *actr10* mutants (Figure 4G,H). As
velocity of movement was largely normal, these data suggest a role for Actr10 in the attachment of mitochondria to the retrograde motor for transport.

**Actr10 functions autonomously in neurons to regulate axon morphology and mitochondrial localization**

*actr10* is ubiquitously expressed, albeit enriched in the nervous system during larval stages (Figure 5A–C). As neuronal activity and the presence of growth factors can modulate mitochondrial transport (Chada and Hollenbeck, 2004; Chen and Sheng, 2013), it was possible that changes in the axonal environment in *actr10* mutants could alter mitochondrial movement. This led us to ask if Actr10 functions autonomously in axons to regulate mitochondrial transport. To test for a neuron-specific function for Actr10 in mitochondrial localization, we expressed monomeric red fluorescent protein (mRFP) tagged Actr10 in individual pLL neurons using zygotic injection of a 5kbneurod:mRFP-*actr10* DNA plasmid and assessed axon terminals. While mRFP-Actr10 expression in wild-type axons had no apparent effects (Figure 5D; n = 6 in 3 biological replicates), it rescued axon terminal morphology and mitochondrial localization in all mutants analyzed (Figure 5E–G; n = 5 mutants in 3 biological replicates). These experiments confirmed a neuron-specific function for Actr10 in mitochondrial localization in axons.

**Dynein localization and motility does not rely on Actr10**

Previous work on Actr10 function assayed mitotic spindle positioning during cell division in cultured fibroblasts and nuclear positioning in fungi to show that Actr10 depletion phenocopied dynein loss of function (Lee et al., 2001; Yeh et al., 2012; Zhang et al., 2008). In association with structural work implicating Actr10 in Arp1 filament capping (Urnavicius et al., 2015), these data could imply that loss of Actr10 impacts dynactin integrity and, subsequently, all dynein function. However, the aforementioned work could also be interpreted to mean that
appropriate dynactin-nuclear membrane interaction requires Actr10 function. Supporting this possibility, Actr10 has been shown to regulate dynactin-membrane association (Clark and Rose, 2006). With this data in mind, we next wanted to determine if loss of Actr10 phenocopied dynein or dynactin loss of function in mature neurons, the cell type that displayed specific defects in mitochondrial retrograde transport in our studies. First, we analyzed the localization of dynein and dynactin in actr10 mutant axons at 4 dpf. Neither Dynein heavy chain (DHC) nor p150 were significantly mislocalized in actr10 mutants, though we did observe a trend of increased DHC in actr10 mutant axon terminals (Figure 6A–F). Next, to determine if dynein was able to move in the retrograde direction, we assayed dynein motility by tagging the core motor complex using expression of an mRFP tagged variant of dynein light intermediate chain (5kbneurod: dync1li1V2-mRFP; Figure 6G,H). Kymograph analysis revealed no change in the parameters of retrograde dynein movement, though we did note a 42% reduction in the distance and a 26% reduction in the velocity of anterograde dynein transport. Despite these defects in anterograde dynein transport, there was no change in the proportion of dynein-positive puncta moving in the anterograde or retrograde direction in actr10 mutants at 4 dpf (Figure 6I–M). Additionally, we analyzed reversal frequency to determine if there was a defect in persistence of dynein movement in actr10 mutants but found no difference in the average number of puncta that reversed direction during our imaging sessions (wildtype: 1.62/100 mm*min ±0.37; actr10 mutants: 1.74/100 mm*min ±0.44; ANOVA; p=0.844). These experiments demonstrated that loss of Actr10 does not impede dynein-dynactin complex localization or dynein retrograde movement in axons.

We then analyzed the stability of dynein-dynactin interaction using co-immunoprecipitation from whole larval extracts. Immunoprecipitation of endogenous proteins
was not possible due to lack of zebrafish-specific antibodies, so we turned to overexpression of tagged dynein components using zygotic injection of *in vitro* synthesized mRNA. First, we confirmed that a GFP tagged version of dynein intermediate chain 2b (i2b), a core dynein protein, could integrate into the motor complex. mRNA encoding i2b-GFP was injected into zygotes and anti-GFP antibodies were used to immunoprecipitate the complex. Extracts were subjected to western blot analysis and probed with anti-DHC antibodies. i2b-GFP can integrate into the core dynein complex (Figure 6N). We then used this approach to determine if loss of Actr10 impacts dynein-dynactin interaction. i2b-GFP was expressed in *actr10* mutants and wild-type siblings and larval extracts were subjected to GFP-based immunoprecipitation at 4 dpf. Western blot of larval extracts revealed no change in p150 interaction with the core dynein motor (Figure 6O). Together, these experiments confirmed that loss of Actr10 does not impact dynein-dynactin interaction.

To further explore the effect of loss of Actr10 on retrograde cargo transport, we analyzed the movement of additional dynein cargos in axons. First, we visualized peroxisome transport in lateral line axons. Kymograph analysis of peroxisome transport at 4 dpf demonstrated small changes in anterograde transport velocity and distance of retrograde transport bouts (Figure 7A–G). A slight, but not significant decrease in the proportion of peroxisomes moving in the retrograde was apparent as well (Figure 7G; ANOVA; p=0.07). Upon further investigation, we found that previous studies revealed a surprising relationship between mitochondria and peroxisomes, including a large number of shared membrane proteins, such as Drp1 (reviewed in Schrader, 2006). Our work implicates Drp1 in Actr10-dependent mitochondrial localization (see Figure 12), making the impact of Actr10 loss of function on peroxisome transport complex. Therefore, we analyzed the transport of a third cargo, Lamp1-labeled vesicles, which are
composed of late endosomes and lysosomes. Analysis of Lamp1-positive vesicle movement at 4 dpf demonstrated that this cargo is transported normally in actr10 mutant axons (Figure 7H–L). These live imaging experiments, in conjunction with the immunolabeling of known retrograde cargos (Figure 2) and our TEM studies (Figure 3), argue that loss of Actr10 does not hinder the transport of all dynein-dependent, retrograde cargos in axons.

Since we observed small changes in retrograde peroxisome transport, we wanted to further investigate the relationship between loss of Actr10 and loss of dynein/dynactin. To do this, we analyzed the ability of DHC and p150 null mutants to phenocopy actr10 mutants. First, we compared actr10 mutants to a previously isolated zebrafish DHC mutant, dyn1h1<sup>mm20</sup> (hereafter referred to as dyn1h1; Insinna et al., 2010). Unlike actr10 mutants (see Figure 1), dyn1h1 mutants displayed rapid degeneration of pLL axons by 4 dpf, with pLL nerves extending less than half-way to the tail at this time-point (Figure 8A,B). Mitochondria do accumulate in distal axons of dyn1h1 mutants, as expected with global disruption of retrograde transport (Figure 8F,G). The disparity between actr10 and dyn1h1 mutant axonal phenotypes argues that loss of Actr10 does not impact all dynein function in axons.

