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Regulation Of Cytoplasmic Dynein By LIS1 And Adenomatous Polyposis Coli

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DEDICATION

This work is dedicated to my parents who have supported me in every endeavor I've attempted. Well, at least the ones that didn't get me into trouble.

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years in supporting me. I would like to thank all my other friends and family who I have not explicitly mentioned, as well.

ABSTRACT

Cytoplasmic dynein 1 (dynein) is a microtubule motor that plays a role in mitosis, cell migration, and minus-end directed microtubule-based transport. The lissencephaly protein, Lis1, and its binding partner, Ndel1, are critical regulators of cytoplasmic dynein (Niethammer et al., 2000). In humans, haploinsufficiency of Lis1 leads to lissencephaly, a devastating developmental neurological disorder characterized by severe brain malformation, leading to cognitive and motor defects, and progressively worsening seizures (Dobyns et al., 1993). While Lis1 is known to play a role in regulating dynein-dependent functions such as neuronal migration and mitotic spindle orientation during development, the protein is still highly expressed in the adult mouse nervous system, suggesting an important postdevelopmental role in neurons. Indeed, it has been established that Lis1 and Ndel1 regulate dynein-dependent axonal transport in mature neurons (Niethammer et al., 2000; Smith et al., 2000; Pandey and Smith, 2011; Klinman and Holzbaur, 2015). To further elucidate the importance of Lis1 in the adult nervous system, we generated a tamoxifen-inducible Lis1 knockout (KO) mouse to remove the gene post-developmentally. Using an actin promoter to drive expression of a tamoxifen-inducible cre recombinase, homozygous Lis1 KO caused the rapid onset of neurological symptoms, such as hind limb clasping and spinal kyphosis. Administration of tamoxifen resulted in dose-dependent onset of a severe phenotype, which correlated with the extent of recombination observed

in the midbrain/hindbrain. Chromatolysis, a sign of axonal dysfunction, was observed in neurons of brainstem cardiorespiratory nuclei of Lis1 KO animals. Additionally, transport defects, axonal swellings, and altered neurofilament distribution were observed in cultured DRG neurons from Lis1 KO mice. Restricting Lis1 KO to cardiomyocytes resulted in no observed symptoms, indicating loss of Lis1 in the heart is not the cause of the Lis1 KO phenotype. Thus my work suggests that Lis1 plays a vital role in autonomic neurons and disrupted axonal transport is the primary cause of the Lis1 KO phenotype.

Dynein has also been shown to interact with adenomatous polyposis coli (APC). This interaction is regulated by insulin signaling, specifically through phosphorylation by glycogen synthase kinase 3 (GSK3) (Gao et al., 2015; Gao et al., 2017). In wild-type (WT) cells, inhibition of GSK3 causes release of dynein from APC, leading to accumulation of dynein at the centrosome. In cells with the multiple intestinal neoplasia (MIN) mutation of the APC gene, GSK3 is unable to regulate the dynein-APC interaction. Using western blot analysis, I found that the insulin-signaling pathway remains functional in MIN cells. Since APC binds to microtubules, which are the tracks for dynein-dependent transport, I looked for changes in overall microtubule morphology and posttranslational modifications. No difference was observed in microtubule morphology, but there was less detyrosinated tubulin in MIN cells. However, this is unlikely to cause the observed phenotype, as dynein motility is *decreased* on detyrosinated microtubules (McKenney et al., 2016). Finally, using coimmunoprecipitation, I found that dynein interacts with the C-terminus of APC, which is absent in MIN

cells, and not the N-terminus. My work indicates that the absence of the C-terminus of APC, and not alterations of the microtubule cytoskeleton or insulin-signaling pathway, is responsible for the inability of GSK3 to regulate dynein in MIN cells.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 CYTOPLASMIC DYNEIN

Cytoplasmic dynein 1 (dynein) is a highly conserved minus-end directed microtubule motor that plays a role in a variety of cellular processes. The multi-subunit holoenzyme is comprised of heavy chains (DHC), which contain the motor and microtubule-binding domains and intermediate chains (DIC), light intermediate chains (DLIC), and light chains (Tctex1, roadblock, and LC8), which serve to regulate dynein-cargo interactions (Vale, 2003). This assortment of subunits, along with other interacting proteins, allows the motor to carry out diverse cellular processes.

Early work in budding yeast and filamentous fungi implicates dynein in nuclear migration and mitotic spindle orientation (Eshel et al., 1993; Li et al., 1993; Plamann et al., 1994; Xiang et al., 1994). Dynein is also critical for mitotic spindle formation and orientation in mammalian cells (Vaisberg et al., 1993; O'Connell and Wang, 2000). Dynein also facilitates nuclear envelope breakdown and localizes to kinetochores during mitosis, where it plays a role in microtubule capture, chromosome segregation, and progression through the spindle assembly checkpoint (Pfarr et al., 1990; Steuer et al., 1990; Sharp et al., 2000;

Howell et al., 2001; Wojcik et al., 2001; Salina et al., 2002; Bader and Vaughan, 2010).

During neurodevelopment, dynein is also important for migration of neurons and progenitors. Dynein and another microtubule motor, kinesin 3, mediate interkinetic nuclear migration (INM) of radial glial progenitor cells in the developing mammalian cortex (Tsai et al., 2007; Tsai et al., 2010; Hu et al., 2013). Newly formed neurons in the intermediate zone have a multipolar morphology, but soon transition to a bipolar morphology by extending one neurite toward the ventricle and another toward the cortical plate. This process is impeded by knockdown or mutation of dynein (Tsai et al., 2005).

One of dynein's most pertinent functions for this project is to mediate microtubule minus-end directed intracellular transport. In the axons of neurons, microtubules have a uniform "plus-end out" polarity, which means dynein is responsible for transport of cargoes from the axon terminal back toward the cell body – a process known as retrograde axonal transport (Baas et al., 1988; Vallee et al., 1989). The cargoes transported by dynein (and other motors) vary widely and include ribonucleoproteins (RNPs), membrane-bound vesicles, mitochondria, and cytoskeletal components.

Since dynein has such a diverse array of cellular functions, its activity must be tightly regulated. This is done primarily via a combination of protein-protein interactions, such as with Lis1, Ndel1, or APC, and posttranslational modifications, specifically phosphorylation of dynein and its regulators by protein

kinases, such as GSK3 and CDK5 (Allan, 2011; Gibbs et al., 2015). These regulatory mechanisms will be discussed in the following sections.

1.2 The Role of Lis1 and Ndel1 Outside of Axonal Transport

In humans, haploinsufficiency of the Lis1 gene causes lissencephaly, a neurodevelopmental disorder characterized by a smooth cerebral cortex, cognitive and motor deficits, and progressively worsening seizures. Lis1 binds directly to DHC and regulates many dynein-dependent processes both during and after development (Sasaki et al., 2000; Smith et al., 2000; Tai et al., 2002). In fact, decreased Lis1 expression in lissencephaly patients impairs dynein-dependent processes in mitosis and migration of neural progenitors, which has been implicated as the major mechanism underlying the disease (Sasaki et al., 2000). Depletion of Lis1 alters mitotic spindle assembly and orientation, impeding proliferation of neural stem cells, leading to depletion of the stem cell population (Tsai et al., 2005; Wynshaw-Boris, 2007). Aberrant migration is another consequence of decreased Lis1 expression, which results in ectopic neuronal positioning, which could contribute to the epileptic seizures seen in lissencephaly patients (Hirotsune et al., 1998; Tsai et al., 2005; Wynshaw-Boris et al., 2010).

Ndel1, a binding partner of Lis1, operates in a similar capacity during development through its interaction with DHC via its C-terminal domain, as well as DIC and Lis1 via independent binding sites on its N-terminal coiled-coil domain (Sasaki et al., 2000; Tai et al., 2002; Wang and Zheng, 2011; Zylkiewicz et al., 2011). This interaction with dynein is mediated by phosphorylation of Ndel1

by cyclin-dependent kinase 5 (CDK5) (Niethammer et al., 2000; Sasaki et al., 2000). In fact, hypomorphic Ndel1 mutant mice (expressing ~20% of WT levels) displayed neuronal migration defects and blastocysts of Ndel1 null mice had cell proliferation defects causing embryonic lethality, similar to Lis1 mutant mice (Hirotsune et al., 1998; Cahana et al., 2003; Sasaki et al., 2005). Lis1 and Ndel1 are crucial for these processes because they increase dynein force production, enabling transport of large loads, such as nuclei and chromosomes (McKenney et al., 2010).

1.3 Axonal Transport

As mentioned previously, dynein is the primary mediator of microtubule minus-end directed transport. Kinesins are a family of primarily plus-end directed microtubule motors (Vale et al., 1985; Vale, 2003). Since microtubules in axons have a uniform “plus-end out” orientation, dynein is responsible for transport of cargoes from the axon terminal or growth cone back toward the cell body (retrograde transport), while kinesins predominantly mediate transport of cargoes from the soma toward the end of the axon (anterograde transport) (Vale et al., 1985; Baas et al., 1988; Vallee et al., 1989; Vale, 2003).

These motors are capable of transporting a wide variety of cargoes to carry out the diverse processes required to support the axon. Cytoskeletal components, such as neurofilaments and vimentin, are transported along axons to support their structure and growth (Lee and Cleveland, 1996; Shea and Flanagan, 2001; Shim et al., 2008). mRNAs are transported in axons as

ribonucleoproteins (RNPs) where they can be locally translated in response to certain stimuli, such as neurotrophin signaling or axon injury (Ben-Yaakov et al., 2012; Gomes et al., 2014; Villarin et al., 2016). Mitochondrial transport is an essential process for sustain adequate energy levels for the axon, which may extend up to one meter in humans. Maintenance of mitochondria along the axon requires not only the transport of healthy mitochondria to regions of high energy demand, but also removal of damaged mitochondria through a process called mitophagy (Misgeld and Schwarz, 2017). Other membrane-bound organelles, such as endosomes, lysosomes, and autophagosomes are also transported within axons to recycle or degrade cellular components.

Due to the presence of three distinct, highly specialized cellular compartments (dendrites, cell body, and axons), motors must be spatially regulated to ensure transport of cargoes to the proper domain. One distinct region involved in sorting of cargoes to the correct compartment is the axon initial segment (AIS). Most organelles likely have both microtubule motors, as well as the actin-based motor, myosin, associated with them at any time. To facilitate directional movement of these cargoes, there must be a mechanism (or mechanisms) to coordinate the numerous motors attached. The uniform microtubule directionality in the axon allows sorting of axonal and somatodendritic cargoes through motor regulation. Axonal cargoes carried by plus-end directed kinesin motors to proceed through the AIS into the axon, while somatodendritic cargoes are turned away through mechanisms which engage the minus-end directed dynein motor. One such mechanism involves cyclin-

dependent kinase 5 (CDK5)-dependent phosphorylation of Ndel1, which is stably bound to the AIS (Kuijpers et al., 2016; Klinman et al., 2017). This increases Ndel1 binding to dynein and recruits Lis1 to this complex to activate transport of these cargoes out of the AIS toward the cell body (Kuijpers et al., 2016)

Another possible mechanism of regulating directionality of cargoes involves a molecular “tug of war” between opposing motors, where activity of one type of motor influences the activity of another (Levi et al., 2006; Ally et al., 2009; Hendricks et al., 2010). An alternative mechanism involves the scaffold protein, JNK-interacting protein 1 (JIP1), and the dynein regulator, dynactin (Fu and Holzbaur, 2013). JIP1 binding to kinesin heavy chain (KHC) relieves autoinhibition of the motor. JIP1 also interacts with the dynactin subunit, $p150^{Glued}$, which sequesters JIP1 from binding and activating KHC (Fu and Holzbaur, 2013). Phosphorylation of JIP1 by JNK acts as a switch to promote its interaction with KHC, stimulating anterograde transport of amyloid precursor protein-containing vesicles in axons (Fu and Holzbaur, 2013).

Lis1 and Ndel1 regulate dynein activity in retrograde axonal transport. The mechanisms behind this are still under some scrutiny, but it is apparent that Lis1 and Ndel1 are critical for proper transport of various cargoes. Previous research from our lab showed that CDK5 acts as a regulatory switch by phosphorylating Ndel1 and increasing its interaction with dynein, thereby increasing retrograde transport of Lysotracker labeled organelles (Pandey and Smith, 2011). Conversely, phosphorylation of dynein by glycogen synthase kinase 3 (GSK3) impairs Ndel1 binding, thus decreasing minus-end directed transport (Gao et al.,

2015). Expression of a dominant-negative form of CDK5 or an Ndel1 mutant which cannot be phosphorylated by the kinase dramatically reduced transport. Additionally, knockdown of Lis1 or expression of Lis1 mutants that could not bind dynein or Ndel1 disrupted retrograde transport (Pandey and Smith, 2011). Others, however, have observed disruption of dynein-dependent transport of various organelles when CDK5 activity is increased by overexpression of the CDK5 activator, p25, which would increase Ndel1 binding to dynein (Klinman and Holzbaur, 2015). Likewise, there have been contradictory results regarding the role of Lis1 in the transport of mitochondria. Knockdown of Lis1 in ageing Drosophila wing neurons augmented mitochondrial transport, while knockdown of Lis1 or Ndel1 disrupted mitochondrial transport in cultured rat hippocampal neurons (Shao et al., 2013; Vagnoni et al., 2016).

One explanation for the different effects observed is that regulation of dynein by Lis1 and Ndel1 is cargo-specific. For example, Lis1 and Ndel1 appear to play a crucial role in transport of large vesicles in axons, perhaps due to their ability to increase dynein force production (McKenney et al., 2011; Pandey and Smith, 2011). Another argument is that the type of regulation is dependent on the number of Lis1 molecules bound to a dynein motor. A single Lis1 protein binding to dynein appears to induce tighter binding to microtubules, perhaps conferring its ability to transport high-load cargoes, while binding of two Lis1 molecules produces weaker microtubule binding, allowing faster dynein-dependent transport (DeSantis et al., 2017). Additionally, Lis1's effects on dynein motility

may depend on whether another regulatory protein complex, dynactin, is bound to the motor (Baumbach et al., 2017; Gutierrez et al., 2017).

Dynactin can also stimulate dynein-dependent processes, including nucleokinesis and organelle transport, directly via its interaction with DIC (Schroer, 2004). Additionally, dynactin can facilitate dynein's interaction with cargoes via adaptor proteins, such as Bicaudal D homologue 2 (BICD2), BICD related-1 (BICDR1), and HOOK3 (Urnavicius et al., 2018). Interaction of dynactin with BICDR1 and HOOK3 can recruit two dynein motors to a single complex to increase force production and velocity (Urnavicius et al., 2018).

While all of the regulatory elements of axonal transport are not currently known, it is clear that precise control of motor activity and localization are critical to maintaining the integrity of the nervous system.

1.4 Link Between Neurodegenerative Disease and Axonal Transport

Due to the fundamental importance of intracellular transport, disruption of this process can have dire consequences, particularly in neurons, whose axons can extend over distances of up to 1m in humans. In fact, many neurodegenerative and later-onset neurological diseases have been linked to defective axonal transport.

For example, impaired retrograde transport of NGF and BDNF have been linked to Alzheimer's and Huntington's diseases, respectively. Endosomal trafficking and mitochondrial transport are commonly disrupted processes in a variety of degenerative diseases, such as Parkinson's disease, upper motor

neuron diseases, Charcot-Marie-Tooth (CMT), and amyotrophic lateral sclerosis (ALS) (Hinckelmann et al., 2013; Millecamps and Julien, 2013). Interestingly, many of these defects are seen in patients with mutations in genes which are not directly related to axonal transport or microtubule motors. For this reason, it is not currently known whether disrupted transport is causative in the progression of the diseases, or merely a symptom due to defects in other processes (Hinckelmann et al., 2013; Millecamps and Julien, 2013).

Mutations in the dynein motor itself, as well as direct regulators of dynein-dependent transport, have also been linked to several later-onset neurological disorders. These mutations can affect various aspects of transport, including motor dimerization, processivity, and cargo binding.

Mutations in the dynein heavy chain gene (DYNC1H1) that cause spinal muscular atrophy with lower extremity predominance (SMALED) have been shown to disrupt long-distance transport, without affecting cargo binding (Harms et al., 2012; Hoang et al., 2017). Meanwhile, DYNC1H1 mutations found in patients with CMT type 2O (CMT2O) are located in the stem domain of the protein and impair homodimerization (Weedon et al., 2011). Two mouse models of ALS – Legs at odd angles (Loa) and Cramping 1 (Cra1) – also have DYNC1H1 mutations that are thought to prevent homodimerization (Hafezparast et al., 2003). The Loa mutation also affects dynein intermediate chain binding and transport speeds (Hafezparast et al., 2003). Additionally, mutations in the dynein regulator, dynactin, cause Perry syndrome, a late-onset neurological disorder whose symptoms include parkinsonism, weight loss, depression, and

hypoventilation often leading to insufficient respiration and death, or lower motor neuron disease (Farrer et al., 2009; Moore et al., 2009). These mutations are in the CAP-Gly domain of the $p150^{glued}$ subunit of dynactin, which facilitates microtubule binding and affects dynein function (Farrer et al., 2009; Moore et al., 2009).

Interestingly, the mutations found in these diseases do not completely ablate the motor activity, but have a more subtle effect on its function. This could explain why their effects are seen later in life – complete disruption of motor activity would result in a devastating phenotype, but relatively minor alterations in function may lead to accumulation of damaged mitochondria, proteins, or other problematic cellular components over time.

1.5 The Role of APC in Neurons

Adenomatous polyposis coli (APC) is a tumor suppressor gene expressed in a wide variety of cell types. Mutations in APC are known to cause colorectal cancer in humans. It has also been shown to play an important role in the nervous system. APC is involved in Wnt signaling as part of the β-catenin destruction complex. It is also a microtubule plus-end binding protein, which enables it to regulate microtubule dynamics. These functions give rise to its role in regulating cell polarity and migration (Barth et al., 2008; Purro et al., 2008; Eom et al., 2014). These are particularly important processes in neurons, as they contain multiple compartments with distinct polarity and must migrate long distances in the developing brain.