We then compared the actr10 mutant phenotype to loss of dynein-dynactin interaction using zebrafish p150 mutants. There are two orthologs of p150 in zebrafish, p150a and p150b. These paralogues are highly similar at the amino acid level but have slightly different expression patterns: while both are ubiquitously expressed, p150b is enriched in developing (1 and 2 dpf) and mature (4 dpf) neurons while p150a is enriched only in mature neurons 4 dpf (Figure 9A–F). To determine whether loss of p150 phenocopies the actr10 mutant, we assayed single and double p150 mutants using a p150a mutant described previously (Del Bene et al., 2008; Wehman et al., 2005) and a novel p150b mutant we engineered (p150b<sup>nl16</sup>). Loss of p150a alone has no
discernable effect on axons or mitochondrial localization (Figure 8C,H). We used CRISPR-Cas9 technology (Hwang et al., 2013) to create a deletion in exon 3 of p150b, which resulted in a frame-shift and premature stop site. p150b single mutants are indistinguishable from wild-type siblings at 4 dpf, with normal axon terminals and no abnormalities in mitochondrial localization (Figure 8D,I). This strain does not have the small eye phenotype observed in dynclh1 and p150a mutants (Figure 8A–D; Del Bene et al., 2008; Insinna et al., 2010; Wehman et al., 2005). Unlike single p150 mutants, p150a/b mutants are phenotypically similar to dynclh1 mutants, with truncated pLL axons and mitochondrial accumulation at axon ends (Figure 8E,J). This confirms the reliance of mitochondria on the dynein-dynactin complex for retrograde movement and demonstrates that actr10 mutants do not phenocopy the axon truncation phenotype of dynein or dynactin loss of function mutants.

We then analyzed the effect of p150 loss of function on mitochondrial transport in pLL axons. Mitochondria were tagged with TagRFP using zygotic injection of the 5kBneurod:mito-TagRFP plasmid and kymograph analysis was performed on videos acquired through live imaging (Figure 8K-N). Unlike loss of Actr10, p150a/b double mutants had an almost complete cessation of mitochondrial movement in the anterograde and retrograde directions (Figure 8N,O). In addition, we noted a dramatic loss of mitochondria from the central portion of axons in p150a/b double mutants, likely because of the strong defect in anterograde mitochondrial transport (Figure 8P). Analyses of transport parameters revealed no change in the distance or velocity of the residual mitochondrial movement in either direction (Figure 8Q,R). The dramatic loss of all mitochondrial movement in p150a/b mutants, specifically reductions in both the anterograde and retrograde pools, is not observed in actr10 mutants, which have only a reduction in the retrograde mitochondrial pool (see Figure 4). Furthermore, two cargos known to localize
normally in actr10 mutants, pJNK and Lamp1 (see Figure 2), accumulate in in p150a/b mutant axon terminals (Figure 10A–D), indicating that the retrograde transport of these cargos is perturbed by loss of dynein-dynactin interaction. Therefore, our phenotypic, in vivo transport, immunolabeling and biochemical data fail to support the argument that loss of Actr10 impacts all dynein-dynactin function in axons and, instead, supports a specific requirement for Actr10 in mitochondrial retrograde transport in this neuronal compartment.

**Mitochondria fail to attach to the dynein-dynactin complex in actr10 mutants**

The specific effect of Actr10 depletion on retrograde mitochondrial transport frequency led us to ask if Actr10 is necessary to attach this organelle to the dynein motor complex. We addressed this question using mitochondrial fractionation from whole larvae (Figure 11A; Prudent et al., 2013). Analysis of fractions revealed equal levels of p150 in the input and heavy fractions between wildtype and actr10 mutants. Strikingly, in actr10 mutants, p150 was largely lost from the mitochondrial fraction with a concomitant increase of p150 to the light fraction, which contains all cellular components not pelleted under low centrifugation speeds (Figure 11B,C). This result demonstrates the necessity of Actr10 for mitochondrial attachment to dynactin.

To better define the mechanism of dynactin-Actr10-mitochondrial interaction, we identified the domain in Actr10 necessary for association with the dynactin complex using immunoprecipitation of deletion constructs (overlapping deletion of 40 amino acid regions) from HEK293T cells. Whereas Actr10△7–△10 deletions showed reduced binding to dynactin, Actr10△7 most consistently failed to immunoprecipitate dynactin in all experiments (Figure 11E,F; n = 4). As this result was somewhat variable in HEK cells, we confirmed the necessity of this domain for Actr10-dynactin interaction in vivo using immunoprecipitation of Actr10 and
Actr10△7 from embryo lysates. This revealed a loss of interaction between Actr10△7 and dynactin, demonstrating the reliance of Actr10 on this region for interaction with dynactin (Figure 11G; n = 3). Finally, if Actr10 is a part of the scaffold necessary for mitochondria-dynactin interaction, a separate mitochondrial binding domain likely exists in Actr10. If this is the case, Actr10 lacking the dynactin binding domain should maintain its interaction with mitochondria. To address this, we assayed the ability of Actr10△7 to bind mitochondria using mitochondrial fractionation. mRFP-Actr10 and mRFP-Actr10△7, expressed using zygotic microinjection of respective mRNAs, were both present in the mitochondrial fractionation (Figure 11D,H; n = 2), arguing that separate protein domains exist in Actr10 which are necessary for interaction with dynactin and mitochondria. This data supports a role for Actr10 in binding mitochondria to dynactin for retrograde mitochondrial transport.