APC also plays a role in synapse formation and axon growth. APC is involved in synapse formation and maturation by mediating PSD-95 and AMPA receptor clustering at the post-synapse (Shimomura et al., 2007). Additionally, deletion of APC impairs learning and memory and produces an autism-like phenotype in mice (Mohn et al., 2014). Deletion of APC also disrupts cytoskeletal organization in the axon and induces aberrant branching of cortical axons (Chen et al., 2011). It was recently discovered that APC can act as an RNA-binding protein and spatially regulate translation of mRNAs, including tubulin isoforms, in growth cones of extending axons (Preitner et al., 2014). This additional function may contribute to its role in axon growth.

In addition to interacting with microtubules directly, APC has been shown to interact with microtubule motors. The localization of APC at the periphery of fibroblasts and in growth cones of neurons is regulated by kinesin motors (Ruane et al., 2016). Our lab also found that APC directly interacts with and regulates the motility of cytoplasmic dynein and mutations in APC abrogate this regulatory mechanism (Gao et al., 2017). I will discuss my contributions to these studies in Chapter 4.

1.6 Goals of the Project

The overarching goal of the project is to determine how dynein-dependent transport is regulated, with an emphasis on axonal transport. Since axonal transport is disrupted in a variety of neurodegenerative diseases, understanding the mechanisms that influence this critical cellular process could provide new

avenues of research and targets for therapeutics for these diseases. Our hypothesis for these projects is that disruption of dynein-dependent axonal transport will produce signs of axon degeneration, which will impair neural function. By studying different pathways, using several techniques (e.g., genetic approaches, pharmacological manipulations, etc.), we hope to produce a more comprehensive model for how dynein-dependent transport is regulated and how dysregulation can lead to axonal pathology.

Additionally, one of the future directions for the lab is to identify the roles of these dynein regulatory pathways on axon regeneration following nerve injury. Chapter 2 discusses the role of spatial signaling after axon injury and during regeneration. While this chapter provides insight into this area of research, there are still many unanswered questions with regard to the role of dynein and its regulators during axon growth and regeneration.

CHAPTER 2

NEURONAL TRANSPORT AND SPATIAL SIGNALING MECHANISMS IN NEURAL REPAIR[1]

2.1 INTRODUCTION

Neurons form the circuits that control and coordinate all animal behavior. These circuits are vital for normal function of the brain, spinal cord, and peripheral nerves. Intercellular communication generated by transfer of electrical activity be-tween neurons and from neurons to target tissues is needed for every movement, sensation, and thought produced. A typical neuron is composed of three parts: a cell body, several dendrites, and an axon. The dendrites traverse short distances (≤ 2 mm) and bring activity to the cell body, while the axons are much longer and transmit activity away from the cell body. A neuron uses membrane depolarization to rapidly convey the information that is encoded by neural activity, from 'post-synaptic' sites in dendrites to the 'presynaptic' sites at the 'termini' of axons (i.e., intracellular communication). Arrival of such an 'action potential' at the axon terminus triggers release of neurotransmitters to propagate information across neural circuits or directly to the neuron's target (i.e., inter-cellular communication). The human neural circuits that connect motor cortex to

[1] Kalinski AL*, Hines TJ*, Smith DS, and Twiss JL. *Neuronal transport and spatial signaling mechanisms in neural repair*. Encylopedia of Cell Biology, Elsevier Press, Eds. Bradshaw RA and Stahl P. Reprinted here with permission from the publisher. ***indicates equal contributions**

spinal cord to muscle cover a distance approaching 2 m in some individuals. It is the axons of the motor cortex neuron and the spinal motor neuron that cover these long distances in the spinal cord and peripheral nerves, respectively. Disruption of axons through injury or disease blocks normal transmission of this neural activity and these long axonal processes are particularly vulnerable to damage.

While propagation of action potentials across these distances occurs on a millisecond time frame, neurons also use slower forms of intracellular communication by conveying organelles, protein complexes, and RNA–protein complexes anterogradely and retrogradely in axons and dendrites. Axonal transport has been particularly well-studied, and has historic-ally been separated into fast and slow components based on the speed of labeled proteins moving within axons. The fast component travels around 100 mm day^{-1}, while the slow component travels around 1 mm day^{-1} (Shah and Cleveland, 2002). The cargos that move in the fast component are important for the growing tips of developing axons (‘growth cones’) and for the proper function of mature synapses. These include organelles, membranes, proteins, and RNAs. Fast axonal transport also conveys signals to the cell body of the neuron that can regulate gene expression. The slow component of axonal transport ferries cytoskeletal polymers and other soluble proteins for maintenance of axon integrity (Shah and Cleveland, 2002). Movement of organelles and complexes within axons and dendrites is essential for neural function and plays key roles in neural repair.

‘Molecular motors’ or ‘motor proteins’ play the role of pack animals and messenger pigeons to move cargos to and from the neuron’s cell body along axons and dendrites. There are several types of motors but all share certain attributes, being multisubunit protein complexes that harness the energy of ATP hydrolysis to move along ‘cytoskeleton’ tracks in axons and dendrites. The rates at which motors ferry cargo depends on a number of criteria, but in vitro speeds of individual mammalian motors and also membrane bound vesicles in axons have been clocked at up to 4 mm s^{-1}. This speed is probably not maintained consistently, as transport cargos are frequently observed to pause and even reverse directions within axons and dendrites. Nonetheless, the movement of cellular components over very long distances in relatively short amounts of time is a rather herculean task that requires a significant expenditure of energy. Regulation of motor protein motility, cargo composition, and the organization of the cytoskeleton tracks within neurons can dramatically influence neuronal function, particularly during the repair of neural connectivity after injury. Motor-dependent transport mechanisms are also prime targets for dysregulation in disease processes that affect the nervous system.

This chapter will cover the contributions of this subcellular trafficking to neural repair, focusing on the axon. During development, axon growth is characterized by a rapid elongation phase until targets are reached. At this point an alternate mode of arborizing growth allows axons to contact target cells and initiate synaptogenesis. This short-distance sprouting of axons may also underlie a presynaptic contribution to synaptic plasticity. It should be noted that

development and regeneration of axonal processes share many common mediators, so much of this chapter will also be relevant to events in developmental growth. Although we focus on the repair of axons because of their fundamental importance in long-range communication in the nervous system, many of the mechanisms outlined here are also relevant for repair of dendritic processes.

2.2 Constituents for Intracellular Communication and Trafficking in Neurons

2.2.1 The Neuronal Cytoskeleton Provides Structure, Highways for Communication, and Growth Functions

The three components of the neuronal cytoskeleton, micro-filaments, intermediate filaments, and microtubules, provide structural integrity for mature axons and dendrites. Their continued maintenance throughout the life of the animal supports normal axonal and dendritic function. During developmental and regenerative events the cytoskeleton itself is highly dynamic; polymerization and depolymerization of microtubules and microfilaments in the distal ends of axons (and dendrites) contributes to both growth and pathfinding for axons and dendrites (Lowery and Van Vactor, 2009). Posttranslational modifications to the cytoskeleton and associated proteins can contribute to neuronal cytoskeletal dynamics – for example, cyclin-dependent kinase 5 (CDK5) impacts all three components of the neuronal cytoskeleton (Smith, 2003). Motor proteins can contribute to cytoskeletal dynamics by moving short segments of cytoskeletal

polymers or oligomers as cargo (Shah and Cleveland, 2002). This is particularly relevant during axon growth and arborization, but even after synapses are formed, transport of cytoskeletal components is likely to be important for maintenance of axons. The bulk of this movement appears to occur much more slowly than the speeds typically associated with fast axonal transport. However, this slow movement (and perhaps all of the slow component of axon transport) is thought to be driven by 'fast' motors that move the cargo intermittently (Li et al., 2012).

Microfilaments

Microfilaments are composed of two strands of actin monomers twisted into helical filaments that have intrinsic polarity. All cells, including neurons, have a microfilament-rich, mesh-like network on the cytoplasmic side of the plasma membrane. This 'cortical actin' has been proposed to provide a tethering platform for non-translocating but active motors, allowing them to function in unison to move small microtubules and attached cellular components within axons and dendrites (Myers et al., 2006). Polymerization and depolymerization of microfilaments at the distal end of growing axons and along the axon shaft, respectively, also play key roles in axon growth and branching. A large number of proteins have been identified that regulate actin dynamics and the spatial organization of actin filaments in growth cones (Lowery and Van Vactor, 2009).

Spectrin is another filamentous protein that is concentrated beneath the plasma membrane, providing a cross-link for the actin cytoskeleton and anchor for complexes of signaling and structural proteins, including membrane-linked

protein complexes (Machnicka et al., 2014). Different spectrin isoforms are concentrated in subdomains of the axon (e.g., axon initial segment, nodes of Ranvier) that are needed for neural function (Eshed-Eisenbach and Peles, 2013). Axonal spectrins can be rapidly proteolyzed following axonal injury (Buki et al., 1999), so reconstruction of these axonal domains and repopulating the axon with spectrin is a key event in transitioning a regrown axon into a functional axon.

Intermediate filaments

Neuronal intermediate filaments do not provide tracks for motors, but these proteins have important roles in both axon function and growth. Neuron-specific intermediate filaments provide structural support for growing axons, and accumulation of one class of filaments, the neurofilaments, dictates the final caliber of an individual axon (Lee and Cleveland, 1996). The pathophysiology of some human diseases is accompanied by alterations in neurofilament deposition, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, and several neuropathies (Perrot and Eyer, 2009). Posttranslational modification of the poly-peptides that make up neurofilaments contributes to their axonal delivery (Szaro and Strong, 2010). The intermittent 'slow component' of microtubule-based transport of neurofilaments may contribute to the precise deposition of intermediate filaments along the axon (Shea and Lee, 2013). High levels of neurofilament mRNA and protein levels are typically associated with more mature neurons. In fact, synthesis of neurofilaments protein falls after axotomy of peripheral nerves and stays relatively lower during regeneration (Hoffman and Cleveland, 1988). In some cases phosphorylated neuro-filaments

accumulate in the soma of regenerating axons, suggesting that transport into growing axons is reduced (Goldstein et al., 1987).

Another type of intermediate filament protein in neurons, peripherin, is expressed primarily in peripheral neurons. In contrast to neurofilament expression, peripherin expression increases during regenerative growth, falling back to basal levels as axons near their target tissues (Reid et al., 2010). The axonal inclusions seen in some ALS model mice have been shown to include peripherin, and overexpression of the ALS-linked protein TDP43 has been shown to cause a pro-longed increase in peripherin expression after peripheral nerve injury (Swarup et al., 2012). This implies that the ALS model has an altered course of neural repair.

Microtubules

Microtubules are the largest of the cytoskeletal polymers, and like microfilaments, have intrinsic polarity and the potential to be highly dynamic. Each microtubule is made of 13 parallel protofilaments, which are polymers of α- and β-tubulin heterodimers (Prokop, 2013). The organization of the heterodimers imparts the polarity to microtubules, with the α-subunit nearer to the 'plus end' and the β-subunit is nearer to the 'minus end.' In axons, microtubules have uniform polarity with distal plus ends oriented away from the cell body, but dendrites have mixed microtubule polarity (Baas and Lin, 2011). In motor-based transport this polarity is important because motor proteins typically prefer to move in either a plus-end or minus-end direction (Figure 2.1A). Therefore, the

polarity of microtubules creates a situation in which motors exhibit unidirectional movement in axons and bidirectional movement in dendrites.

Microtubules shrink and grow due to dimer addition and loss at their plus ends in a process called dynamic instability (Mitchison and Kirschner, 1984). Plus ends are the most dynamic, and have a cadre of associated proteins, known as 'plus end tracking proteins' (+TIPs), that regulate their behavior (Schuyler and Pellman, 2001). Growth and shrinkage occur less frequently at minus ends, in part because minus-end binding proteins can both protect against microtubule depolymerization as well as prevent addition of tubulin dimers (Goodwin and Vale, 2010). With neural injury, the microtubules and neurofilaments are depolymerized in the axon near the injury site on the proximal side (Figure 2.1B).

The importance of microtubules in axon growth has been known for decades. The expression of tubulins increases during regeneration, recapitulating developmental expression levels (Hoffman and Cleveland, 1988). Microtubule polymerization is a critical and highly regulated process in axon growth; and drugs that inhibit polymerization can block axon growth in cultured neurons (Tanaka et al., 1995). Interestingly, stabilization of microtubules with taxol can augment spinal cord regeneration (Hellal et al., 2011). Microtubule acetylation has been linked to microtubule dynamics and to regeneration (see below) (Cho et al., 2013). Microtubule-associated proteins (MAPs) also modify microtubule structure (e.g., 'bundling' of microtubules along the axon shaft), microtubule stability, and motor protein processivity (Hirokawa, 1994).

2.2.2 The Growth Cone Is Uniquely Suited for Its Job

The growing ends of developing and regenerating axons form a specialized structure called the 'growth cone' (Figure 2.1C). The shape and behavior of the growth cone is dictated by both extrinsic and intrinsic factors (Gomez and Letourneau, 2014). Examples of extrinsic factors are substrate molecules in the extracellular milieu or matrix (ECM) present along the growth trajectory, and molecules secreted locally into the environment by neighboring cells. Intrinsic factors include cytoskeletal dynamics that are often regulated by proteins within the growth cone like the Rho, Rac, and CD42 small GTPases, as well as the spatial distribution of receptors for sensing environmental cues. Generation of a growth cone after an axon is injured is generally thought to be essential for regenerative growth. One of the initial events in establishing a growth cone after injury is the entrance of calcium into the damaged axon – as outlined in subsequent sections, this triggers a number of changes that not only allow this important structure to form, but also facilitates signaling to the neuronal cell body (Bradke et al., 2012).

While growth cones can vary greatly in shape and behavior, a typical advancing growth cone is composed of three major domains: central (C-), transition (T-), and peripheral (P-) domains (Gomez and Letourneau, 2014). The C-domain is made up of stable microtubule bundles that come from the axon shaft. These microtubules tend to be acetylated in the axon shaft and as microtubules enter the growth cone, tyrosination becomes the prominent posttranslational modification on the tubulins (Figure 2.1C). Acetylated tubulin is

associated with stable polymers, while tyrosinated tubulin is associated with more dynamic polymers (Janke, 2014). The C-domain typically contains the more dynamic microtubules that can extend linearly in sync with the advancing edge of the growth cone. However, microtubules are responsive to environmental cues, and can shrink back or become curved in order to pause advancement or to reorient direction of growth (Lowery and Van Vactor, 2009). The P-domain at the very leading edge of the growth cone is composed of actin-based projections called lamellipodia and filopodia. Microfilaments in lamellipodia form a dynamic mesh, with new filaments being added to growing edge and old filaments being broken down at the back of the mesh near the C-domain. Force-generating myosin motors (see below) control the contractility of this meshwork (Medeiros et al., 2006). The filopodia, are finger-like projections outward from lamellipodia that extend and retract to sample their environment. They contain dynamic bundles of actin filaments, which are thought to form through the concerted action of myosin-driven 'retrograde flow' of the actin mesh in lamellipodia and the extension of actin polymers in the filopodia (Medeiros et al., 2006).

The dynamic nature of the cytoskeleton in growth cones is what allows these structures to respond to environmental cues, and growth cones ultimately must transition into synaptic endings or other types of functional axon termini. When the growth cone adheres to a substrate such as laminin, the actin cytoskeleton becomes anchored to receptors, preventing retrograde movement of actin, which causes the filopodia of the growth cone to extend or protrude via distal actin polymerization (Mitchison and Kirschner, 1988). As this happens,

myosin motors create tension between F-actin filaments and an F-actin arc at the base of the growth cone closest to the axon shaft. This allows microtubules of the C-domain to move forward, leading to an engorged growth cone; after this, the F-actin arc depolymerizes and the membrane collapses around the stable microtubule bundles, consolidating this region into an extension of the axon shaft (Lowery and Van Vactor, 2009). It should be noted that localized synthesis of β-actin protein and actin-interacting proteins in growth cones may also contribute to actin dynamics in the growth cone, as well as along the axon shaft where collateral branches form (Zhang et al., 1999; Spillane et al., 2013). Motor protein regulators (e.g., lissencephaly protein (Lis1) and nuclear distribution E-like 1 (Ndel1), see below) are prominent in growth cones of regenerating axons in culture (Niethammer et al., 2000; Smith et al., 2000), and can play an active role in promoting growth cone motility.

2.2.3 Molecular Motors Ferry Cargo to and from Axons and Provide Force Generation for Axon Growth

Molecular motors transport of a wide range of cargos in axons including organelles, vesicles, mRNA, and signaling molecules. The mRNAs move as RNA–protein complexes termed ‘ribonucleoproteins’ (RNPs) (Gomes et al., 2014). Cargo may be deposited along axons, or trafficked anterogradely to synapses and growth cones or retrogradely back to the cell body. Transport along microtubules is carried out by kinesins, which for the most part are plus-end directed, and cytoplasmic dynein, which is minus-end directed (Hirokawa et al., 2010). Cytoplasmic dynein also has the capacity to become tethered to the

cortical actin cytoskeleton within axons and growth cones. The force generating heads that project into the axon away from the cortical actin are then able to 'kick' short microtubule segments, causing them to move with their plus-ends leading (Mazel et al., 2014). In addition to microtubule motors, axons contain myosin motors that ferry cargo on microfilament tracks. While these three classes of motors share common features, including motor domains that require ATP, they are also quite distinct with respect to molecular organization as well as motility regulatory mechanisms.