**Drp1 functions with Actr10 in mitochondrial retrograde transport**

It is unlikely that Actr10 binds directly to mitochondria as it does not have predicted transmembrane or other membrane-associated domains. Rather, similar to Kinesin-1-mitochondrial attachment, there are likely partner proteins that facilitate this interaction (Glater et al., 2006; Guo et al., 2005). Literature searches revealed a particularly strong association between Drp1 (Dynamin related protein 1), a GTPase associated with mitochondrial fission, and mitochondrial localization. Specifically, a lysine to alanine mutation at amino acid 38 in Drp1 (mimicking a constitutively GDP-bound form) causes clustering of mitochondria in the perinuclear region of fibroblasts in a microtubule-dependent manner (Smirnova et al., 2001; Varadi et al., 2004). Using immunoprecipitation, we demonstrated that Drp1 interacts with Actr10, with strongly enhanced interaction between Actr10 and the Drp1K38A variant (Figure 12A; n = 3 replicates in independent experiments; (Smirnova et al., 2001, 1998).
Next, we asked whether Drp1 works with Actr10 to regulate mitochondrial retrograde transport \emph{in vivo}. To address this, we examined the genetic interaction between Drp1 and Actr10 in mitochondrial localization in pLL neurons. We reasoned that if Drp1 and Actr10 function in the same pathway, Drp1 manipulation would impact the location of mitochondria in wildtype but not \emph{actr10} mutant axons. We expressed Drp1-mRFP, Drp1\textsuperscript{K38A}-mRFP, and cytoplasmic mRFP (control) in pLL neurons using injection of a plasmid encoding the respective open reading frame under an inducible promotor. Mitochondria were labeled by \emph{5kbneurod:mito-EGFP} co-injection. In wild-type larvae, expression of Drp1\textsuperscript{K38A}-mRFP caused mitochondrial clustering in the perinuclear region, phenotypically similar to the results observed in cultured fibroblasts (Figure 12B,C; Smirnova et al., 2001; Varadi et al., 2004). In contrast, Drp1\textsuperscript{K38A}-mRFP expression did not alter mitochondrial localization in \emph{actr10} mutant pLL neurons (Figure 12D,E). If Drp1\textsuperscript{K38A} mediates retrograde mitochondrial movement, we predicted its expression would induce movement of mitochondria from the proximal axon into the cell body, leading to loss of mitochondrial occupation of the axon. Indeed, expression of Drp1\textsuperscript{K38A} caused a substantial decrease in the number of mitochondria per micron in wildtype but not \emph{actr10} mutant axons (Figure 12F–J). Notably, \emph{actr10} mutants already have reduced mitochondrial occupation of pLL axons which could impact the fraction of mitochondria capable of motility in this assay. More definitive analyses of Drp1’s role in Actr10-dependent mitochondrial motility will be the subject of continued investigation. Together, the biochemical and genetic interaction between Actr10 and Drp1\textsuperscript{K38A} support a model in which Drp1, in its GDP-bound state, modulates dynein-based mitochondrial localization via an Actr10-dependent mechanism.
B.4 Discussion

Using genetics, immunolabeling, biochemistry, and *in vivo* imaging of cargo movement, we have identified Actr10 as a mediator of retrograde mitochondrial transport. In the absence of this dynactin pointed end protein, mitochondrial retrograde transport frequency is selectively disrupted, leading to accumulation of this organelle in axon terminals. Importantly, the anterograde transport of mitochondria, the localization and transport of other cargos assayed, the localization of dynein-dynactin components as well as dynein retrograde movement are all largely intact in the absence of Actr10, arguing for a role for Actr10 in mitochondrial retrograde transport in axons. Additionally, Actr10 maintains its ability to interact with mitochondria without being incorporated into the dynactin complex, suggesting a specific role for this protein in linking this organelle to the retrograde motor. Finally, we demonstrated that Actr10, perhaps through interaction with Drp1, links mitochondria to the dynactin complex. Our study brings us a step closer to understanding how unidirectional mitochondrial transport is mediated in axons to maintain a functional neural circuit.

**Actr10s role in mitochondrial retrograde transport**

We provide multiple pieces of evidence supporting a function for Actr10 in mitochondrial transport. First, mitochondria are the only cargo analyzed whose localization or retrograde transport is significantly perturbed with loss of Actr10. If dynein-mediated cargo movement was generally disrupted, other cargos, including lysosomes and dynein itself, would fail to move in the retrograde direction as well. Second, the axonal phenotype of dynactin and dynein loss-of-function mutants is vastly different from *actr10* mutants: *dync1h1* and *p150a/b* mutants display pLL nerve degeneration at 4 dpf while *actr10* mutants do not. Third, loss of dynactin-dynein interaction in *p150a/b* mutants impedes all mitochondrial transport, a phenotype
that is not observed in actr10 mutants. Fourth, neither the localization of dynein and dynactin nor the retrograde movement of dynein are perturbed with loss of Actr10. Lastly, we identified a domain in Actr10 (△7) that is essential for interaction with the dynactin complex but inconsequential for interaction with mitochondria. This piece of data is critical as it demonstrates that Actr10 can interact with this organelle independently of dynactin, arguing for a direct role for this protein in mitochondria-dynactin interaction. Together, our data argue that Actr10 is necessary for dynactin-dependent, retrograde transport of mitochondria in axons.

Actr10 could directly link mitochondria to dynactin or potentially facilitate this interaction through other dynactin pointed end proteins, such as p62. p62 is of particular interest in this regard as it is predicted to rely on Actr10 for binding to the dynactin complex (Urnavicius et al., 2015). Based on our current data, we cannot rule out a role for this protein in mitochondrial transport. Furthermore, Actr10 interacting proteins, including p62, could have additional impacts on mitochondrial transport parameters, including transport distance. As noted above, actr10 mutants have slight deficits in the distance moved by mitochondria in both the anterograde and retrograde directions. While we cannot definitively ascertain the underlying mechanism of this disruption, at least three possibilities exist. First, bidirectional transport distance could be affected by the abnormal localization of mitochondria. Mitochondrial localization directly affects local levels of ATP, which in turn could compromise the processive activity of ATP-dependent motors necessary for long distance transport. Another possibility is local deficits in mitochondrial activity: if mitochondrial health is compromised, this could account for the bidirectional transport deficits of mitochondria. Finally, it is possible that Actr10 or its interacting proteins, such as p62, regulate motor engagement. It is largely thought that cargos typically have anterograde and retrograde motors attached simultaneously and direction
of transport is attributed to regulation of motor engagement. More frequent oscillations in motor engagement due to loss of Actr10 or Actr10 interactors could account for the shortened distances moved by mitochondria in both directions. At this point, we do not have a full explanation for these transport parameter alterations but they are a topic of continued interest.