Kinesin

There are 14 classes of kinesin superfamily proteins organized in part by where the motor domain is located within the polypeptide (Hirokawa et al., 2009). 'Conventional' kinesins in the Kinesin-1 group are heterotetramers. Two 'heavy chains' (kinesin heavy chain, KHC) form a dimer with globular head domains that interact with microtubules and undergo force-producing conformational changes in response to ATP binding and hydrolysis. KHC stalk regions contain the dimerization domains, while the C-terminal tails interact with 'light chains' (kinesin light chain, KLC) that confer cargo binding (Marx et al., 2006). Though the motor domains are well conserved, substantial variability in other parts of the heteromer, including KLCs, can confer cargo selectivity. Kinesins move a variety of cargo toward axon termini, so they are critical for maintenance of mature axons and synapses, as well as for delivery of construction materials, receptors, adhesion proteins, and membranes to growing axons (Figures 2.1(a) and 1(b)). Different kinesins in this large superfamily are responsible for anterograde

transport of specific cargo types within axons. Some of the specificity is likely derived from distinct heavy chains, but cargo-specific transport by the same heavy chain can be modulated by associated light chains (Hirokawa et al., 2009).

Dynein

Although a few kinesins (e.g., KIFC2) may be capable of minus end-directed transport (Hirokawa et al., 2009), the pre-dominant minus end-directed motor in axons is cytoplasmic dynein (Figures 2.1A and B). This huge protein complex contains 12–14 subunits and has 2 identical heavy chains (dynein heavy chain, DHC) that form a dimer (Vale, 2003). The large ring-shaped motor domains at one end of DHC are ATPases, and are the force-generating component of the protein complex. A stalk extending from each DHC motor domain interacts with microtubules. DHC's stem interacts with an array of other 'chains.' These include two intermediate chains, 2–3 light intermediate chains and 3 distinct light chains. It is generally believed that the accessory subunits bind to adaptors that confer cargo specificity and/or control dynein processivity (Allan, 2011). The cytoplasmic dynein holoenzyme is fairly uniform in subunit composition. However, multiple genes encode related but distinct isoforms of each accessory subunit. Moreover, alternative splicing and post-translational modifications can alter subunit composition to optimize dynein motors for different tasks (Pfister et al., 1996). Because axonal dynein moves retrogradely toward the cell body, it is important for molecular communication between axon endings and the nucleus. In intact axons, dynein carries information related to

synapses and target tissues, and convey signals that prevent continued axon elongation after developmental growth has ceased (Smith and Skene, 1997).

Myosin

Myosin is a force-generating ATPase that underlies muscle contraction. The classic muscle myosin remains tethered in place and brings about muscle contraction by exerting tugging forces on actin filaments. Some myosins in neurons also function by exerting forces on the subcortical actin network (Figure 2.1C) – these are particularly prominent in regulation of growth cone dynamics needed for development and re-generation (Lowery and Van Vactor, 2009). Other members of the myosin superfamily are processive and can ferry cargo, using microfilaments as transport tracks. Some myosins (e.g., myosin Va) are plus-end directed, and others (e.g., myosin VI) are minus-end directed (Kneussel and Wagner, 2013). Myosin transport is typically thought to occur over shorter distances than the long-range axonal transport associated with microtubule motors (i.e., at branch points, in growth cones, in synapses, etc.).

Regulation of motor proteins – Activation, inhibition, and cargo selectivity

Much of what we know about motor regulation has come from in vitro studies with purified proteins or cultured cells. However, studies in neuronal cultures suggest that these mechanisms are likely relevant to nerve repair. Because of their force-generating capacities, their plethora of cargos and the many cellular processes influenced by motors, a strictly enforced regulatory network must be in place to coordinate motor behaviors and modulate their behaviors in response to environmental cues and intrinsic cues. At the most

basic level, ATP must be in good supply for all motors to function properly. Perhaps intuitively, mitochondrial ATP was shown to be the source of ATP for fast axon transport of mitochondria (Zala et al., 2013). However, the source of ATP for transport of some vesicular cargos appears to be from glycolytic machinery directly associated with the vesicle (Zala et al., 2013). Thus, the cargo itself can impact activity of the motor protein carrying it, and there is a growing understanding that complexes of motor proteins, scaffolds, and regulatory proteins can modulate motor processivity and force generation. The selection of a motor's cargo is in part regulated by cargo adaptors, proteins that bind to both the motors and to a specific type of cargo (Fu and Holzbaur, 2013). An interesting new development is that mammalian cytoplasmic dynein is incapable of moving along microtubules in vitro in the absence of either cargo or cargo adaptor proteins (McKenney et al., 2014; Schlager et al., 2014). It remains to be determined if this is also the case within the confines of the axon shaft.

Knowledge of motor protein regulation has been advanced by studies of neurological disorders. For example, mutant forms of huntingtin (HTT) protein that cause Huntington's disease form aggregates in axons and dendrites that can block transport by motors (Lee et al., 2004). Wild-type HTT can bind directly to dynein intermediate chain (DIC) subunits (Caviston et al., 2007), while an HTT binding partner HTT-associated protein 1 (HAP1) interacts with KLC (McGuire et al., 2006). Moreover, HTT is involved in linking the glycolytic machinery to cargo vesicles to provide a ready source of ATP for fast axonal transport (Zala et al., 2013). While pathogenic HTT remains capable of interacting with motor

complexes, it reduces motor association with microtubules through a c-Jun N-terminal kinase 3 (JNK3)-mediated phosphorylation of kinesin-1 (Morfini et al., 2009).

Most organelles have all three classes of motors associated with them, usually more than one of each kind of motor. Interactions between motors may occur via scaffolding systems (Fu and Holzbaur, 2013). There is growing evidence that motors of one class can affect activity of other classes of motors (Levi et al., 2006; Ally et al., 2009). While many of these interactions have been observed, how functions are coordinated to regulate cargo movement in the same axons has not been extensively studied. The precise trafficking of an individual cargo likely involves regulation of the stoichiometry of motor association, a tug of war between opposing motors of all three classes, the proximity of transport tracks, as well as posttranslational modification of proteins and cargos (Kardon and Vale, 2009; Fu and Holzbaur, 2013). Each of these mechanisms is operant in the mature as well as injured and regenerating axon. One known molecular control mechanism involves the JNK-interacting protein 1 (JIP1) that acts as a scaffold protein that binds to both KHC and the dynein activator, dynactin (Fu and Holzbaur, 2013). JIP1 binding to KHC relieves an intramolecular autoinhibition of kinesin motor activity. The dynactin subunit $p150^{Glued}$ competes with KHC for binding to JIP1 so that KHC is inactive when JIP1 is bound to p150Glued. Kinesin disinhibition and dynactin binding by JIP1 is regulated by JNK-dependent phosphorylation, which can lead to directional changes in movement of amyloid precursor protein-containing vesicles in axons

(Fu and Holzbaur, 2013). JIP1 is also responsible for directional selectivity of autophagosomes in axons. Autophagosomes are retrogradely transported by dynein, and are presumed important for axonal maintenance and growth. Dual mechanisms appear to prevent activation of kinesin on these organelles (Fu et al., 2014). JIP associates with autophagosomes through the adaptor, microtubule-associated protein light chain 3 (LC3), and LC3 prevents JIP binding to KHC.

Other proteins can bind directly to motors and regulate force generation and/or processivity in vitro and also impact axon transport in cultured cells. For example, loss of Lis1 protein reduces retrograde transport of acidic vesicles and Lis1 overexpression increases speed and run lengths of retrogradely moving vesicles in axons (Pandey and Smith, 2011). Lis1 seems to function with dynein to promote microtubule advance during growth cone remodeling and fast axon growth (Grabham et al., 2007). During embryogenesis, Lis1 contributes to neuronal migration (Reiner and Sapir, 2013), and it interacts with two paralogous dynein-binding proteins Ndel1 and Nde1 (Bradshaw et al., 2013). Ndel1/Nde1 also serves as a platform for modulation of motor activity by posttranslational modification. Ndel1/Nde1 strengthens the Lis1 association with dynein and increases force production by promoting cooperation between multiple dynein motors (McKenney et al., 2010). Interestingly, the neuronally enriched kinase Cdk5, phosphorylates Ndel1/Nde1 (Niethammer et al., 2000). Phosphorylation of Ndel1 by Cdk5 is important for dynein-dependent organelle transport in growing axons extended by adult sensory neurons in culture (Pandey and Smith, 2011).

Recent evidence suggests that Cdk5 is an important regulator of membrane vesicle dynamics in growth cones (Hsiao et al., 2014; Tojima et al., 2014). Other kinases that contribute to regulation of axonal transport have also been observed to impact regeneration. For example, glycogen synthase kinase 3 (GSK-3) exerts a negative influence on axon transport in squid and fly axons (Morfini et al., 2002). GSK-3 has shown varying effects on axon regeneration, but recent work indicates that sustained activation of GSK-3 supports regeneration of axons in peripheral nerve (Gobrecht et al., 2014). Clearly, the interplay between signals regulating axon transport and those regulating axon growth is complex, and just beginning to be understood.

How extracellular stimuli regulate motor proteins is also not well understood, but some pathways are beginning to emerge. For example, activation of semaphorin receptors along growing axons stimulates both anterograde and retrograde movement of axonal vesicles (Li et al., 2004). Semaphorin stimulation of distal axons shifts α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit localization to dendrites through a coordinated retrograde signaling in axons and subsequent anterograde transport in dendrites (Yamashita et al., 2014). Neurotrophins also impact transport. These ligands increase endosome binding to dynein through DIC phosphorylation, thereby stimulating retrograde transport (Mitchell et al., 2012). Interestingly, neurotrophin receptors (Trks) are endocytosed in axons along with their ligands and can be retrogradely transported as a ‘signaling endosome’ that carries downstream effector kinases to the cell body (Schmieg et al., 2014).

During development, many neuronal populations compete for binding to target-derived neurotrophins and other trophic agents. The neurons that do not secure an appropriate source of trophic support are eliminated through naturally occurring cell death. Culture preparations in which the neurons' cell body and axons are compartmentalized have shown qualitative differences in the response to neurotrophic stimulation of distal axons compared to the cell body (Campenot and MacInnis, 2004). This suggests that the signaling endosome conveys a signal different from Trk receptors endocytosed along the cell body.

Activation of Trk receptors along the axon has been shown to modulate transport of RNPs into axons, with accumulation and translation of mRNAs adjacent to the trophic stimulus along axons (Willis et al., 2007). Target-derived nerve growth factor (NGF) in sympathetic neurons was recently shown to coordinate transcriptional responses in the cell body with delivery of mRNA encoding a survival promoting protein, B-cell lymphoma w (Bclw), to the axons (Cosker et al., 2013). Given that peripheral nervous system (PNS) injury results in increased expression of neurotrophins and other trophic factors by Schwann cells in the severed nerve stump (Funakoshi et al., 1993), it is intriguing to hypothesize that signaling endosomes moving retrogradely to the cell body would be used to coordinate anterograde delivery of macromolecules to the regenerating axons. The cargos that motor proteins deliver into axons obviously play a role growth and regeneration. The axon needs new cytoskeleton and membrane components to grow, as the severed segment of the axon distal to injury site undergoes Wallerian degeneration in mammals (see below). It was

also recently recognized that protein products of axonally transported mRNAs support regeneration in the PNS (Donnelly et al., 2013), and there is some evidence that this may occur after spinal cord injury as well (Willis et al., 2011).

2.3 Neural Repair Mechanisms

2.3.1 Injury Changes Axon Structure and Axonal Transport to Initiate a Regeneration Program

With damage and severing of an axon, the segment distal to the site of injury (i.e., the portion no longer connected to the cell body) is actively degraded through Wallerian degeneration. In the portion proximal to the site of injury, influx of Ca^{2+} from extracellular spaces promotes membrane sealing and locally activates proteases (Bradke et al., 2012). Proteases degrade axonal proteins including cytoskeletal elements, and this is necessary for the initiation of axonal growth (Ziv and Spira, 1997). The increase in axoplasmic Ca^{2+} also triggers retrograde signaling through transport mechanisms (Rishal and Fainzilber, 2010). This shift in retrograde transport ultimately helps to turn on expression of genes that support growth (regeneration-associated genes (RAGs)) and turn off expression of genes associated more with mature neuronal functions (Rishal and Fainzilber, 2010). For example, axotomy in the PNS increases the expression of growth-associated protein-43 (GAP-43) and Tα1-tubulin, but decreases expression of neurofilaments. Injury to the mammalian brain or spinal cord, in contrast to peripheral nerves, often does not activate expression of RAGs, and this may in part, underlie the reduced growth capacity of mammalian central nervous system (CNS) neurons after injury (Hoffman, 2010). Many strategies to

increase axon regeneration after central injuries have focused on altering the 'intrinsic growth capacity' of CNS neurons through changing gene expression programs (Mar et al., 2014). ECM glycoproteins and glial-derived proteins in the injured brain and spinal cord also actively block axon growth, and over-coming these 'roadblocks' is often a complimentary strategy to support CNS neural repair (Geoffroy and Zheng, 2014).

Some of the mechanisms that underlie the shift in retrograde axonal transport are known. Axonal JNK3 is locally activated after injury, with a resulting increased association of a complex of JNK-interacting proteins 3 (JIP3) and activated JNK3 with the p150Glued subunit of dynactin, and increased retrograde transport of activated JNK3 (Cavalli et al., 2005). This leads to activation of c-Jun in the neuronal cell body that presumably supports expression of genes needed for regeneration (Ruff et al., 2012). Interestingly, JIP3 binding to KHC can also increase kinesin motility (Sun et al., 2011). It is not clear whether the JIP1-dependent retrograde transport of autophagosomes mentioned above contributes to neural repair; however, it is intriguing that both JIP1 and JIP3 can coordinate anterograde and retrograde transport (Fu and Holzbaur, 2014). This provides a powerful mechanism to coordinate transport to meet physiological needs of the neuron and potentially to recycle proteins between axons and cell body.

Calcium influx coordinates the injury response

The influx of Ca^{2+} into injured axons also triggers translation of locally stored mRNAs (Perry and Fainzilber, 2014). These mRNAs are transported into

axons as RNPs and it remains unclear exactly how Ca^{2+} influx activates translation locally in axons, but it likely includes a commensurate release of Ca^{2+} stores from sites in axons (Vuppalanchi et al., 2012). Axonal mRNAs known to be translated by increased axoplasmic (Ca^{2+}) include Importin β1, Ran specific binding protein 1 (RanBP1), and vimentin (Gomes et al., 2014). These encode proteins that contribute to a retrograde injury signal in a complex highly orchestrated process (Rishal and Fainzilber, 2010). The newly translated Importin β1 in axons heterodimerizes with Importin α3 (Hanz et al., 2003). Localized synthesis of RanBP1 facilitates this by releasing α3 from an anterograde transport complex (Yudin et al., 2008)). Interaction of the Importin β1/α3 complex with dynein is needed for the transport of proteins containing nuclear localization signal (NLS) to the nucleus. The cargo of the dynein-Importin β1/α3 complex includes other proteins that are locally synthesized with injury (e.g., signal transducer and activator of transcription 3α, Stat3α) as well as proteins that are locally activated through injury-induced posttranslational modifications (e.g., extracellular-regulated kinase, Erk 1/2) (Perlson et al., 2005; Ben-Yaakov et al., 2012). Vimentin has been considered as a glial intermediate filament protein in the nervous system, but vimentin can be expressed in neurons, and its mRNA has been shown to localize to axons (Willis et al., 2007). Curiously, proteolytic cleavage of vimentin in injured axons generates a 'scaffold peptide' for activated Erk 1/2, a mitogen-activated protein kinase (MAPK), to bind to Importin β1/α3 dimer. This kinase is transported to the cell body with dynein, where it activates Elk1 (and possibly other) transcription factors (Perlson et al.,

2005). It is likely that such a posttranscriptional mechanism is shared with other physiological responses of axons, since neurodegenerative stimuli, neuronal survival promoting stimuli, and a neurotropic virus are now recognized to couple events in the distal axons to cell body responses through translation of axonal mRNAs (Cox et al., 2008; Cosker et al., 2013; Koyuncu et al., 2013; Baleriola et al., 2014). These and other studies indicate that axonal injury induces a localized, integrated response to shift retrograde transport toward cargos that assist in mounting a regenerative response. The full extent of these cargos has yet to be determined.

Calcium influx transforms the injured axon to support regenerative growth

Much of what we know about axonal transport and localized growth mechanisms have been derived from studies in cultured neurons, where events can be spatially and temporally monitored with high precision. Neurons cultured from invertebrate organisms have provided a platform for visualizing cytoskeletal dynamics in growing and injured axons. For example, the initial studies on Ca^{2+} signaling after axonal injury resulted from work in Aplysia neurons (Ambron and Walters, 1996). The Ca^{2+} gradient that forms in these axons after injury occurs in both the proximal (intact) and distal (severed) segments of the injured axon, but only the proximal end undergoes regeneration (Ziv and Spira, 1997). The Ca^{2+} dependent events including membrane sealing and cytoskeletal restructuring controlled by calcium-activated calpain, syntaxin, synaptotagmin, and synaptobrevin are critical for neural repair (Bradke et al., 2012). Indeed, activation of calpain through a Ca^{2+}-dependent mechanism is required for growth

cone formation (Gitler and Spira, 2002). Proteolysis of cytoskeletal elements in the cut axon results in a reorganization of microtubule polarity in Aplysia (Sahly et al., 2006). This may generate a 'trap' for capturing both anterogradely and retrogradely transported vesicles, which are then available to fuse with the plasma mem-brane for efficient and effective formation of a growth cone (Erez and Spira, 2008). Work in vertebrate neurons indicates that activation of localized protein synthesis in the distal cut axon is also needed for growth cone formation (Verma et al., 2005). Though the proteins synthesized were not identified, TC10 and Exoc3 mRNAs, which encode components needed for plasma membrane expansion, are locally translated in growing axons (Gracias et al., 2014). Stimulation of MAPKs and mammalian target of rapamycin (mTOR) converge on protein synthesis machinery and are needed for growth cone formation (Bradke et al., 2012). Thus, localized mRNA translation supports the initial transition of an injured axon into a regenerating axon. Subsequent growth of the axon requires delivery of necessary building blocks of cytoskeleton and membrane components, which is accomplished by anterograde transport of proteins, vesicles, and RNPs.