**Multiple roles for Actr10 in the cell**

Our data does not preclude a function for Actr10 in the retrograde transport of additional cargos in axons or other roles for Actr10, which could vary based on a number of factors including cell type and developmental stage. Of particular interest is a potential role for Actr10 in peroxisome transport through interaction with Drp1. While we did not find a statistically significant difference in the proportion of peroxisomes moving in the retrograde direction, there was a trend towards a decrease in this population. As Drp1 is known to localize to peroxisome membranes as well (Schrader, 2006), it will be interesting to further explore the interplay between these proteins and the transport of these highly related organelles. In addition to cargo transport, one predicted function for Actr10, based on structural and biochemical data, is in capping the Arp1 filament (Urnavicius et al., 2015). In support of this role, knockdown of Actr10 using siRNA in Cos7 cells resulted in dissociation of the dynactin complex pointed end (Yeh et al., 2012). Additionally, studies in the fungi neurospora and aspergillus demonstrated that loss of Actr10 phenocopies the abnormal nuclear positioning observed with loss of dynein (Lee et al., 2001; Zhang et al., 2008). Similarly, zebrafish actr10 mutants display the small eye phenotype observed in dynclh1 and p150a mutants (see Figure 8 and Del Bene et al., 2008; Insinna et al., 2010). Finally, similar to loss of p150, knockdown of Actr10 resulted in multipolar spindle formation in Cos7 cells (Yeh et al., 2012). Together, these data could imply that Actr10 is in fact necessary for all dynein-dynactin activity through stabilizing the Arp1 filament; however, they
could also be interpreted to mean that Actr10 is necessary for regulation of Arp1-membrane interaction. Indeed, Actr10 has been shown to regulate Arp1-membrane interaction (Clark and Rose, 2006) and the zebrafish eye phenotype in the \textit{p150a} mutant is due to a defect in interkinetic nuclear migration (Del Bene et al., 2008). This type of function for Actr10 could also explain the nuclear positioning defect in the \textit{actr10} loss of function studies in fungi (Lee et al., 2001; Zhang et al., 2008). Therefore, rather than purely serving a stabilizing role for the dynactin complex, Actr10 may modulate both Arp1 interaction with the nuclear membrane and mitochondria-dynactin binding in axons. How these functions are disparately regulated in different cell types, in different cellular compartments and/or at varying developmental stages is still unclear.

Given the previous data implicating Actr10 in Arp1 capping and subsequent dynactin stability and dynein function (Urnavicius et al., 2015; Yeh et al., 2012; Zhang et al., 2008), we were presented with the challenge of confirming that Actr10 has a specific function in mitochondrial retrograde transport and reconciling our work with these previous studies. As outlined above, our evidence for a role for Actr10 in mitochondrial transport specifically includes: (1) \textit{actr10} mutant axons are not phenotypically identical to \textit{dync1h1} or \textit{p150} mutants; (2) mitochondrial retrograde, but not anterograde, transport is disrupted in \textit{actr10} mutants while all mitochondrial movement is inhibited in \textit{p150a/b} mutants; and (3) localization and transport of all other cargos are not disrupted in \textit{actr10} mutants. Together, these results argue that loss of Actr10 does not impact all dynein-dynactin function. In addition, a variant of Actr10 lacking the dynactin binding domain retains its ability to interact with mitochondria, further substantiating a direct role for this protein in mitochondria-dynactin interaction. Together, our data argue that Actr10 participates in mitochondrial attachment to the retrograde motor protein complex in
neurons in addition to potentially facilitating Arp1 capping and nuclear positioning in other contexts.

**Drp1 and Actr10 function together to regulate mitochondrial retrograde transport**

The interaction between Drp1 and Actr10 in mitochondrial retrograde transport may provide a link between mitochondrial movement and mitochondrial fission. Originally, we investigated the role of Drp1 in retrograde mitochondrial transport as previous studies revealed the unique ability of this GTPase to elicit mitochondrial localization to microtubule minus ends (Smirnova et al., 2001, 1998). Upon further investigation, it became apparent that a select number of proteins implicated in mitochondrial fission and fusion, including Drp1, have been shown to be essential for mitochondrial transport. Specifically, the fusion-related protein Mitofusin binds to the Miro-Milton complex and is necessary for mitochondrial movement (Misko et al., 2010). Similar to Miro and Milton, loss of Mitofusin results in decreased anterograde and retrograde mitochondrial transport in cultured neurons (Misko et al., 2010). Interestingly, the inner mitochondrial membrane protein Opa1, also necessary for mitochondrial fusion, does not participate in transport (Misko et al., 2010). Thus, eliminating mitochondrial fusion itself does not impact mitochondrial movement; rather mitochondrial outer membrane proteins may have dual roles in fusion-fission dynamics and transport.

Similarly, our work in conjunction with other studies supports the argument that Drp1 participates in both mitochondrial fission and retrograde mitochondrial transport. Drp1 translocates to mitochondria where it can bind to receptors Fis1 and Mff (LosonLosón et al., 2013). In its GTP-bound form, it then oligomerizes, forming a constrictable collar necessary for fission during which GTP is exchanged for GDP (Ingerman et al., 2005). We found that a Drp1 variant incapable of binding GTP stimulates mitochondrial accumulation at the nuclear
periphery, linking fission with transport. Our genetic interaction data suggest that Actr10 functions with Drp1 in this process, though further experiments are necessary to confirm this. Rather than Actr10 working with Drp1 to regulate mitochondrial localization, it is also possible that loss of Actr10 results a decrease in the motile pool of mitochondria in axons upon which Drp1\(^{K38A}\) could work. We cannot differentiate between these possibilities at this point. Nevertheless, given the intriguing data generated here, it is tempting to speculate that, upon the GTP-GDP transition, not only do mitochondria undergo fission but Drp1 also recruits the retrograde motor protein complex through interaction with Actr10 to facilitate separation of these daughter mitochondria. The precise nature of Drp1-Actr10 interaction and the role of this interaction in mitochondrial retrograde transport is a subject of current investigation.

**B.5 Conclusions**

The coordinated regulation of mitochondrial transport is necessary for the formation and maintenance of active neural circuits. Our work identifies Actr10 as a crucial protein for mitochondrial interaction with the dynein-dynactin complex, regulating retrograde transport. In addition, we argue that the role of Actr10 is specific and not due to a general loss of dynactin stability or dynein function. The strongest pieces of evidence supporting this assertion are the ability of Actr10 to bind mitochondria in the absence of dynactin and the disparate nature of the \textit{actr10-dync1h1} and \textit{actr10-p150a/b} mutant phenotypes in axons. In addition, we identified Drp1, a GTPase implicated in mitochondrial fission, as an Actr10 interactor with a potential role in mitochondrial retrograde transport. Together with previous studies on mitochondrial motility, a complex picture is emerging in which mitochondrial transport and fission/fusion are dependent on a core group of proteins, with an independent set of proteins regulating mitochondrial docking. In conclusion, further work is crucial to understanding how mitochondrial transport and
dynamics are orchestrated in axons, which will ultimately allow us to better understand how this critical organelle is positioned and maintained. As mitochondrial transport and dynamics have been implicated in neurological disorders, a more complete mechanistic understanding of their regulation will provide insight into disease pathology.
B.6 Figures

![Figure 1](image)