2.3.2 Unexpected Roles for Proteins in Growing Axons

Axonal functions for nuclear proteins

With technical advancements in subcellular imaging, protein/ RNA detection and analyses, and study of signal transduction mechanisms, previously unrecognized functions have been ascribed to proteins and protein networks that support neural repair. For example, epigenetic regulation is now well recognized as a

means to modulate expression of entire gene families, and there is clear potential for such chromatin-based modulation to contribute to neural repair (Finelli et al., 2013). However, recent work from two groups have uncovered intra-axonal functions for histone deacetylase (HDAC) proteins that are classically viewed as modifiers of chromatin structure and, hence, transcription. The injury-induced Ca^{2+} entry into axons noted above produces a retrograde 'Ca^{2+} wave' that triggers exit of HDAC5 from the nucleus in a protein kinase C-dependent pathway. In keeping with classic functions of HDACs, the loss of HDAC5 from the nucleus supports expression of genes whose encoded proteins have roles in re-generation (e.g., c-Fos, c-Jun, KLF4, KFL5, and Gadd 45a; see Cho et al., 2013 and references within). However, HDAC5 is also anterogradely transported into the injured axon, where it supports deacetylation of microtubules. This decreases stability of microtubules in the cut axon that is needed for growth cone formation just proximal to the injury site. Curiously, this anterograde movement of HDAC5 only occurred after peripheral nerve injury and not after optic nerve injury, a lesion of CNS axons (Cho et al., 2013), pointing to another distinction between the central and PNS with axonal injury (Cho et al., 2013). HDAC6 protein has also been shown to function in axons, but inhibition of HDAC6, rather than activation, brings growth-promoting effects (Rivieccio et al., 2009). For HDAC6 inhibition, this allows for growth on nonpermissive substrates including myelin-associated glycoprotein and CSPGs that block axonal growth after spinal cord injury. These observations suggest that HDAC5 and HDAC6 have antagonistic functions in neural repair. However, HDAC6 may contribute to axon health

because it facilitates the retrograde transport of protein aggregates for destruction in autophagosomes (Lee et al., 2010). It will be intriguing in the coming years to discover the mechanism of action and targets of these deacetylases in developing, regenerating the mature axons.

New functions for adenomatous polyposis coli protein, a protein associated with colorectal cancer

Other proteins have shown multifunctionality that impacts axon development and likely axon regeneration. Adenomatous polyposis coli protein (APC) is a large, multidomain microtubule-interacting protein initially discovered because truncating mutations lead to colorectal cancer in humans (Vogelstein and Kinzler, 2004). APC has also been linked to axon growth through promoting microtubule polymerization (Chen et al., 2011). In addition to its function as a +TIP, APC interacts with many different proteins including β-catenin and GSK-3β from the canonical Wnt signaling pathway (Clevers and Nusse, 2012). Initial studies in fibroblasts raised the possibility that APC's actions may also extend to inter-action with mRNAs (Mili et al., 2008). More recent work has shown that APC binds directly to more than 250 neuronal mRNAs (Preitner et al., 2014). Interestingly, nearly half of those mRNAs were known to localize into growing axons of PNS sensory neurons, including the Importin β1 and β-actin mRNAs that are mentioned above. Preventing APC binding to a tubulin isoform mRNA (TUBB2B2) decreased axonal levels of this mRNA and caused growth cones to shrink, which could significantly hamper the growth cone's ability to interact with growth substrates (Preitner et al., 2014). Though it is not clear whether APC is

needed for transport or translation of these mRNAs (or both), the protein is perfectly positioned in the growth cone to serve as a scaffold concentrating mRNAs and translational machinery near the processive tips of micro-tubules. There is work in developing neurons indicating that localized mRNAs accumulate along with translation factors and ribosome subunits adjacent to cytoplasmic tails of axon guidance receptors (Tcherkezian et al., 2010). It will be interesting to see if APC also contributes to transport or translation of mRNAs in injured axons where localized protein synthesis is known to contribute to regeneration.

A protein kinase that can regulate both axon regeneration and Wallerian degeneration

Genetic screens in model organisms, including flies, worms, and mice, identified the 'dual leucine zipper kinase' (DLK) as regulator of both axon regeneration and degeneration (Tedeschi and Bradke, 2013). DLK has also been called 'ZPK' in mice and 'Wallenda' in Drosophila. Loss of DLK delays degeneration of axons but also attenuates RAG transcription in the cell body (Shin et al., 2012a). Activation of DLK can lead to downstream activation of both JNK and 38 kDa mitogen-activated protein kinase (p38MAPK). As emphasized above, JNK activation is important in neural repair, and downstream c-Jun dependent transcription activates expression of RAGs in the cell body after axonal injury (Shin et al., 2012a). However, blocking JNK activation in severed axons distal to the injury site delays Wallerian degeneration, indicating dual functions for both DLK and JNK (Ferraris et al., 2013). In addition to JNK's roles in axonal transport mediated through JIPs, JNK is known to target microtubule binding proteins that

regulate dynamic stability of these cytoskeletal elements, which can influence both axon formation and regeneration (Tedeschi and Bradke, 2013). JNK phosphorylation triggers degradation of super cervical ganglion clone 10 (SCG10, also called Stathmin-2) in axons (Shin et al., 2012b). SCG10 is a membrane bound protein that destabilizes microtubules (Riederer et al., 1997). Degradation of SCG10 in the severed axon is linked to Wallerian degeneration, but SCG10 is rapidly replenished in the proximal axon segment proximal to the injury and this is needed for regeneration (Shin et al., 2014). Destabilization of axonal microtubules through SCG10's actions near the growth cone contributes to axon motility. JNK is also known to phosphorylate microtubule-associated protein 1b (MAP1b) in axons, which conversely increases stability of microtubules (Chang et al., 2003). Interestingly, the mRNA encoding a protein kinase that can be an intermediate between DLK and JNK, MKK7, or mitogen-activated protein kinase kinase 7 (MAP2K7), localizes to proximal neurites of differentiating NIE-115 cells and primary hippocampal neurons (Feltrin et al., 2012). This may provide a means to compartmentalize the functional outcome of JNK's activation in growing axons, by concentrating substrates that differentially modify microtubule dynamics to different sites along the axon (e.g., growth cone for destabilization vs. axon shaft for stabilization).

Regulation of microtubule motor proteins can directly support axon regeneration

Interestingly, kinesin and dynein motors have recently been shown to have unexpected roles in attenuating axon growth. The levels and/or activity of kinesin and dynein motors may contribute to axonal growth by providing a gauge

for how much material the axon needs in order to grow (Albus et al., 2013). In cultures of adult sensory neurons, partial depletion of kinesin and/or dynein heavy chains with siRNA actually increases axon length over time (Rishal et al., 2012). Legs at odd angles (Loa) mice that have reduced levels of dynein heavy chain 1 (Dync1h1) show increased axon length in developing limb buds indicating that this length sensing mechanism is also relevant for axon growth in vivo (Rishal et al., 2012). These data suggest that continual anterograde and retrograde signaling, analogous to a radar signal, contributes to axon growth rates (Kam et al., 2009). However, the cargos that are needed for this, both those carried anterograde into the axons and those carried retrograde back to the cell body, are not clear

Work from the Baas group using depletion of kinesins also suggested that localized interactions of kinesin and dynein motors could affect axon length (Liu et al., 2010). They observed that partial depletion of kinesin-5 or kinesin-12 increased axon length in cultured neurons, but attributed this to a shift in the tractile forces along the distal axon to favor increased growth (Myers and Baas, 2007; Liu et al., 2010). They hypothesized that loss of either kinesin allows cytoplasmic dynein to pull microtubules in the opposite direction, moving more microtubules from the axon shaft into the growth cone, resulting in both increases in axon length and the inability of the growth cone to turn properly without spatial control over the microtubules in the growth cone (Liu et al., 2010). Regardless of whether this is a cargo-dependent signaling mechanism or localized force differentials through altered motor stoichiometry, the end result of decreasing

motor protein activity results in the counter-intuitive effect of increasing axon length. It will be of substantial interest to determine how these mechanisms can be integrated with the injury-dependent and regeneration-associated changes in axonal trafficking out-lined above that are essential for repair of neural connectivity.

2.4 PERSPECTIVES

Despite all that has been learned of neural repair mechanisms and the potential for using trafficking to encourage axons to regrow after injury, even is the PNS where regeneration is relatively robust, regenerating axons only progress at 1–2 mm per day. Thus, finding a means to increase growth rates should bring huge benefits. However, whether increased growth rate can be accomplished be modulating axon transport dynamics in vivo remains to be determined. Interesting potential targets for regulating axon growth through trafficking include the motors themselves, their regulatory partners, kinases, cargo adaptors, and specific cargos.

In addition to overt axon elongation driven by growth cone advance, developing neurons must also undergo a different kind of axon growth even after synapse formation. Small-scale axon growth and remodeling of synaptic connections occur throughout adult life and underlies learning and memory. But this does not compare to increased length that nerves and axon tracts accomplish during growth from a late stage embryo to late adolescent, and is unlikely to be the mechanism underlying this elongation. There is some evidence that neurons switch to a stretch-induced interstitial growth as organisms increase

their rostral-to-caudal dimensions (Suter and Miller, 2011). Cultured sensory neurons have been 'stretched' in an attempt to model this process, and within limits, stretching results in increased rates of axon growth while maintaining electro-physiological function (Pfister et al., 2006). The mechanisms underlying this increased growth are not clear, but it would seem that the axon must lay down more cytoskeleton and membrane or suffer compromised integrity. Adult neurons also seem to tolerate this axon stretching less well than embryonic neurons, which could relate to age acquired cytoskeletal modifications or loss of developmental gene expression programs (Loverde et al., 2011). Still the concept of stretch-induced growth may be a promising lead for future neural repair strategies once the mechanism(s) can be uncovered, particularly if this might complement other growth-promoting strategies.

There are intersections between neural repair, axonal transport, and neurodegenerative mechanisms that warrant mention. As indicated above, DLK and SCG10 have dual roles in regeneration and axonal degeneration. The severed axon has lost its cell body source of proteins, RNPs, and organelles. Consequently, this severed axon segment needs to survive on existing entities, or gather what it needs from its local environment (e.g., glia). To some extent, defects in axonal transport can result in a similar situation. Reduced anterograde delivery of axonal components produced in the cell body and reduced transport of signals originating in distal targets can compromise the health of the neuron. Indeed, transport defects can be a key factor in neurological diseases and mutations or depletion of motor proteins or their regulators are emerging as

causative agents in disease. Beyond Huntington's disease mentioned above, defects in axon transport in age-related dementias such as Alzheimer's disease (Kanaan et al., 2013). The distances that motor axons traverse may make them particularly vulnerable to transport deficits. Consistent with this notion, a KHC mutation that disrupts microtubule binding has been found in patients with hereditary spastic paraplegias, mutations in mouse DHC lead to an ALS-like motor neuron degeneration (Fichera et al., 2004; Ikenaka et al., 2012) and a mutation in the p150Glued dynactin subunit has been found in an autosomal dominant form of lower motor neuron disease (Puls et al., 2003).

Symptoms of neurodegenerative diseases including ALS have been linked to loss of synapses that may be initiated by 'dying back' of the distal axon (Adalbert et al., 2012). Blocking the degeneration of their axons can prevent the death of neurons in some neurodegenerative diseases. For example, deletion or inhibition of DLK is neuroprotective in cellular and animal models of Alzheimer's disease, Parkinson's disease and glaucoma (Ferraris et al., 2013). Expression of axonally targeted Ube4b-NMNAT1 fusion protein (Wallerian degeneration slow protein, 'Wlds') can delay Wallerian degeneration and prevent neuron loss in several different animal models of neurodegeneration (Coleman and Freeman, 2010). Delayed degeneration occurs because the Wlds protein maintains nicotinamide adenine dinucleotide (NAD+) levels in the severed axons. Normally axons contain NMNAT2 that fulfills the same enzymatic function to maintain NAD+, but NMNAT2 has short a half-life and must be continually replenished by anterograde transport (Gilley and Coleman, 2010). If transport becomes

compromised, the neuron can be placed in the vulnerable position of losing its axon. Intriguingly, work in ALS animal models pointed to a shift in the retrogradely transported cargos from 'pro-survival' cargos to 'stress-related' cargos with increasing severity of neurodegeneration (Perlson et al., 2009). The similar responses in physical injury of the axon and neurodegenerative diseases suggest the possibility that neurons in both cases may be mounting, or attempting to mount, a regenerative response, although few studies have addressed this possibility. Coordination of several signal transduction pathways and intracellular trafficking/signaling is needed to mount and maintain a regenerative response. Small alterations in these pathways could effectively alter function, growth, and maintenance of the axon.

Axon injury/repair and Lis1

While the bulk of this chapter is a review beyond the scope of the work I've completed during my doctoral degree, it is relevant to some future directions we are beginning to explore in the lab and covers some of the signaling pathways involved in regulating transport, which plays a crucial role in axon regeneration. Specifically, the sections regarding the role of motor proteins in injury signaling and how transport of cellular components (cytoskeletal proteins, synaptic proteins, etc.) is critical to regeneration of an injured axon are intimately related to the work I have published and present in this dissertation.

Additionally, our lab has begun investigating the role of Lis1 in nerve regeneration. There are multiple aspects of injury and repair that Lis1 may regulate. First is the retrograde injury signaling – is Lis1 involved in the transport

of pSTAT3 and other injury signals from the site of injury to the nucleus? Additionally, Lis1 and its regulation of dynein could have effects on axon growth itself through transport of cellular components and its effects on cytoskeletal dynamics. Indeed, Figure 3.2I in Chapter 3 shows data indicating Lis1 knockout may disrupt growth of axons from cultured adult DRG neurons. Future experiments will attempt to determine the mechanism through which this occurs.

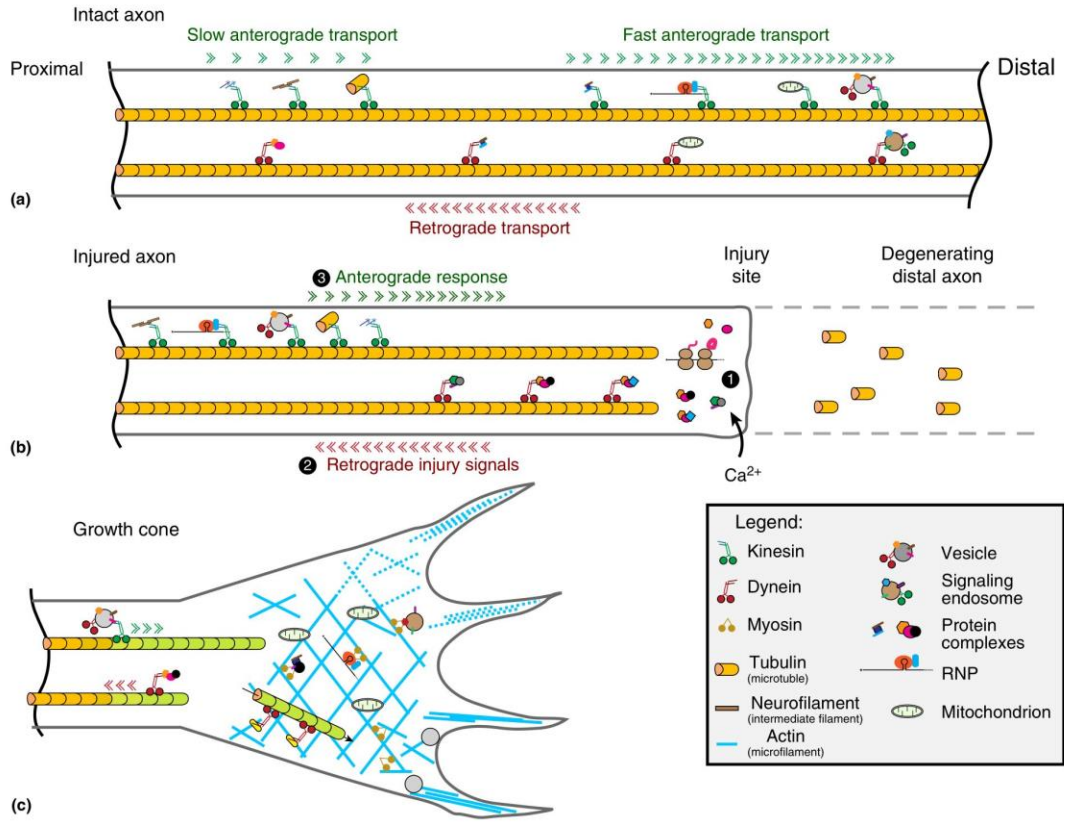

Figure 2.1: Schematic of neural trafficking in mature, injured, and regenerating axons. A, Schematic of mature axon with proximal on left and distal on right; microtubules show characteristic polarity with plus ends oriented distally. With this polarity, kinesin is used for anterograde transport and dynein is used for retrograde transport. Intermediate filaments and cortical microfilaments characteristic of the mature axon are not shown here. **B**, With injury of the axon, the higher extracellular (Ca^{2+}) results in Ca^{2+} influx (1) that triggers membrane sealing, proteolysis, and translation of resident mRNAs encoding injury-associated gene (IAG) products in axons. Injury results in a shift in retrograde transport (2), including signaling proteins that are 'activated' locally after injury as well as newly translated proteins encoded by the some of these IAG mRNAs in axons. Transcription in the neuronal cell body is altered by this shift in transport resulting in increased transport of regeneration-associated gene (RAG) products (3) into the injured axon to support neural repair. **C**, The repaired end of the injured axon is transformed into a growth cone that is rich in actin microfilaments and contains dynamic microtubules that support growth. This figure is adapted from (Kalinski et al., 2015a).