**Figure 1. actr10 mutants have swollen axon terminals.**

(A) Wild-type larva (pigment free) at 4 dpf carrying the *neurod:egfp* BAC transgene. By 4 dpf, pLL axons are fully extended and functional synapses have formed with hair cells of primary neuromasts. (B) *actr10* mutant axons fully extend but terminals display large swellings. Posterior lateral line ganglion (pLLg) and innervation of the third neuromast (NM3) are indicated. Insets in (A) and (B) show magnified NM3 axon terminals. Arrows in inset point to swellings. Asterisks label areas of the pLL nerve obscured by pigment in the mutant. (C) The mutant axon terminal swelling phenotype can be suppressed by exogenous expression of mRFP-Actr10 in a dose-dependent manner. Proportion of mutants with axon terminal swellings is depicted. (D–F) Injection of TALENS targeting exon 1 of the *actr10* genomic locus phenocopies the *actr10* mutant axon terminal swelling phenotype in F0 injected larvae (arrows). Axons are labeled by the *neurod:egfp* BAC transgene (white). Scale bars in A, B = 100 µm
Figure 2: Loss of Actr10 causes mitochondrial accumulation in swollen axon terminals.

(A,B) At 4 dpf, swollen axon terminals in actr10 mutants (B) have high levels of Cytochrome c immunolabeling (arrow; NM3 shown) relative to a wildtype sibling (A). (C,D) TagRFP-labeled mitochondria accumulate in actr10 mutant (arrow), but not wildtype, axon terminals at 4 dpf. (E) Mean fluorescence intensity (background subtracted) of Cytochrome C in axon terminals of A and B. (F,G) Mean fluorescence intensity (background subtracted) in axon terminals of H–K show comparable levels of Lamp1 and pJNK fluorescence intensity between wildtype and actr10 mutant terminals (ANOVA; mean ± SEM shown). (H–K) Lamp1 and pJNK immunolabeling in NM3 axon terminals (arrows). Lamp1 and pJNK are in red in (H–K) and white in H’–K’. (L–O) Mitochondrial accumulation in mutant axon terminals, assayed using Cytochrome c
immunolabeling (white), can be suppressed by mRNA-mediated expression of mRFP-Actr10. Arrows point to axon terminal regions for comparison. Scale bars = 10 µm.
Figure 3: TEM analysis of axon terminal swellings reveal mitochondrial accumulation.

(A) Wild-type axon terminals innervating lateral line neuromasts showed no swellings (N = 3). (B) All neuromasts assayed in actr10 mutants had mitochondrial laden axon terminal swellings (N = 4). A’ and B’ are higher magnification views of the areas outlined in A and B. Arrowheads in B’ point to mitochondria.
Figure 4: Mitochondrial retrograde transport is specifically disrupted in actr10 mutants.

(A,B) Stills from Videos 1 and 2 of mitochondrial transport in single pLL axons of a wildtype (A) and actr10 mutant (B). Yellow and red arrowheads point to mitochondria moving in the anterograde and retrograde directions respectively. (C,D) Kymograph analyses of mitochondrial transport in wildtype (C) and actr10 mutants (D). (E,F) Distance and velocity of mitochondrial transport in actr10 mutants (Ant-anterograde; Ret-retrograde; ANOVA). (G) The number of mitochondria moving in the retrograde direction is significantly reduced in actr10 mutants (ANOVA; p<0001). (H) The proportion of mitochondria moving in the retrograde, but not anterograde, direction is reduced in actr10 mutants (ANOVA with post-hoc contrasts; p<0.01). Scale bars = 10 µm. Error bars represent mean ± SEM. Number of larvae assayed (biological replicates) is indicated on graphs.
Figure 5: Actr10 functions cell autonomously in axons.

(A–C) Expression of actr10 mRNA was assayed using in situ hybridization with DIG-labeled riboprobes and alkaline phosphatase-mediated NBT/BCIP precipitation. actr10 is ubiquitously expressed at all time points tested, with elevated expression in the nervous system at later stages (3 and 5 dpf). Arrows point to the pLL ganglion in B and C. (D) Neuronal mRFP-Actr10 expression does not alter axon morphology or mitochondrial localization in wildtype larvae at 4 dpf. Mitochondria were visualized by Cytochrome c immunolabeling in NM3 axon terminals. (E) Uninjected actr10 mutants have high levels of Cytochrome c immunolabeling in NM3 axon terminals at 4 dpf. (F) mRFP-Actr10 expression in actr10 mutant neurons suppressed axon terminal swellings and mitochondrial accumulation. Yellow arrowhead points to the region of the axon terminal expressing mRFP-Actr10. Note that mRFP-Actr10 negative axon terminals (red arrowhead) display axonal swelling and high levels of Cytochrome c labeling. Lines in D”, E” and F” indicate regions used for line scan analysis. (G) Line scans show that mRFP-Actr10 expression rescues Cytochrome c levels in actr10 mutant axons (compare pink/red and yellow lines). Pigment cells (*) obscure some nerves. Scale bars = 10 μm.
Figure 6: Dynein-dynactin localization and retrograde movement are intact in actr10 mutants.

(A,B) Dynein heavy chain (DHC) immunolabeling demonstrates normal DHC localization in NM3 axon terminals of actr10 mutants at 4 dpf. (C,D) p150 is normally localized in NM3 axon terminals of actr10 mutants at 4 dpf. (E,F) Analyses of mean fluorescence intensity in axon terminals (background subtracted) showed comparable fluorescence intensity between mutant
and wildtype siblings (ANOVA; mean ± SEM shown). (G,H) Stills from dynein time-lapse imaging sessions (Videos 3 and 4) in wildtype and actr10 mutant pLL axons at 4 dpf. Arrowheads indicate retrograde dynein movement. (I,J) Kymograph analyses of dynein transport in wildtype (I) and actr10 mutants (J). (K–M) Retrograde dynein transport parameters, including the proportion of dynein-labeled vesicles (K), distance moved by vesicles (L) and velocity of movement (M) are unaffected in actr10 mutants (ANOVA; mean ± SEM shown). A reduction of anterograde dynein-positive puncta distance and velocity was noted (ANOVA; mean ± SEM shown). (N) Dynein intermediated chain 2b fused to GFP (i2b-GFP) interacts with the core dynein complex. Immunoprecipitation of i2b-GFP from whole embryo extracts followed by DHC western (top). Whole embryo lysate controls for i2b-GFP (middle; top band not present in wildtype) and DHC (bottom). Bead only (no 1°) immunoprecipitation controls shown. (O) The core dynein complex labeled by i2b-GFP can immunoprecipitate p150 from whole larval lysates derived from wildtype and actr10 mutant larvae at 4 dpf (top). Whole larval lysate control for i2b-GFP (middle) and p150 (bottom). Bead only (no 1°) and uninjected wildtype immunoprecipitation controls shown. Scale bars = 10 µm. Number of larvae assayed is indicated on graphs.
Figure 7: Peroxisome and Lamp1 vesicle transport in *actr10* mutants at 4 dpf.