CHAPTER 3

AN ESSENTIAL POSTDEVELOPMENTAL ROLE FOR LIS1 IN MICE[2]

3.1 INTRODUCTION

LIS1 mutations in humans cause a “smooth brain” mal-formation called lissencephaly (LIS) characterized by severe cognitive and motor impairments and worsening epilepsy, leading to early mortality (Dobyns and Das, 1993; Dobyns et al., 1993; Sapir et al., 1999; Gleeson, 2000; Sicca et al., 2003; Saillour et al., 2009; Reiner and Sapir, 2013; Herbst et al., 2016). Most of the human mutations result in a null allele with 50% reduction of LIS1 protein levels, which profoundly impacts the developing nervous system. Other mutations can produce a milder phenotype, but the phenotype/genotype correlation is complex. A classic mouse study made it clear that gene dosage is relevant, as progressive reduction of Lis1 protein levels caused progressively more severe phenotypes (Hirotsune et al., 1998). Deletion of a large portion of one Lis1 allele in mice, resulting in a null allele, delays neuronal migration and differentiation, but unlike humans, mature mice show mild neurologic abnormalities and are viable and fertile (Hirotsune et al., 1998; Gambello et al., 2003). Cre-mediated knockout (KO) in

[2] Hines TJ, Gao X, Sahu S, Lange MM, Turner JR, Twiss JL, Smith DS. (2018). *An Essential Post-Developmental Role for Lis1 in Mice*. eNeuro. doi: 10.1523/ENEURO.0350-17.2018. Reprinted here with permission from the publisher.

specific subpopulations of developing neural cells in mice impacts mitosis and nucleokinesis, causing developmental delay (Tsai et al., 2005; Tsai et al., 2007; Yingling et al., 2008; Youn et al., 2009; Hippenmeyer et al., 2010).

Lis1 is a highly conserved regulator of the minus-end directed microtubule motor protein, cytoplasmic dynein 1; together they regulate neural stem cell spindle orientation, nucleokinesis, and nuclear envelope breakdown during brain development (Vallee et al., 2001; Wynshaw-Boris and Gambello, 2001; Gambello et al., 2003; Shu et al., 2004; Tsai et al., 2005; Vallee and Tsai, 2006; Tsai et al., 2007; Hebbar et al., 2008; Schwamborn and Knoblich, 2008; Yingling et al., 2008; Youn et al., 2009; Hippenmeyer et al., 2010; Moon et al., 2014). In fact, mutations in the dynein heavy chain gene DYNC1H1 can also cause cortical malformations in humans (Vissers et al., 2010; Willemsen et al., 2012; Poirier et al., 2013).

Of particular interest are reports that DYNC1H1 mutations cause later onset neurologic disorders, including forms of spinal muscular atrophy (SMA) and Charcot-Marie-Tooth disease (Weedon et al., 2011; Harms et al., 2012). Additionally, mutations in genes encoding two other dynein regulators DCTN1 and BICD2, cause Perry syndrome and SMA (Wider and Wszolek, 2008; Neveling et al., 2013; Oates et al., 2013; Peeters et al., 2013). The extent to which Lis1 functions post-developmentally, especially in minimally proliferative tissues like adult brain, has not been studied extensively. Hunt et al. (2012) found that heterozygous Lis1 KO in six-week-old mice altered synaptic function in the hippocampus in the absence of altered laminar granule cell architecture, but the

mechanisms underlying the altered activity are not known (Hunt et al., 2012). It has been shown that Lis1 manipulations impact dynein-dependent axon transport in sensory neurons cultured from adult rats (Smith et al., 2000; Pandey and Smith, 2011). These results suggest that Lis1 is a positive regulator of dynein-based axon transport in adult mammals. This was also found in adult mouse DRG neurons (Klinman and Holzbaur, 2015). Although axon transport studies suggest a role for Lis1 in cultured adult neurons, these neurons do not form synaptic connections, so its involvement in synapse formation and maturation is currently unknown. If Lis1 indeed regulates axon transport in the mature nervous system, Lis1 mutations could have deleterious effects on circuitry in mature brains. We have addressed this fundamental question using a tamoxifen-inducible Cre-Lox system to disrupt Lis1 selectively in adult mice. We show that Lis1 is indispensable in adult mice, and describe unexpected temporal and spatial recombination patterns and how they impact the phenotype of Lis1 KO in adult animals. Our data point to a vital role for Lis1 in cardiorespiratory nuclei in the hindbrain.

3.2 Results

3.2.1 Lis1 is expressed in adult mouse tissues, predominantly in the nervous system

As detected by automated capillary immunoblotting, levels of Lis1 protein were only modestly lower in adult brain than embryonic brain protein samples (Fig. 3.1). Substantial Lis1 was also observed in adult spinal cord and dorsal root ganglia. While Lis1 could be detected in adult heart, liver, kidney, lung, and

diaphragm, the levels were much lower in all non-nervous tissues tested than in brain, spinal cord and DRG. Therefore, Lis1 may function in other tissues, but is either present in fewer cells or at lower amounts per cell.

3.2.2 KO OF LIS1 BY CRE-MEDIATED RECOMBINATION CAUSES AXON TRANSPORT AND GROWTH DEFECTS IN CULTURED ADULT SENSORY NEURONS

All mouse strains used in these studies are described in Table 1. To test the effectiveness of the Act-Cre-ER model for inducing recombination in neurons and reducing Lis1 expression, DRGs from Lis1 KO mice and the no Cre controls were dissociated and cells cultured for 24 h. Cultures were treated with 4-OHT for an additional 72 h. No tamoxifen-induced recombination was observed in the no Cre control cultures, as detected by prominent tdTomato fluorescence but no GFP (Fig. 3.2A). In contrast, substantial recombination was observed in the majority of neurons in the Lis1 KO cultures, detected by the presence of bright GFP fluorescence (Fig. 3.2B). GFP was observed in both axons and cell bodies. Also, Lis1 protein levels in extracts prepared from Lis1 KO cultures was greatly reduced relative to no Cre control cultures (Fig. 3.2C). Together these findings demonstrate effective KO of Lis1 by bath-applied 4-OHT in cultures of Lis1 KO mice but not of no Cre controls.

Intraperitoneal injection of tamoxifen into adult Lis1 KO mice also resulted in recombination in DRGs. For this experiment we injected 8 mg of tamoxifen on two consecutive days (2 x 8 mg regimen; see Materials and Methods) and harvested DRGs on day 4 after the first injection. Bright GFP fluorescence was

observed in neuronal plasma membranes and satellite cells in DRG sections from these animals (Fig. 3.2D). No GFP was detected in DRGs from no Cre control animals injected at the same time (data not shown). Dissociated cultures prepared from no Cre control DRGs showed only tdTomato fluorescence (Fig. 3.2E), while Lis1 KO cultures exhibited bright GFP fluorescence indicative of substantial recombination (Fig. 3.2F). We first immunostained these cultures for NF to label axonal processes specifically so that images could be analyzed using automated software algorithms. However NF immunoreactivity was much more prominent in axon endings in Lis1 KO axons, possibly reflecting altered NF transport (Fig. 3.2E, F). Because of this we used the GFP and tdTomato signals to manually count varicosities. As predicted, the KO neurons had significantly more varicosities than the no Cre controls (Fig. 3.2G). Although varicosities occur normally at sites of growth cone pausing or sites of branch formation (Rees et al., 1976; Malkinson and Spira, 2010) an increased number is often associated with axonal blockages due to transport defects (Liu et al., 2012). Indeed, reduced retrograde transport of acidic organelles in living axons was observed in Lis1 KO axons compared to no Cre controls (Fig. 3.2H) consistent with other studies in adult rat DRG neurons where Lis1 was depleted using siRNA transfections (Pandey and Smith, 2011). We also observed that Lis1 KO axons appeared shorter. To quantify this we measured total axon length per neuron using a mixture of neuron-specific antibodies to ensure uniform labeling of axons (Fig. 3.2I). On average, Lis1 KO neurons had significantly shorter axons. There were

also fewer neurons that had extended axons on day 2 after plating, indicating a delay in the onset of axon regeneration (Fig. 3.2I).

3.2.3 Lis1 KO causes a severe phenotype in adult mice

Surprisingly, the 2 8 mg regimen caused a rapid decline in health of Lis1 KO animals, with spinal kyphosis (Fig. 3.3A) and hind leg clasping (Fig. 3.3B) observed 4 d after the first injection. None of the control animals showed this phenotype. In early experiments animals died within a week after the first injection, and mice were subsequently killed as soon as they began to exhibit symptoms, typically on days 3–5. Animals that were given a different regimen of tamoxifen, 2 mg injected for five consecutive days (5 x 2 mg regimen), remained non-symptomatic for nearly two weeks, after which they exhibited similar symptoms as observed in the 2 x 8 mg regimen. Figure 3.3C shows symptom-free survival duration plots for these animals. No differences were observed between male and female animals. Most animals were 2 months old at the time of injection, but similar responses were observed in older animals (4-5 months). Control animals did not exhibit any symptoms but were typically killed at the same time as KOs to be able to compare tissues for extent of recombination and Lis1 expression levels. However, six no Cre control and six CAG-cre/Esr1 animals that received the 2 x 8 mg tamoxifen regimen lived for over a month with no detectable symptoms (Fig. 3.3C).

3.2.4 Temporal differences in tamoxifen-induced recombination in distinct brain regions of Lis1 KO mice

Lis1 KO mice and no Cre control strains were given the 2 8 mg tamoxifen regimen. As expected, the no Cre control brains only exhibited tdTomato fluorescence on day 4 after the first injection (Fig. 3.4A, left). In contrast, bright GFP fluorescence was detected in Lis1 KO mice, but surprisingly, primarily in midbrain and hindbrain regions, with little fluorescence detected in the cortices (Fig. 3.4A, right). A similar result was observed in no flLis1 control so the recombination pattern is similar regardless of whether or not the mice carry the floxed Lis1 alleles.

Although the severity of the phenotype in Lis1 KO mice prevented examination of recombination at later times, no flLis1 control animals and Lis1 KO hets remained symptomless for at least three weeks after the 2 x 8 mg tamoxifen regimen. These animals showed substantial recombination observed across the entire brain, including the cortex, demonstrating variable rates of recombination in different brain regions with this 2 x 8 mg tamoxifen regimen (shown for Lis1 KO het in Fig. 3.4B).

In Lis1 KO mice recombination was observed throughout the medulla pons, midbrain and cerebellum and into the spinal cord, but only sparsely in the cortex and hippocampus (Fig. 3.4C). At this level of analysis, the most prominent recombination in the cerebellum occurs in the molecular layer in linear profiles reminiscent of Bergmann's glia. Substantial recombination was also ob-served in the olfactory bulb and choroid plexus (Fig. 3.4C). GFP-positive cells in the

brainstem appeared stellate in shape (Fig. 3.4D). White matter tracks in the brainstem and cervical spinal cord were also GFP-positive (Fig. 3.4E). This GFP distribution correlates with the reduced Lis1 expression observed in brainstem and cerebellum but not in cortex (Fig. 3.4F).

3.2.5 Lis1 KO occurs in both neurons and glia

In cryosections of Lis1 KO DRGs (2 x 8 mg regimen), recombination was observed in both neurons and satellite cells (Fig. 3.2C). Membrane-targeted GFP was observed along neuronal membranes in the brainstem, Purkinje cells in the cerebellum, and motor neurons in the spinal cord (Fig. 3.5A–F). It was more difficult to distinguish neuronal processes from glia or axons from dendrites in the neuropil. Since Lis1 depleted DRGs neurons show altered axon transport and DRGs cultured from Lis1 KO animals showed signs of altered axon transport, we examined cross sections of phrenic, vagus, and sciatic nerves, as well as spinal cord ventral roots. Two concentric rings of GFP were typically observed around myelinated axons (Fig. 3.5G–J). The outer ring flanked the periphery of the myelin sheaths (Fig. 3.5I) that stained for myelin basic protein (Fig. 3.5J). GFP was not observed in the tightly packed myelin sheath itself, so the outer ring likely represents the plasma membrane of the myelinating glial cell. The inner ring was juxtaposed along the axoplasmic membrane of the ensheathed axon, and we interpret this as representing tamoxifen-induced recombination in axons of Lis1 KO neurons. This interpretation is strengthened by the observation that only an outer GFP ring was observed in some myelinated axons (Fig. 3.5I), which would be unlikely if the inner GFP-positive ring was also part of the

myelinating Schwann cell. Unmyelinated C-fiber bundles in the sciatic nerve are ensheathed by membranes of Schwann cells that do not form myelin. These “Remak bundles” can be observed by EM (Fig. 3.5K). Red and green fluorescent rings were often observed in the same Remak bundles indicating that some, but not all axons in the Remak bundle had undergone recombination (Fig. 3.5L). Together these data support the in vitro finding that recombination, and by inference, Lis1 KO, occurs in both neurons and glia. The preponderance of recombination in the midbrain, hindbrain, and PNS, coupled with reduction in Lis1 protein levels in these regions, suggest that Lis1 KO in neurons and glia in these regions contributes to the observed neurologic phenotypes.

3.2.6 Brainstem neurons show chromatolysis in Lis1 KO mice

Figure 3.6A shows GFP expression in a coronal section through the hindbrain, with a dense concentration of GFP-positive cells in the ventral hindbrain. This area contains nuclei that are vital for cardiorespiratory function, and complete functional loss of these neurons would result in rapid death (Melov et al., 1998; Quintana et al., 2012). Impairment of axonal transport or other dynein-dependent processes in this region could account for the severe phenotype in Lis1 KO animals. Experimental axotomy and diseases that involve axonal dysfunction can produce the cell body response of chromatolysis (Cragg, 1970; Hanz and Fainzilber, 2006). Though the mechanisms underlying chromatolysis remain hypothetical, the process is characterized by nuclear swelling and nuclear acentricity, both of which were observed in GFP-rich regions in coronal sections through the brainstem of Lis1 KO mice (Fig. 3.6C).

The nuclei were significantly larger and more acentric than controls providing evidence for axonal dysfunction in these neurons (Fig. 3.6D–G).

3.2.7 Lis1 loss in the hindbrain is the most likely cause of the KO phenotype

Lis1 KO mice receiving the 2 x 8 mg tamoxifen regimen had a much more rapid onset of symptoms compared to the 5 x 2 mg regimen (Fig. 3.3C). Indeed, at a time when the 2 x 8 mg mice were severely affected (day 5), the 5 x 2 mg animals had no overt symptoms. Substantially more recombination was observed in the brains of 2 x 8 mg animals compared to 5 x 2 mg animals, which correlates with the onset of severe symptoms (Fig. 3.7A) and the level of Lis1 mRNA in the brainstem (Fig. 3.7B). In contrast, recombination in the heart was similar in both sets of mice (Fig. 3.7C), as were Lis1 mRNA levels, which were reduced equally with both regimens (Fig. 3.7D). This indicates that Lis1 KO in the heart is less likely to be responsible for the early onset of symptoms. Other tissues with sporadic GFP (lung, liver, and kidney) also showed a similar degree of recombination with both regimens, supporting the idea that the hindbrain loss of Lis1 contributes significantly to the KO phenotype.

We performed another experiment to directly test contributions of Lis1 KO in the heart to the phenotype observed in Lis1 KO mice by generating an inducible KO in which Cre-ER is driven by a Myh6 promotor (Myh6 KO; (Sohal et al., 2001)). As expected, tamoxifen injections (2 x 8 mg) reduced Lis1 expression in the heart but not in the brainstem of these mice (Fig. 3.7E). Moreover, robust GFP expression, and thus Myh6 KO-dependent recombination, was observed in

the heart, but not in the brain (Fig. 3.7F). Despite significant recombination in the heart, 0/12 mice showed any detectable phenotype, and all lived apparently symptom free, until killed four weeks later. Together these data provide evidence that loss of Lis1 in midbrain/hindbrain neurons is responsible for severe phenotype in Lis1 KO mice.

3.3 Discussion

We show that Lis1 KO by tamoxifen-induced recombination causes neuropathology and lethality in adult mice. The finding that Lis1 continues to play a vital role after the majority of mitosis and migration in the brain has occurred supports the idea that Lis1 regulates additional cellular processes such as axonal transport. Given the preponderance of evidence that Lis1 regulates cytoplasmic dynein coupled with the observed temporal and spatial pattern of Cre-mediated recombination, the simplest explanation for the severe phenotype caused by Lis1 depletion is that defective axon transport results in pathologic changes in midbrain, hindbrain, spinal cord, and DRG neurons. That these areas are critical to the phenotype is supported by the correlation between the location and extent of recombination (and thus Lis1 loss), and the onset of symptoms using different tamoxifen dosing regimens. At the onset of severe neurologic symptoms (4 – 5 d) the 2 x 8 mg tamoxifen regimen produced substantial recombination in cells in those regions. At the same time point in animals exposed to the 5 x 2 mg tamoxifen regimen no symptoms were present and much less recombination was observed in these areas. Unlike the nervous system, other tissues (heart, lung,

liver, kidney) had a similar degree of recombination using both regimens, arguing for an important nervous system contribution to the Lis1 KO phenotype. This is further supported by evidence of chromatolytic neurons in the brainstem and axon transport defects and varicosities in DRG neurons cultured from Lis1 KO mice. Axons of the vagus, phrenic, sciatic nerves and ventral roots contained the Cre reporter (GFP) indicating that recombination had occurred in neurons. Lis1 depletion in neurons with axons running in those nerves could contribute to the Lis1 KO phenotype. For example the vagus nerve contains autonomic axons emerging or converging on the nucleus ambiguous, solitary nucleus, dorsal nucleus of vagus, and spinal trigeminal nucleus, and is critical for cardiorespiratory control. The phrenic nerve contains sympathetic, sensory and motor axons innervating the diaphragm, mediastinal pleura, and pericardium. Brainstem perturbations in animals can lead to death due to cardiorespiratory disruption (Talman and Lin, 2013; Piroli et al., 2016; Sun et al., 2017). Thus, the idea that Lis1 KO in brainstem neurons is a lethal event in mice is not too farfetched.

Sensory and motor neurons with axons in the sciatic nerve are less likely to contribute to the death of the animals, but may contribute to the leg clasping and kyphosis observed in 100% of Lis1 KO mice. Also, the finding that axon growth by DRG neurons in culture is compromised by Lis1 depletion indicates that peripheral nerve regeneration in vivo could be affected by changes in Lis1 expression. The role of microtubule motors in developmental axon growth and axonal regeneration after injury is complex. In some studies, motor activity

appears to be a negative regulator of growth. Retrograde target derived signals prohibit growth in mature connected neurons (Smith and Skene, 1997). Transport-dependent length-sensing signals have an inverse relationship with axon length, in those studies dynein or kinesin knockdown or a specific dynein mutation caused adult DRG axons to be longer (Kam et al., 2009; Rishal et al., 2012). Finally, stimulation of dynein by a BICD2 mutation resulted in shorter axons in cultured hippocampal neurons (Huynh and Vale, 2017).

On the other hand, there are also many studies that indicate that both anterograde and retrograde signals promote regenerative growth (Mar et al., 2014; Kalinski et al., 2015b). For example, a pro-growth injury signal that is required for axonal regeneration in the sciatic nerve requires retrograde transport by dynein (Ben-Yaakov et al., 2012). Dynein can push the cytoskeleton forward during axon initiation and elongation in developing axons (Dehmelt et al., 2006; Roossien et al., 2014), and DHC knockdown reduced microtubule movements into growing axons and stunted axon outgrowth in cultured adult PNS neurons (Ahmad et al., 2006). While our data focused on regeneration of adult axons, others have also found that depletion of Lis1 leads to decreased developmental axon growth (Grabham et al., 2007; Kumamoto et al., 2017). Taken together, these data suggest that Lis1 knockdown could reduce transport of regenerative signals and also disrupt dynein-dependent cytoskeletal changes required for axon growth. Because Lis1 overexpression stimulated dynein-dependent organelle transport (Pandey and Smith, 2011), it will be interesting to determine whether Lis1 overexpression also negatively impacts axon growth.

While it is critical to know the organs, tissues and cells that contribute to the severe Lis1 KO phenotype, it is equally important to determine which are less affected by Lis1 depletion. With respect to the midbrain/hindbrain, spinal cord, and DRG neurons, we cannot state that some neuronal subtypes are more affected by Lis1 KO than others, and in fact, suspect that disruption in all of them could be a contributing factor to the phenotype, as suggested above. With respect to other brain regions, including the cerebral cortex, hippocampus, striatum, pallidum, and hypothalamus, Lis1 KO animals died before significant recombination occurred in these regions. However, we expect that Lis1 is important in all axons; signs of pathology may have become apparent in these regions had Lis1 KO animals survived long enough for recombination to occur. Our heterozygous Lis1 KO animals exhibited substantial recombination in all brain regions by three weeks after 2 x 8 mg tamoxifen injection but did not exhibit leg clasping, kyphosis or lethality. It remains to be determined whether signs of axonal dysfunction will arise in cortical regions of heterozygous Lis1 KO mice as animals age. Another group used a similar inducible system to examine hippocampal function following heterozygous Lis1 KO in six-week-old mice (Hunt et al., 2012). They found an increase in excitatory synaptic input to granule cells in the absence of neuronal positioning defects. The molecular and cellular underpinnings of this are not known, but our data suggest that it might involve axonal transport disruption. In our study, a much more limited Lis1 depletion was accomplished by stereotaxic injections of 4-OHT into lateral ventricles. This did not produce any obvious symptoms (leg clasping, kyphosis or death) probably

because recombination had occurred very sparsely and mainly in glial cells (data not shown). Ultimately, more selective examination of the role of Lis1 in adult neuronal circuits will require using Cre-driver(s) specific for different neuronal populations. Some glial cells exhibited Cre-mediated recombination in our Lis1 KO mice. Glial cells in cortical, hippocampal and DRG cultures express Lis1 (Smith et al., 2000), and there was substantial recombination in Bergmann's glia, astrocytes and Schwann cells, so depletion of Lis1 in any of these cells could theoretically contribute to Lis1 KO phenotypes. Glial specific Lis1 KO may allow us to answer this question.

With respect to other tissues in the mouse, we can state unequivocally that the severe Lis1 KO phenotype was not due to Lis1 depletion in cardiomyocytes. Depletion in liver, lung, and kidney was likely highly mosaic at the time when symptoms were severe because recombination was sparse in these tissues, and was similar on day 5 using both tamoxifen regimens. Thus, while our data suggest that Lis1 depletion in these tissues does not produce the rapidly lethal phenotype, to fully answer this question, we will need to use organ or cell specific Cre-ER drivers.

While our studies unequivocally demonstrate a vital role for Lis1 in adult mice and strongly support a role in axon transport, Lis1 and dynein both function in dendrites, at least during development. For example, dynein-based transport occurs in dendrites in rodent hippocampal cultures (Kapitein et al., 2010; Ayloo et al., 2017), and signs of transport defects are observed in motor neurons in the adult Loa dynein mutant mouse (Wiggins et al., 2012).

Interestingly, Lis1 knockdown by shRNA expression disrupted the translocation of excitatory synapses in developing interneuron dendrites in hippocampal cultures and organotypic slices (Kawabata et al., 2012). Moreover, two-photon microscopy showed altered spine morphology dynamics in three-week-old Lis1 mice (Sudarov et al., 2013). Together, these reports suggest that Lis1 dysfunction in dendrites in the midbrain, hindbrain, and spinal cord could contribute to the Lis1 KO phenotypes.

Uncovering a role for Lis1 in axonal or dendritic transport in post-mitotic, connected neurons is interesting for several reasons. First, if axonal transport is compromised in LIS, it could contribute to the seizures (which become increasingly frequent and severe) and the early lethality typical of the disorder. Axon transport defects, unlike defects that arise because of brain malformations that occur in utero, might be ameliorated with drugs that target the dynein regulatory machinery. Second, dynein-related proteins are linked to many neurodegenerative diseases (Rees et al., 1976; Wider and Wszolek, 2008; Weedon et al., 2011; Harms et al., 2012; Neveling et al., 2013; Oates et al., 2013; Peeters et al., 2013). In fact, respiratory problems in the late-onset disorder, Perry Syndrome, are the cause of lethality in humans (Wider and Wszolek, 2008). To treat such disorders, it is critical to understand the mechanisms regulating dynein in axons. In most cell culture studies, Lis1 overexpression stimulated, and Lis1 disruption reduced processivity (Liu et al., 2000; Smith et al., 2000; Pandey and Smith, 2011; Shao et al., 2013; Klinman and Holzbaur, 2015; Villarin et al., 2016). However, one transport study indicated

that Lis1 knockdown increased mitochondrial transport (Vagnoni et al., 2016), and several in vitro biophysical studies showed that Lis1 inhibited processivity of purified dynein (Yamada et al., 2008; McKenney et al., 2010; Huang et al., 2012). More recent assays using purified proteins are beginning to reveal how this might occur at the molecular level in the context of other dynein regulators like dynactin and BICD2 (Baumbach et al., 2017; DeSantis et al., 2017; Gutierrez et al., 2017). In those studies, Lis1 dramatically increased dynein processivity. Interestingly BICD2 mutations that cause SMALED stimulate dynein processivity, so motor activity must be finely tuned (Huynh and Vale, 2017). Kinase pathways that impact dynein function have been identified. CDK5, mutations in which have been linked to LIS (Magen et al., 2015; Parrini et al., 2016) and other kinases phosphorylate and regulate the Lis1- and dynein-interacting protein Ndel1 (Hebbar et al., 2008; Pandey and Smith, 2011). Phosphorylation also regulates motors directly (Gibbs et al., 2015). We recently reported that insulin-dependent inhibition of GSK3b, a kinase with a growing list of neurologic disease links (Dell'Osso et al., 2016), phosphorylates dynein and regulates its interactions with Ndel1 and APC (Gao et al., 2015; Gao et al., 2017). It will be interesting to determine whether these pathways can be manipulated to alter the severity of the Lis1 KO phenotype, and if they can be used in trying to alleviate symptoms of patients with diseases caused by transport defects.

3.4 Materials and Methods

Mice

All animal experiments were conducted under a protocol approved by the Animal Care and Use Committee of the University of South Carolina. Males and females were used in experiments; no differences were observed in outcomes between males and females. Four mouse strains were used to generate the inducible Lis1 KO mice (Table 3.1). (1) 129S-Pafah1b1tm2Awb/J (The Jackson Laboratory 008002, RRID:IMSR_JAX:008002): loxP sites flank exons 3 – 6. Homozygous mice are viable and fertile, but have mild hippocampal abnormalities and express 75% of WT Lis1 levels (Hirotsune et al., 1998); (2) Tg(CAG-cre/ Esr1)5Amc/J (The Jackson Laboratory 004453, RRID: IMSR_JAX:004453), a chicken β-actin promotor drives expression of Cre recombinase fused to a modified estrogen receptor; (3) Tg(Myh6-cre/Esr1)1Jmk/J (The Jackson Laboratory 005650, RRID:IMSR_JAX:005650), expression of Cre-ER is under the control of a cardiac-specific a-myosin heavy chain promoter so that tamoxifen stimulates recombination only in cardiac cells; and (4) Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (The Jackson Laboratory 007576, RRID:IMSR_JAX:007576), a Cre re-porter mouse with loxP sites flanking a membrane-targeted tdTomato cassette that is positioned upstream of a membrane-targeted EGFP cassette. All cells in these mice exhibit membrane-associated red fluorescence until the tdTomato cassette is deleted by Cre recombinase for expression of membrane-associated EGFP fluorescence, allowing visualization of both recombined and non-recombined cells in the same

tissue (Muzumdar et al., 2007). Table 3.1 shows the crosses that were used to generate experimental animals, and shows the descriptive names used for each throughout the chapter. All strains used in experiments were homozygous for the Cre reporter. Genotyping of all animals was performed using primers and protocols recommended by The Jackson Laboratory. Primers are available on request.

Tamoxifen administration

Numbers of animals are provided below (Experimental design and statistical analysis). Tamoxifen was delivered by intraperitoneal or intracerebroventricular injections in adult mice (two to five months old). For intraperitoneal delivery, mice were injected with 40 mg/ml tamoxifen (Sigma-Aldrich) dissolved in 10% ethanol and 90% corn oil (Sigma). Two different daily tamoxifen dosage regimens were tested based a previous study using this Cre-ER strain (Hayashi and McMahon, 2002): regimen 1 (5 x 2 mg), 2 mg for five consecutive days (total 10 mg); and regimen 2 (2 x 8 mg), 8 mg injected on two consecutive days (total 16 mg). For intracerebroventricular delivery, mice were anesthetized with an isoflurane/oxygen vapor mixture (5% induction, 2–3% maintenance) and placed in a stereotaxic device (Kopf Instruments). Five microliters of 50 mM (Z) 4-hydroxytamoxifen (4-OHT; Sigma) dissolved in 100% ethanol was infused into the left lateral ventricle at a rate of 0.4 ml/min using a 5 ml Hamilton syringe. The needle was left in place for one additional min before removal to allow for diffusion from the injection site. Coordinates (-1.0 mm posterior from Bregma, 1.0 mm mediolateral, and -2.5 mm ventral to skull

surface) were determined using the atlas of Paxinos and Franklin (Franklin and Paxinos, 2001).

Analysis of Cre-mediated recombination by tdTomato/EGFP fluorescence

Images shown in all figures are representative of data acquired from at least N = 3 animals per experiment. Animals under deep isoflurane anesthesia were perfused transcardially with ice-cold PBS, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4). Before sectioning, tissues were cryoprotected by immersion overnight in 15% sucrose, followed by 24 h in 30% sucrose in PBS. Tissues were then frozen in OCT compound (Fisher) using a beaker of 2-methylbutane chilled in dry ice. Ten- or 50-mm-thick cryosections were stored at 80°C until use. Whole brains and hearts were imaged immediately after dissection using an Olympus SZX-12 with an SZX-RFL2 coaxial fluorescence attachment. Cryosections were imaged using a Leica TCS SP8X confocal microscope equipped with LAS X software and a 63 oil immersion objective (1.4 N/A). Some images were obtained using a Zeiss Axiovert 200 inverted microscope equipped with AxioVision software and a Plan-Neo 100 Å /1.30 and Plan-Apo 63 Å /1.40 oil-immersion objectives (Immersol 518F; Carl Zeiss, Inc.) or a Plan-Neofluor 20 dry objective.

Protein isolation and immunoblotting

All blots are representative of at least N = 3 repeats. Tissues were dissected quickly from CO_2-killed mice and frozen in liquid nitrogen, followed by Dounce homogenization in ice-cold RIPA lysis buffer with protease and phosphatase inhibitors (Thermo). Total protein in extracts was determined using

a BCA assay (Thermo). Automated capillary electrophoresis and immunoblotting (Figs. 3.1, 3.2) was performed with the Wes Simple Western system using the manufacturer's protocol (Protein Simple). One microgram of lysate was loaded for each sample. Anti-mouse (ERK1), anti-rabbit (Lis1), and total protein detection modules were used per manufacturer's instructions. Blots were analyzed using Compass Software (Protein Simple). For traditional Western blotting (Figs. 3.4, 3.7) 10 mg of each sample were separated on 10% acrylamide gels, then transferred to PVDF membrane. Blots were probed with antibodies against Lis1 and dynein intermediate chain and proteins were detected by chemiluminescence.

Sciatic nerve transmission electron microscopy

While anesthetized with isoflurane, WT mice were perfused with PBS, and then buffered 2.5% glutaraldehyde. Nerves were removed and fixed overnight in 2.5% glutaraldehyde, then sectioned and stained with osmium tetroxide for imaging on a JEOL 200CX Transmission Electron Microscope.

Antibodies

Primary antibodies used are as follows: Lis1 rabbit polyclonal 484/485 (Smith et al., 2000; WB: 1:500); Lis1 rabbit polyclonal (Wes: 1:25, WB: 1:500; Santa Cruz Biotechnology sc-15319, RRID:AB_2159891); ERK1 rabbit polyclonal (Wes: 1:100; Abcam ab109282, RRID: AB_10862274); a mix of the pan-axonal neurofilament (NF) mouse monoclonal cocktail (IF: 1:500; BioLegend 837904, RRID:AB_2566782); NF light, medium, and heavy chain chicken polyclonals (IF: 1:500; Aves NFL, NFM, and NFH, RRIDs: AB_2313553,

AB_2313554, AB_2313552); and the NF 200-kDa mouse monoclonal, clone RT97 (IF: 1:500; Millipore CBL212, RRID:AB_93408) were used to label NFs; GAP43 rabbit polyclonal (IF: 1:500; Novus Biologicals, NB300-143, RRID: AB_10001196); beta-III tubulin chicken polyclonal (IF: 1:500; Millipore AB9354, RRID:AB_570918); peripherin chicken polyclonal (IF: 1:500; Abcam ab39374, RRID:AB_777207); choline acetyl-transferase goat polyclonal (IF: 1:100; Millipore AB144P, RRID:AB_11214092); MAP2 chicken polyclonal (IF: 1:100; Abcam ab5392, RRID:AB_2138153); myelin basic protein chicken polyclonal (IF: 1:500; Aves MBP, RRID:AB_2313550); a-tubulin mouse monoclonal (WB: 1:2000; Sigma-Aldrich T5168, RRID:AB_477579); and dynein intermediate chain mouse monoclonal (WB: 1:1000; Santa Cruz Biotechnol-ogy sc-13524, RRID:AB_668849). Secondary antibodies used: HRP-conjugated goat anti-rabbit and mouse (WB: 1:50 000; Millipore 12-348 and 12-349, RRIDs: AB_390191 and AB_390192); Cy5-conjugated donkey anti-chicken, mouse, and goat (IF: 1:250; Jackson ImmunoResearch 703-175-155, 715-175-150, and 705-175-147, RRIDs:AB_2340365, AB_2340819, and AB_ 2340415); and DyLight 405-conjugated donkey anti-chicken (IF: 1:250; Jackson ImmunoResearch 703-475-155, RRID: AB_2340373).

Preparation of DRG cultures

Cultures were generated from two- to five-month-old Lis1 KO and no Cre or no flLis1 control mice. In some experiments mice were exposed to the 2 x 8 mg tamoxifen intraperitoneal regimen then neurons harvested on day 4 after the first injection. In other experiments, 4-OHT (2 μM) was added directly to the DRG

cultures with these same genotypes without previous intraperitoneal injections. DRGs were harvested (20 per mouse), dissociated in type XI collagenase (Sigma) for 1 h at 37°C, and then triturated through a flamed Pasteur pipet. Dissociated neurons incubated in 0.05% trypsin (Invitrogen) at 37°C for 15 min. After a second trituration, cell suspensions were centrifuged through a 12.5% BSA solution to remove myelin fragments. Cells were then plated onto sterile, German glass coverslips (Fisher) coated with 10 mg/ml poly-D-lysine (300 kDa; Sigma) and 10 mg/ml laminin (Millipore). Cells were cultured in DMEM/F12 medium (Corning) with 25 mM HEPES, GlutaMAX (Thermo Fisher), N-2 supplement (Life Technologies), and 10% horse serum (HyClone). In some experiments, 100 mM cytosine arabinoside was added to reduce non-neuronal cell proliferation.

Axonal varicosity analysis

DRG neurons obtained after intraperitoneal injection were maintained in culture for 4 d and then fixed for 10 min in 4% PFA. Coverslips were mounted on glass slides using Prolong Gold Antifade (LifeTechnologies). Neurons were imaged using the ImageXpress XLS high content imaging system (Molecular Devices) equipped with a 20 objective. A segmented line (one-pixel width) was used to trace the axon using ImageJ software. Axonal swellings that protruded visibly beyond the one-pixel line on both sides were counted as varicosities. tdTomato-positive axons from N = 3 no Cre cultures (45 mm of total axon length) and EGFP-positive axons from N = 3 Lis1 KO cultures (27 mm of total axon length) were measured.