(A,B) Stills from peroxisome imaging sessions at 4 dpf from Videos 5 and 6. Yellow and red arrowheads point to anterograde and retrograde peroxisome movement respectively. (C,D) Kymographs analyses of peroxisome transport in wildtype (C) and *actr10* mutants (D). (E–G) Peroxisome transport parameters were similar between *actr10* mutants and wildtype siblings, though a slight reduction in retrograde distance and anterograde velocity were noted (ANOVA; mean ± SEM shown). The proportion of peroxisomes moving in the retrograde direction was reduced as well, though not significantly (ANOVA; mean ± SEM shown; p=0.07). (H,I) Kymographs of Lamp-labeled vesicle movement at 4dpf. (J–L) Lamp1 vesicles were transported similarly between wildtype and *actr10* mutants. An increase in the proportion of these vesicles in mutants that were stationary during imaging was noted (p<0.01; ANOVA; mean ± SEM shown). Number of larvae assayed is indicated on graphs. Scale bars = 10 μm.
Figure 8: *dyne1h1* and *p150a/b* mutants fail to phenocopy *actr10* mutants.

(A) Wildtype larva at 4 dpf with full pLL axon extension into the tail. End of pLL nerve indicated by arrowhead. (B) Loss of DHC in *dyne1h1* mutants results in pLL axon degeneration, leading to a truncated pLL nerve at 4 dpf. Small eyes are also apparent (red dotted line), as has
been reported previously. (C) \( p150a \) mutants have small eyes (red outline) but pLL nerves are identical to wildtype siblings. (D) \( p150b \) mutants at 4 dpf with normal pLL nerves and no change in eye diameter. (E) \( p150a/b \) double mutants have truncated, thin pLL nerves with swellings along the length of axons and in terminals and are indistinguishable from \( dync1h1 \) mutants. \( p150a/b \) mutants also show small eyes similar to \( dync1h1 \) and \( p150a \) mutants. (F,G) \( dync1h1 \) mutants display axon terminal swellings and mitochondrial accumulation compared to wildtype controls at 4 dpf. Arrows point to mitochondria rich, axonal swellings. Mitochondria are labeled by expression of \textit{mito-TagRFP} (red on top F-J, white below F'-J'). (H,I) \( p150a \) and \( p150b \) single mutants have axon terminal morphology and mitochondrial localization in axon terminals similar to wildtype siblings. (J) Loss of both \( p150 \) paralogues (\( p150a/b \); J) results in swollen axon terminals with increased mitochondrial density similar to \( dync1h1 \) mutants (G). (K–N) Kymograph analyses of mitochondrial transport in \( p150 \) mutants. (O) The proportion of mitochondria moving in both the anterograde and retrograde direction in pLL axons is significantly decreased in \( p150a/b \) double mutants (ANOVA with Tukey HSD post-hoc contrasts). (P) The number of mitochondria in pLL axons is decreased in \( p150a \) mutants compared to wildtype siblings (ANOVA with Tukey HSD post-hoc contrasts; \(*p<0.05\)). The number of mitochondria in pLL axons of \( p150a/b \) double mutants is dramatically reduced compared to wildtype siblings (ANOVA with Tukey HSD post-hoc contrasts; mean±SEM shown). (Q,R) Distance and velocity of limited residual mitochondrial movement is not significantly altered in \( p150a/b \) mutants compared to wildtype siblings (ANOVA with Tukey HSD post-hoc contrasts; mean ± SEM shown). Scale bars in A-E = 100 µm. Scale bars in F-J = 10 mm. Number of larvae assayed is indicated on graphs.
Figure 9: *p150a* and *p150b* expression in zebrafish embryos and larvae.

(A,C,E,E’)* p150a in situ* hybridization shows ubiquitous expression at 1 and 2 dpf, with a slight enrichment in neurons of the pLL ganglion at 4 dpf. (B,D,F,F’)* p150b* is enriched in developing neurons with low level expression ubiquitously at all stages assayed. pLL and anterior lateral line ganglia are indicated by an arrowhead or arrow respectively in all. *=ear. Higher magnification
views of the ear, pLL ganglia, and anterior lateral line ganglia shown in insets in B’, D’, E’, and F’. Scale bars in A-F = 100 µm. Scale bars in B’ and D’-F’=20 µm.
Figure 10: pJNK and Lamp1 accumulate in p150a/b pLL axon terminals.

(A,B) pJNK accumulates in axon terminals at 4 dpf in p150a/b double mutants. NM3 shown.
(C,D) Lamp1 accumulates in NM4 axon terminals at 5 dpf in p150a/b double mutants. Arrows point to axon terminals in wildtype and p150a/b double mutants in all. Axons are labeled by the
*neurod:egfp* BAC transgene (green). Lamp1 and pJNK are show in red in A-D and white in A’-D’. Scale bars = 10 µm.
Figure 11: Actr10 is essential for mitochondria-dynactin interaction.

(A) Schematic of whole embryo mitochondrial fractionation. (B) While the level of p150 in the input and heavy fractions are comparable between wildtype and actr10 mutants, p150 is shifted from the mitochondrial to light fraction in actr10 mutants. ATPβ western blot from the same extracts (bottom) serves as a mitochondrial loading control. (C) Quantification of p150 intensity (normalized to mitochondrial loading) in actr10 mutants relative to wildtype. (D) Quantification of p150 intensity (normalized to mitochondrial loading) in mRFP-Actr10Δ7 expressing embryos relative to those expressing mRFP-Actr10. (E) Schematic and sequence of the 47 region in Actr10. (F) Immunoprecipitation of EGFP-Actr10 deletion constructs from HEK293T cells identified the Δ7 region as critical for Actr10’s interaction with the dynactin complex (n = 4 replicates). (G) In zebrafish embryos, mRFP-Actr10Δ7, expressed by zygotic injection of in vitro synthesized mRNA, was unable to interact with dynactin (top). mRFP-Actr10 and mRFP-Actr10Δ7 (middle) and p150 (bottom) are all present at similar levels. Bead only (no 1°) immunoprecipitation controls shown. (H) Actr10Δ7 is able to interact with mitochondria in zebrafish embryos as assayed by mitochondrial fractionation. ATPβ mitochondrial loading control (bottom).
Figure 12: Drp1 functions with Actr10 in mitochondrial retrograde transport.