Axon growth/length analysis

DRG neurons obtained after intraperitoneal injection were maintained in culture for 2 d and then fixed for 10 min in 4% PFA. Neurons were processed for immunofluorescence using a cocktail of neuron-specific antibodies: chicken anti-peripherin, chicken anti-b III tubulin, chicken anti-NF (light, medium, and heavy chains), and rabbit anti-GAP43 to ensure all axons were uniformly labeled. Neurons were imaged using the ImageXpress Micro XLS system and axon lengths were measured using WIS-Neuromath software. N = 5 Lis1 KO mice, and N = 4 no flLis1 control mice were analyzed; n = 122 control and n = 70 KO neurons were measured.

Organelle movement

Cultured DRG neurons obtained after intraperitoneal injection were exposed to 100 nM Lysotracker-Red (Millipore Inc.) for 20 min. Coverslips were transferred into fresh medium containing OxyFluor (Oxyrase Inc.) and 25 mM HEPES (pH 7.4), and placed in a custom-built water-heated microscope stage warmed to 37°C. Organelles were imaged using a Zeiss Axiovert 200 microscope equipped with a C-Apo 63x /1.2 W/0.2 water-immersion objective. Images were acquired at 0.5-s intervals for 2 min using a Zeiss AxioCam HRm charge-coupled camera and linked AxioVision 4.7 software. Kymographs were generated from time-lapse movies using ImageJ soft-ware. N 2 mice of each genotype were analyzed, with a total of 27 axon segments analyzed from each genotype. A total of n 521 no flLis1 control and n 699 Lis1 KO organelles were included. Direction

of movement was determined by locating the cell body before imaging. Net displacement of 5 μm toward the cell body was categorized as retrograde.

Immunofluorescence in DRG cultures and tissue cryosections

Immunofluorescence experiments were performed in triplicate (N = 3 mice per genotype) and representative images are shown in the figures. After permeabilization with 0.1% Triton X-100 for 10 –30 min, samples were blocked in 3% BSA (Fisher), 10% normal goat serum (Sigma), and 0.2% Tween 20 (Bio-Rad) in PBS for 1 h. Cultures and nerve sections were exposed to primary antibodies for 1 h at room temperature. Brain and spinal cord sections were exposed to primary antibody over-night at 4°C. In both cases samples were exposed to fluorophore-conjugated secondary antibodies for 1 h at room temperature. Nuclei were stained with Hoechst dye and samples mounted using Prolong Gold.

Quantifying chromatolysis in brainstem sections

Animals were exposed to the 2 x 8 mg tamoxifen regimen. On day 4, coronal cryosections of brainstem from N = 3 no flLis1 control mice (n = 331 neurons) and N = 4 Lis1 KO mice (n = 583 neurons) were stained with 1% toluidine blue and measured for nuclear enlargement and nuclear acentricity. The Allen Mouse Brain Atlas was used as a guide to select coronal brainstem sections in which the nucleus ambiguus and other cardiorespiratory centers were likely located. Landmarks such as the fourth ventricle and pyramus granular layers were used to identify the proper sections. Comparisons were made from matched sections. Substantial Act-Cre-ER mediated re-combination (GFP

expression) was consistently observed in similar sections following tamoxifen administration. ImageJ was used to determine nuclear and somal areas and centroids. The ratio of the nuclear area to the somal area was calculated to establish a “nuclear enlargement index.” A “centroid displacement index” was calculated by summing the X and Y displacement differences between the nuclear centroid and the somal centroid of each cell.

RNA extraction and analysis

RNA analysis was performed using tissues from 3 mice per treatment and genotype. RNA was isolated from flash frozen tissues using the QIAGEN RNAEasy kit per the manufacturer’s instructions. RNA concentration was determined by fluorimetry using Ribogreen reagent (Thermo Fisher). A total of 100 ng RNA was reverse transcribed with a SensiFast cDNA Synthesis kit (Bioline). These cDNAs were used for quantitative droplet digital PCR (ddPCR) with Evagreen detection reagent and ddPCR Supermix (Bio-Rad). Droplets for ddPCR were made using a QX200 Droplet Generator (Bio-Rad). Results were analyzed using Poisson distribution on the QX200 Droplet Reader. Mouse Lis1 primer sequences (forward, 5’ GCGAACTCTCAAGGGCCATA 3’ and reverse, 5’ CATTGTGATCGTGACCGTGC 3’) were designed using NCBI BLAST (NCBI accession #NM_013625). Mouse b2 micro-globulin (B2M) primers (forward, 5’ TTCTGGTGCTTGTCTCACTGA 3’ and reverse, 5’ CAGTATGTTCGGCTT CCCATTC 3’) were obtained from Harvard Primer Bank (B2M; NCBI accession #NM_009735; https://pga.mgh. harvard.edu/primerbank/).

Experimental design and statistical analysis

Eighty-four Lis1 KO animals were given the 2 x 8 mg tamoxifen regimen; 100% of these animals began to exhibit neurologic symptoms (leg clasping, kyphosis, decreased motility) within a week. Of these, 18 died during that time, and 66 were killed when symptoms became severe; 38 no flLis1 control and 73 no Cre control mice were also given the 2 x 8 mg tamoxifen regimen. None of the controls showed any evidence of neurologic disorder or malaise, but most were killed at the same time as the Lis1 KO animals to compare results. However, six each of the control strains were monitored for four weeks after 2 x 8 mg tamoxifen and showed no symptoms. We also conducted mock injections of vehicle alone in 12 Lis1 KO animals. These controls also showed no symptoms.

Table 3.2 describes the statistical methods used for all quantified experiments. The p-values were obtained using Excel or GraphPad Prism 5.

Table 3.1: List of mouse strains used in Chapter 3.

Founder mouse lines	
Jackson Labs strains	**Descriptive name used in paper**
129S-Pafah1b1^{tm2Awb}/J	Lis1 $^{LoxP/+}$ or Lis1 $^{LoxP/LoxP}$
Tg(CAG-cre/Esr1*)1Jmk/J	Act-Cre-ER (heterozygous)
Tg(Myh6-cre/Esr1*) 1Jmk/J	Myh6-Cre-ER (heterozygous)
Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato, -EGFP)Luo}$/J	Cre Reporter (heterozygous)
All strains below are homozygous for the Cre Reporter	
Lis1 KO strains	**Descriptive name used in paper**
Lis1$^{LoxP/LoxP}$ x Act-Cre-ER (het)	Lis1 KO
Lis1$^{LoxP/LoxP}$ x Myh6-Cre-ER (het)	Myh6 KO
Control strains	**Descriptive name used in paper**
Act-Cre-ER	No flLis1 Control
Lis1$^{LoxP/LoxP}$	No Cre Control
Lis1$^{LoxP/+}$ x Act-Cre-ER	Lis1 KO Het

Table 3.2: Table of statistical tests used in Chapter 3 figures.

	Description	Data Structure	Type of Test	Statistical Value
a	Axonal varicosities	Non-Normal	Mann-Whitney test	$p < 0.0001$
b	Retrograde transport	Normal	t-test	$t_{(52)} = 7.746$, $p = 3.2 \times 10^{-32}$
c	Nuclear Enlargement Index	Normal	t-test	$t_{(912)} = 19.55$, $p = 3.6 \times 10^{-73}$
d	Centroid Displacement Index	Non-Normal	Mann-Whitney test	$p < 0.0001$
e	Lis1 mRNA in brainstem	Normal	ANOVA	$F_{(2, 19)} = 5.033$, $p = 0.0176$
f	Lis1 mRNA in heart	Normal	ANOVA	$F_{(2,16)} = 19.065$, $p = 5.83 \times 10^{-5}$

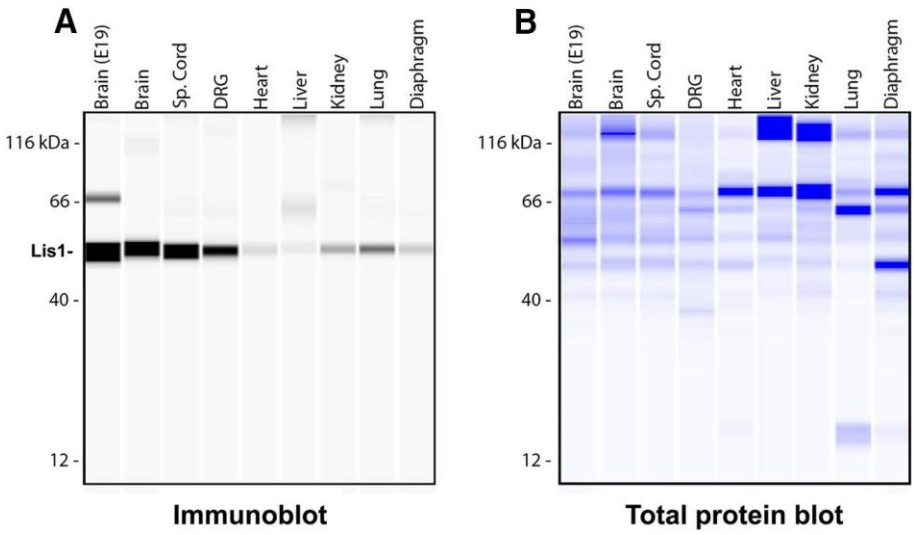

Figure 3.1: Lis1 protein is expressed in adult mouse tissues. A total of 1 µg of tissue lysates was analyzed using the Wes Simple Western System. Brain extracts from E19 were loaded as a positive control. All other extracts are from two-month-old animals. The size-based separation is processed by Compass software and displayed as virtual blots/gels. **A**, Immune detection of Lis1 in protein samples, depicted in a virtual immunoblot generated by the system. **B**, Total protein detection, visualized by a virtual Coomassie gel generated by the system. These blots are representative of three experimental repeats (N = 3).

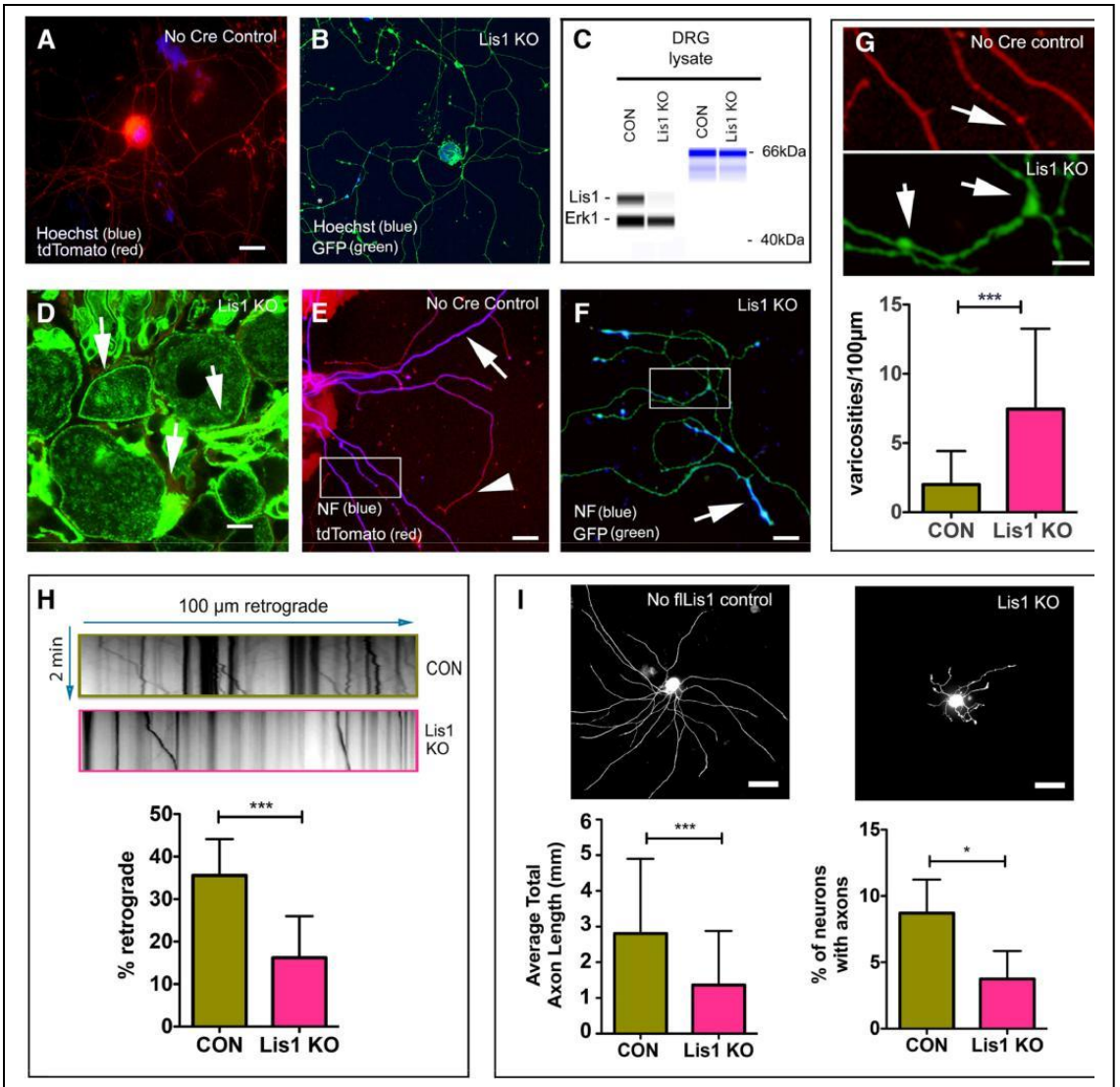

Figure 3.2: Lis1 KO impacts axonal function in adult mouse DRG neurons. A, Cultured DRG neurons from no Cre control exposed to 4-OH tamoxifen for 5 d expressed only tdTomato (red) showing no signs of recombination. **B**, In contrast, Lis1 KO neurons had strong GFP expression (green) demonstrating recombination. **C**, 4-OH tamoxifen reduced Lis1 protein levels in Lis1 KO neurons relative to no Cre control neurons (CON). **D**, Intraperitoneal injection of 2 x 8 mg tamoxifen in Lis1 KO mice resulted in GFP expression in intact DRGs after 4 d. Arrows point to DRG plasma membranes. **E**, Cultured DRG neurons prepared from intraperitoneally injected, no Cre control animals expressed only tdTomato (red). NF (blue) was prominent along axon shafts (white arrow) but less prominent in axon terminals (arrowhead). **F**, DRG neurons prepared from intraperitoneally injected Lis1 KO mice continued to express GFP (green) in culture, and NF (blue) was most prominent in distal axons and enriched in in varicosities (arrow). **G**, Insets from E, F have been digitally enlarged to show axonal varicosities (arrows). The bar graph in G shows the average number of varicosities per 100 µm of axon from N = 3 CON (45 mm total axon length) and three Lis1 KO (27 mm total axon length) mice. **H**, Kymographs were generated from time-lapse movies of LysoTracker labeled organelles in GFP-positive axons. The bar graph shows the percentage moving retrogradely in Lis1 KO and no flLis1 control cultures (CON). A total of 27 100 µm axon segments were analyzed from N = 2 CON and N = 2 Lis1 KO mice. A total of n 521 control and n 699 KO organelles were analyzed. **I**, Cultured DRG neurons prepared from intraperitoneally injected, no flLis1 control and Lis1 KO mice were immunostained with neuron-specific antibodies, and the percentage of neurons with growing axons was determined from N = 4 CON and N = 5 Lis1 KO mice. A total of n = 2219 control neurons and n = 2410 Lis1 KO neurons were analyzed. Bars in G-I indicate mean SD. Significance determined by Mann–Whitney test (G), Student's t test (H, I), p 0.05, p 0.001 (see Table 3.2 for details). Scale bars: 20 µm (A, D, E), 5 µm (B), and 50 µm (I).

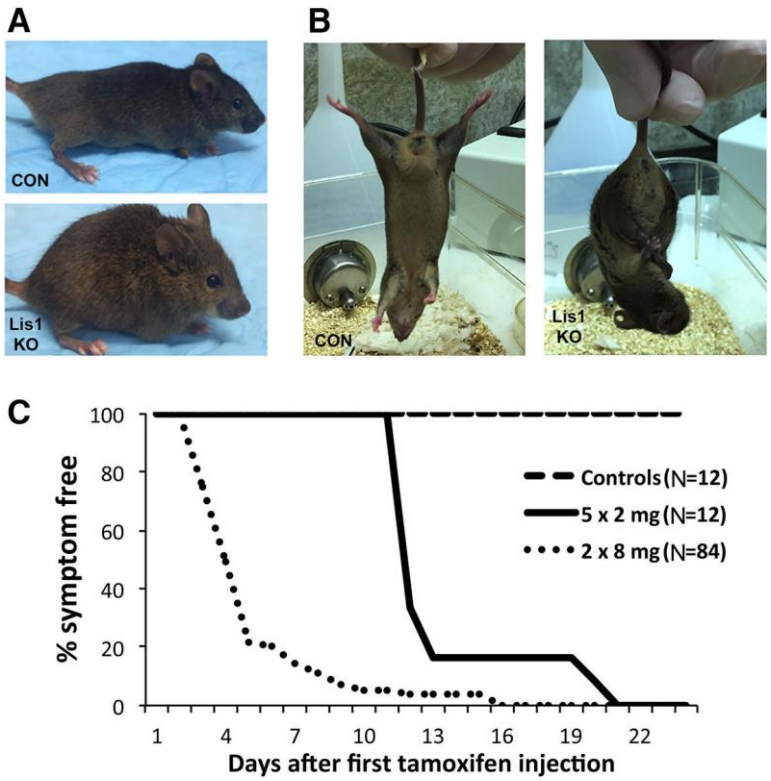

Figure 3.3: Lis1 KO via intraperitoneal tamoxifen injection in adult mice results in a severe phenotype. Lis1 KO mice exposed to tamoxifen invariably displayed spinal kyphosis (**A**, lower panel) and hind leg clasping (**B**, right panel). Neither was observed in control animals (CON) at any time. This includes the no Cre, no flLis1, Lis1 KO het, or mock-injected Lis1 KO animals. Both Lis1 KO and control mice were killed as soon as kyphosis and leg clasping became apparent in the KO animals. Phenotypes arose with latencies depending on the specific tamoxifen-dosing regimen (see Materials and Methods). **C**, Symptom-free survival curves show that the latency is shorter for the 2 x 8 mg regimen (N = 84) compared to the 5 x 2 mg regimen (N = 12). Control mice were killed at the same time as the Lis1 KO mice for recombination and expression studies. However, six no Cre control mice and six no flLis1 control mice receiving the 2 x 8 mg dosing regimen survived symptom free for three weeks before they were killed (total N = 12).