(A) EGFP-Actr10 interacts with myc-Drp1 in HEK293T cells. The K38A mutation in Drp1 strengthens this interaction (n = 3 replicates). (B,C) Expression of Drp1\(^{K38A}\)-mRFP causes mitochondrial clustering in wildtype pLL neuron cell bodies at 4 dpf compared to an mRFP only control (B). (D,E) Though mitochondrial morphology is abnormal in actr10 mutant pLL neuron cell bodies (D), expression of Drp1\(^{K38A}\)-mRFP does not cause clustering of this organelle (E). (F–I) While expression of Drp1\(^{K38A}\)-mRFP in wildtype larvae causes loss of mitochondria from the proximal axon compared to mRFP only controls (F,G), mitochondrial number is unaffected by Drp1\(^{K38A}\)-mRFP expression in actr10 mutants (H,I). Arrowheads point to mitochondria in mRFP-expressing axons. Mitochondria are labeled by expression of a mitochondrial targeting sequence tagged with EGFP (5kbneurod:mito-EGFP; green in B–I, white in B–I’). (J) Quantification of mitochondrial number normalized to mitochondrial size in the proximal axon (ANOVA with post-hoc contrasts; mean ± SEM shown). Scale bars = 10 µm. Number of larvae assayed is indicated on graphs.
B.7 Materials and methods

Zebrafish husbandry and strains

Adult *AB and WIK strains were maintained at 28.5˚C and spawned according to standard protocols (Westerfield, 2000). ENU mutagenesis and mutant screening were performed as described previously (Mullins et al., 1994). Additional strains used include: 

*TgBAC(neurod:egfp)*^nl1^ (Obholzer et al., 2008), *actr10^nl15^*, *dynclh1^mv20^* (Insinna et al., 2010), *p150a* (also known as *mok^s309^*; (Del Bene et al., 2008; Wehman et al., 2005), and *p150b^nl16^*.

RNA-seq based identification of the *actr10^nl15^* mutation and genotyping

RNA-seq-based mapping was performed according to established protocols (Miller et al., 2013). *actr10* heterozygotes (*AB background) were crossed to WIKs to generate a mapping strain. Heterozygous F1 hybrids were identified using pair wise crosses. Eighty wildtypes/heterozygotes and 80 *actr10* mutants were obtained from a single mapping pair. These larvae were lysed at 4 dpf in Trizol according to the manufacturer’s protocol. mRNA was extracted in phenol:chloroform:isoamyl alcohol and twice in chloroform before the pellet was redissolved in 20 mL of RNase/DNase free water. Sequencing was performed by the OHSU Massively parallel sequencing core on an Illumina HiSeq2000, generating approximately 30 million reads per condition. From this RNAseq data, RNA-mapper identified a single nucleotide change in the start codon of the *actr10* gene in the *actr10^nl15^* mutant pool. The mutation (validated by sequencing and restriction digest of individual samples) completely segregated with the mutant phenotype. For experiments, *actr10* mutants were identified by genotyping according to the following protocol: PCR amplification of the region around exon 1 of the *actr10* locus resulted in a 374 bp product (forward primer: 5’-CTGTTTTTCGGATGAACTGCCTG; reverse primer: 5’-
AGATGCTCTTCTCGTCTTCTGGCTA. The actr10 mutation inserts a HaeIII cut site. Digestion with HaeIII generates wildtype (209 bp) and actr10 mutant (195 bp) bands.

**Cloning the zebrafish ortholog of actr10, plasmid production, TALEN synthesis, and mRNA synthesis**

The full-length open reading frame of actr10 was cloned from a 2 dpf zebrafish cDNA library and ligated into the pSC-A-amp/kan vector (Agilent Technologies; forward primer: 5’-AAATGCCCTTG TTTGAA; reverse primer: 5’-TTTCTCAGTGGAAGAAGG). All expression and mRNA synthesis vectors were constructed using Gateway compatible cloning as described (Kwan et al., 2007). For Actr10 expression, the Actr10 open reading frame was PCR amplified with BP arms and inserted into the Gateway compatible 3’-entry vector pDONRP2R-P3 (forward primer: 5’-ggggacaagtgtacaaaaagcagcgtTAATGCCCTTGTGGAA; reverse primer: 5’-ggggaccactttgacaagcagtggtaTTTCTCAG TGGGAAGG). Other constructs used included: 5kbneurod:mito-TagRFP (modified from (Fang et al., 2012); 5kbneurod:mRFP-dync1li1V2 (Drerup and Nechiporuk, 2013); Hsp701:mRFP- polyA; Hsp701:Drp1-mRFP; Hsp701:Drp1K38A-mRFP; and CMV/SP6:EGFP-dync1i2b. They were constructed as described here. The zebrafish dyncl2b ortholog was cloned into the pDONR2P2P3 3’ entry vector using BP-competent primers (forward primer: 5’-ggggacagtctttgtaaagggtgA TGGCTCCTTCTAGAAG; reverse primer: 5’-ggggacacattttgtataataagggtgTCATGCCTCGTC TCTGTC) and a 2 dpf zebrafish cDNA library. The zebrafish Drp1 ortholog was cloned from 2 dpf cDNA, sequence verified, and cloned into the pDONR221 middle entry vector using BP cloning (forward primer: 5’-GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTCAACCGCGATGGAAGGCTCTTA;reverse primer: 5’-GGGGACCACCTTTGTACAAAGAAGCTGGGTACCACAAGTGCGTC). To
generate Drp1\textsuperscript{K38A}, the middle entry vector was mutated to change Lysine 38 to Alanine (forward primer: 5’- CGCAGAGTAGCGGGGCGAGTTCAG; reverse primer: 5’- CCAAAACTGAAGTAAGCCCGGAGTTAC). All expression plasmids were constructed by LR cloning using the 5kbneurod (Mo and Nicolson, 2011), CMV/SP6, or Hsp701 5’ entry vectors and the pDestTol2pA2 destination vector. mRNA was synthesized using the SP6 mMessage Machine kit (Life Technologies). TALENs targeting exon 1 of actr10 were designed using freeware (http://www.talendesign.org) and synthesized as described (Dahlem et al., 2012); Tal1 target: AATTTCTGCTGATAAA ATGC; Tal2 target: GGGCAGCGAGAGAGAGA).

**Generation of the p150\textsuperscript{nl16} mutant and genotyping**

The p150\textsuperscript{nl16} mutant was generated using CRISPR/Cas9-mediated genome editing as described (Hwang et al., 2013; Shah et al., 2015). Guide RNA targeting exon 3 (GGCGAGGGCAGCGC TCCCAC) of the p150b locus was produced using a PCR-mediated scaffold method as described (Gagnon et al., 2014). Cas9 mRNA (Chang et al., 2013) was produced using the SP6 mMessage machine kit. Zygotes derived from p150a (moks\textsuperscript{309}) heterozygous outcrosses were injected with 10 pg guide RNA and 100 pg Cas9 mRNA at the one cell stage. Progeny were raised until adulthood and outcrossed to wildtypes to generate larvae. Larvae were genotyped using PCR amplification of the exon 3 locus (forward primer: 5’-CGTGAGTGAGCTCTTGGTCTG; reverse primer: 5’-CGTGCTATAACCATGTTTGTGACCTTG) and cut with MwoI to identify insertions/deletions. Changes to the guide RNA target result in loss of the MwoI cut site in p150b mutants: wildtype (164 bp + 64 bp) from p150b mutants (224 bp).
**In situ hybridization and antibody labeling**

In situ hybridization and antibody labeling were done according to established protocols (Drerup and Nechiporuk, 2013). The actr10 open reading frame was used for in situ hybridization. An ~800 base pair portion the 3’UTR of p150a and p150b were used for in situ hybridization of these paralogues. Antibodies used were: a-Dynein heavy chain (Protein Tech; #12345–1-AP); a-p150 (BD Transduction Laboratories; #610473); a-Lamp1 (Iowa Hybridoma Bank, #1D4B); a-pJNK (Cell Signaling Technology, #9251S); a-GFP (Aves Labs Inc., #GFP-1020); a-DsRed (Clontech, #632496); a-Cyto- chrome c (BD Biosciences, #556432); a-GFP (Fisher Scientific, #A-11122); a-c-myc (Santa Cruz, #sc-40); and a-ATPb (Abcam; #ab128743).

**Cell culture, immunoprecipitation and mitochondrial fractionation**

HEK293T cells (Sigma Aldrich; #12022001; identity authenticated by STR-PCR) were cultured according to standard protocols in DMEM with 50 U/mL Pen-Strep. Cells tested mycoplasma free (MycoAlert Mycoplasma Detection Kit; Lonza; L07-218). This human cell line was used for its transfection ease and results were confirmed in vivo. Transfection was done in a 6-well plate using 2.5 mg total DNA and 5 mL of Lipofectamine2000 per well according to manufacturer’s protocols (Thermo Fisher;#11668027). The mammalian GFP-Actr10 clone was used previously (Yeh et al., 2012) and obtained from Addgene (plasmid #51398). Drp1-myc and Drp1<sup>K38A</sup>-myc expression constructs were previously described (Smirnova et al., 2001). GFP-Actr10 deletion constructs were made using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent, #200521).

Mitochondrial fractionation was performed according to established protocols (Prudent et al., 2013). Briefly, for each biological replicate 100 wildtype/heterozygous or actr10<sup>0115</sup> mutant larvae were disrupted in 700 mL MB buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA,
10 mM HEPES pH = 7.5 and protease inhibitors) using a 26 gauge needle. After removal of the
input fraction, lysates were subjected to increasing centrifugation speeds to fractionate
mitochondria from a heavy and light fractions. The mitochondrial fraction was washed once in
fresh MB buffer prior to addition of Laemmli buffer to the pellet and heat denaturation. Extracts
were run on a 10% acrylamide gel, transferred to PVDF membrane, and incubated with
antibodies prior to developing with the West Pico Substrate (Thermo Fisher; #34080). After
initial development, membranes were washed in water and PBS/0.1% Tween prior to incubation
with the a-ATPb antibody

**In vivo analysis of axonal transport**

Axonal transport analyses were done as described (Drerup and Nechiporuk, 2013). Briefly,
zygotes were injected with plasmids to express cargos of interest tagged with fluorescent
proteins. At 4 dpf, larvae (each a biological replicate) were screened for pLL ganglion expression
of cargo fusions and imaged using a 63X/NA1.2 water objective on an FV1000 confocal
microscope (Olympus). All imaging was done in a single z-plane, allowing acquisition at 2–4
frames per second, as required by the Nyquist sampling theorem (Nyquist, 1928). Kymograph
analyses of cargo movement were performed using Metamorph (Molecular Devices).

**Transmission electron microscopy**

Zebrafish larvae at 5 dpf were fixed and prepared for TEM analysis using standard protocols
(Czopka and Lyons, 2011). Samples were embedded and sectioned from the tail of the larvae. An
ultramicrotome was used to cut 1000 nm sections, which were then stained with Toluidine Blue
to ensure the presence of neuromasts. Subsequently, 70 nm sections were cut and placed on
copper mesh grids. Grids were stained with uranyl acetate for one hour and then each grid was
cleaned with milliQ water for 45 s. The next day, grids were stained with Sato’s lead stain for 15
min and again cleaned with milliQ water. The Washington University Center for Cellular Imaging Jeol JEM- 1400 (Jeol USA) electron microscope was used to observe samples and an AMT V601 camera captured images.

**Image and statistical analyses**

Volumetric analyses to determine mean fluorescence intensity of immunolabeling was done using Imaris (Bitplane). For this analysis, a surface rendering was done using expression of the *neurod:egfp* transgene in axon terminals and the mean fluorescence intensity was determined. Background from the same channel was subtracted prior to analysis. For quantification of western blots, mean intensity of was determined for signal and background using ImageJ.

Statistical analyses were done in JMP. Prior to parametric analyses, data normality and variance were determined. Parametric data was analyzed using ANOVAs with Tukey-Kramer HSD contrasts for multiple comparisons. Non-parametric data was analyzed using Wilcoxon/Kruskal Wallis Tests. Image analyses and figure preparation was done using ImageJ, Adobe Photoshop, and Adobe Illustrator.

**B.8 Acknowledgements**

We would like to thank members of the Nechiporuk lab for critique of this work, A Forbes and M Culbertson for curating the screen, and C Riso and E Hunt for zebrasfish husbandry. A Miller was instrumental in mapping the *actr10<sup>nl15</sup>* mutation using RNAmapper. We are indebted to S Lusk for her work in genotyping and maintaining the mutant strains used here. C De Palma provided the myc-Drp1 constructs. Funding to CMD (NINDS/NIH: K99NS086903), AVN (NICHD/NIH: R01HD072844 and OHSU Center for Spatial Systems Biomedicine: GBMEN0245A1), ALH (F31 NS096814 and Philip and Sima Needleman graduate student...
fellowship), and KRM (Harry Weaver Scholar of the National Multiple Sclerosis Society) supported this work.

B.9 Author contributions

CMD, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing—original draft, Writing—review and editing; ALH, Formal analysis, Methodology, Writing—review and editing; KRM, Formal analysis, Supervision, Methodology, Writing—review and editing; AVN, Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Investigation, Writing—review and editing
B.10 References