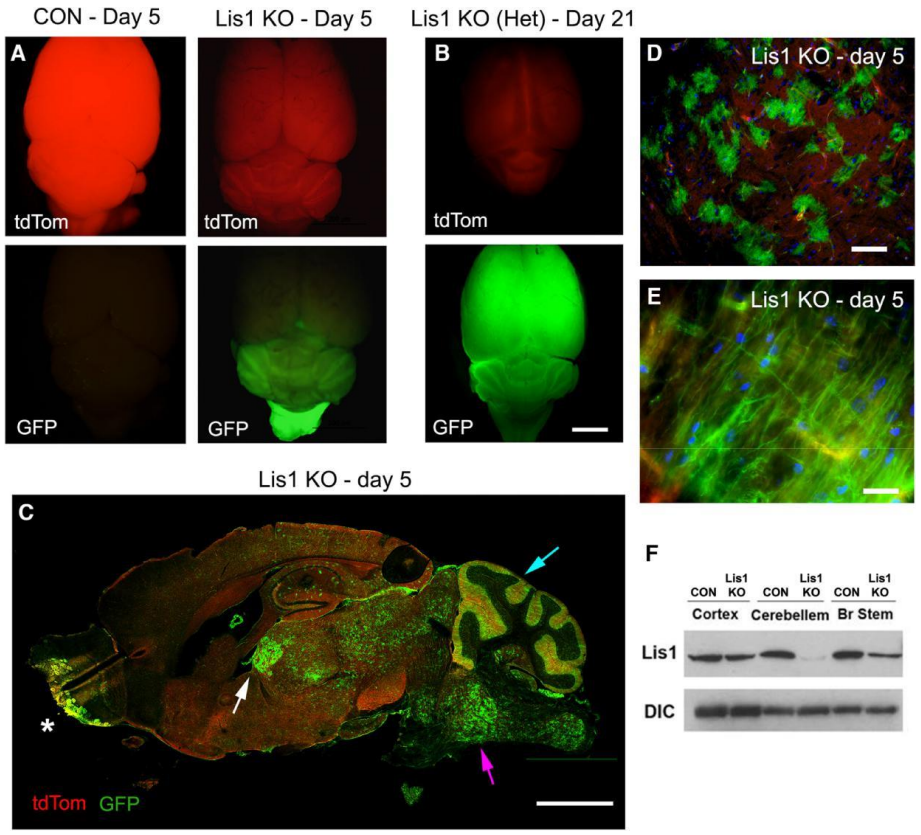

Figure 3.4: Cre-dependent recombination in the brain after tamoxifen injection. All data in this figure are representative of observations from a minimum of N = 4 animals of each genotype. **A**, On day 5 after the 2 x 8 mg tamoxifen regimen, no Cre control brains (CON, day 5) had bright tdTomato fluorescence (top left panel), but no EGFP fluorescence indicative of recombination (lower left panel). Lis1 KO mice (Lis1 KO, day 5) showed reduced tdTomato fluorescence (top right panel) and expressed EGFP primarily in the hindbrain, indicating that Cre activity was pronounced in this brain region (lower right panel). **B**, Lis1 KO het mice [Lis1 KO (Het), day 21], which showed no sign of neurological problems through day 21 after the injection had substantial EGFP expression throughout the brain at that time. **C**, A sagittal section of a Lis1 KO brain on day 5 (Lis1 KO, day 5) shows mosaic recombination in midbrain (white arrow), hindbrain (magenta arrow), and cerebellum (blue arrow), with widely scattered EGFP-positive cells in cortex and hippocampus. Recombination also occurs in olfactory bulb (asterisk). **D**, Using higher magnification, GFP-positive cells in the midbrain can be seen interspersed with cells that have not yet undergone recombination. **E**, Fibers labeled with GFP are clearly visible in the brainstem. **F**, Lis1 expression is reduced in extracts from brainstem and cerebellum of Lis1 KO mice compared to no Cre controls. Scale bars: 5 mm (A–C), 100 µm (D), and 20 µm (E).

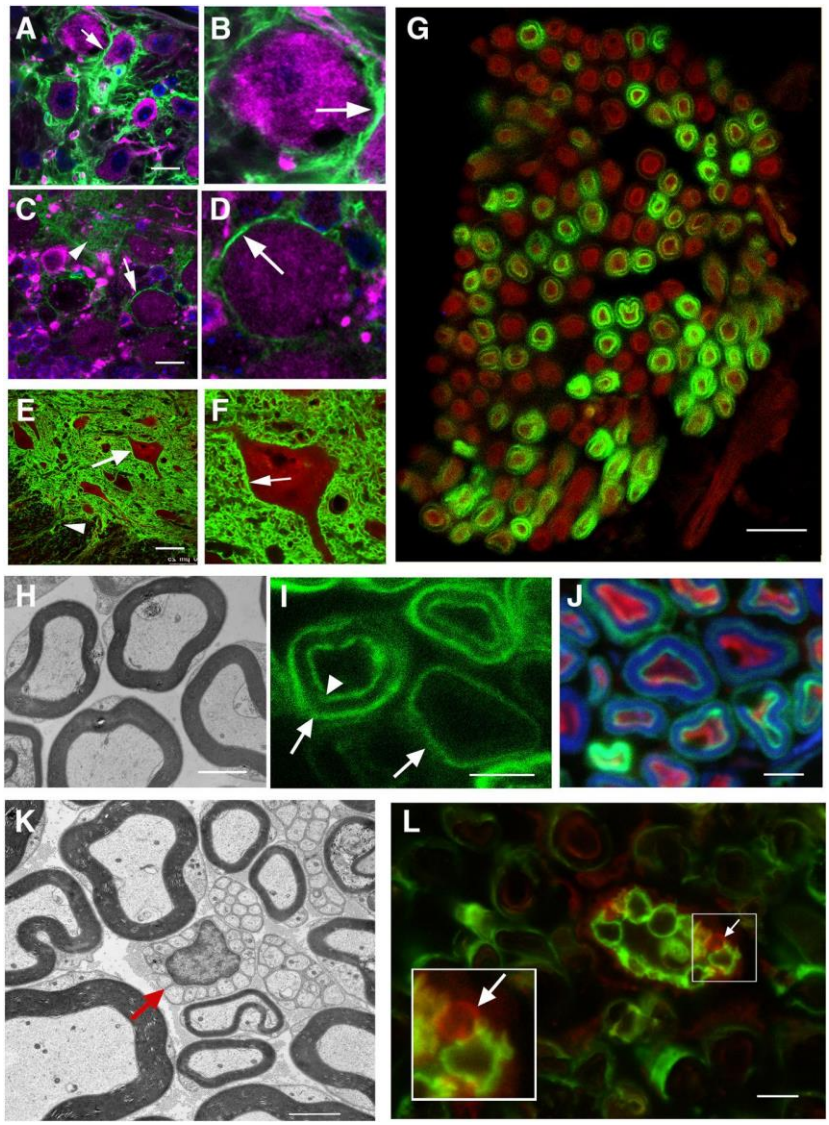

Figure 3.5: Both neurons and glia show evidence Cre-dependent recombination. All data in this figure are representative of observations from a minimum of N = 4 animals of each genotype. **A**, GFP-positive neuropil surrounds MAP2-positive neurons (magenta) in a brainstem region thought to be the nucleus ambiguous in a mouse exposed to the 2 x 8 mg tamoxifen regimen. **B**, The neuron indicated in A has been digitally enlarged to show details. The arrow points to possible neuronal plasma membrane. **C**, Purkinje cells in the cerebellum stained with MAP2 (magenta) also have GFP-positive plasma membranes (white arrow) indicating that recombination occurred in these neurons. Neuropil in the molecular layer is also GFP-positive (arrowhead). **D**, The neuron in C has been digitally enlarged to show detail. **E**, GFP-positive neuropil surrounds a motor neuron (white arrow) labeled with ChAT (red) in the anterior horn of the thoracic spinal cord. GFP-positive fibers (arrowhead) can be seen coursing toward the ventral root. **F**, The motor neuron indicated in E is digitally enlarged to show detail. The white arrow points to apparent neuronal plasma membrane. **G**, A cross section through the phrenic nerve shows concentric rings of GFP around approximately half of the NF-positive axons (red). **H**, EM of a cross section through a WT mouse nerve showing myelinated axons. **I**, GFP can be observed as two concentric rings or single rings (arrows, outer ring, arrowhead, inner ring). **J**, The area between concentric rings is positive for myelin basic protein (blue), while the inside of the inner ring is positive for NF (red). **K**, EM of a cross section of WT mouse sciatic nerve showing Remak bundles of unmyelinated axons surrounded by a single glial cell (red arrow). **L**, Cross section of sciatic nerve from Lis1 KO mouse with a Remak bundle containing some GFP-encircled axons, and some without encircling GFP (arrow, positive for tdTomato only). Inset is digitally enlarged to show an axon without recombination (red, arrow) alongside recombined axons (green). Scale bars: 10 µm (A, C, G), 30 µm (E), and 2 µm (H–L).

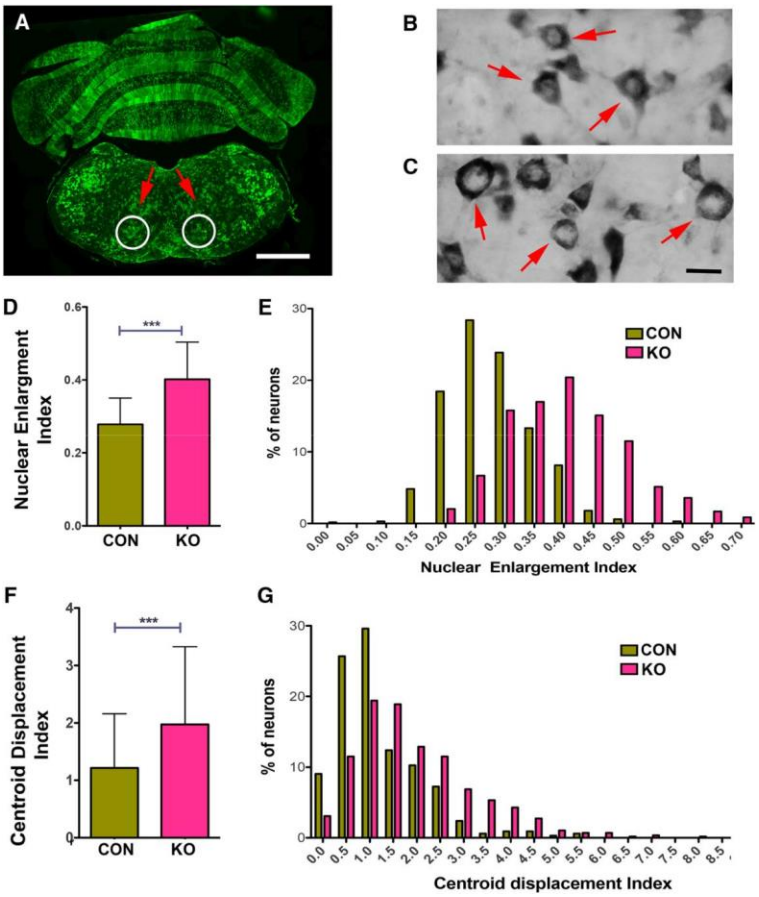

Figure 3.6: Brainstem neurons in Lis1 KO mice exhibit signs of chromatolysis. **A**, A coronal section through the hindbrain on day 4 after the 2 x 8 mg tamoxifen regimen shows extensive recombination in the ventral brainstem containing cardiorespiratory centers. White circles indicate the region used in the analyses of chromatolysis. **B**, **C**, Sections were stained with toluidine blue to determine the size and position of the nucleus in neurons in the indicated regions. The neurons in B are from a no flLis1 control mouse. The neurons in C are from a Lis1 KO animal. **D**, A nuclear enlargement index (see Materials and Methods) was used to compare nuclear enlargement in no flLis1 controls (CON) and Lis1 KO (KO). **E**, The histogram shows the distribution of this index in CON and Lis1 KO neurons. **F**, The position of the nucleus within the soma was also determined using the centroid displacement index (see Materials and Methods). This involves determining the centroid position of both the nucleus and soma and calculating the total displacement distance (µm) of the nuclear centroid from the somal centroid. **G**, Histogram showing the distribution of CDI found in CON and KO neurons. Bars indicate mean +/- SD. Brainstem sections from three no flLis1 control and four Lis1 KO mice were used in the chromatolysis study. This includes analysis of 331 control neurons and 583 Lis1 KO neurons. Significance determined by Student's t test (D), or Mann–Whitney test (F); *** - $p < 0.001$ (see Table 3.2 for details). Scale bars: 1 mm (A) and 10 µm (B, C).

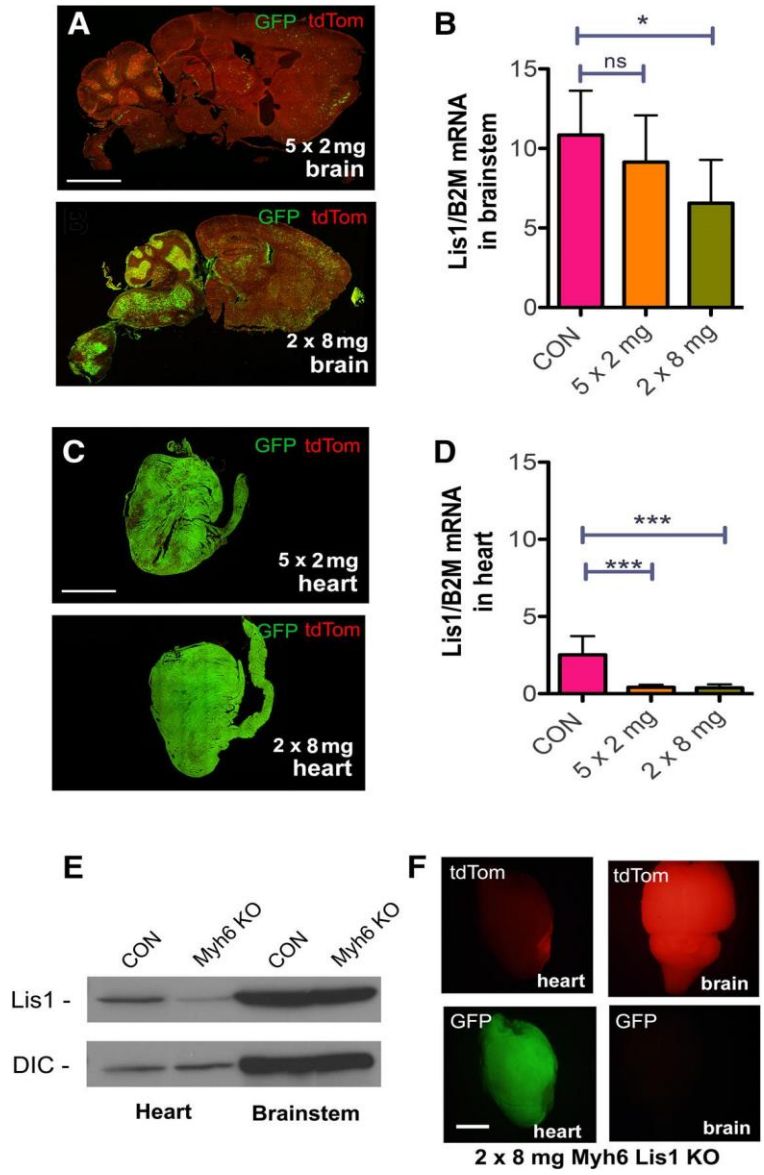

Figure 3.7: Comparing the effect of Lis1 KO in brainstem and heart. A, Sagittal brain sections of Lis1 KO mice 5 d after the initial injection of either five injections of 2 mg tamoxifen (top) or two injections of 8 mg tamoxifen (bottom). The 2 x 8 mg treatment resulted in much higher GFP expression than the 5 x 2 mg treatment, particularly in the brainstem and cerebellum. **B**, Lis1 mRNA levels normalized to B2M mRNA levels from brainstem of no Cre control mice injected with 2 x 8 mg tamoxifen (CON), and Lis1 KO mice injected with either 5 x 2 or 2 x 8 mg tamoxifen. Lis1 mRNA levels were significantly decreased in brainstem of 2 x 8 mg animals, but not 5 x 2 mg animals, relative to no Cre controls, 5 d after initial injection. **C**, Sections of heart from 5 x 2 mg (top)- and 2 x 8 mg (bottom)-treated Lis1 KO mice. Both the 2 x 8 and 5 x 2 mg treatments resulted in similar levels of GFP expression in heart. **D**, Lis1 mRNA levels normalized to B2M mRNA levels from heart of 2 x 8 mg-injected no Cre control (CON)-, 5 x 2 mg-, and 2 x 8 mg-treated mice. Lis1 mRNA levels were reduced significantly in both the 5 x 2 mg- and 2 x 8 mg-treated mice relative to the no Cre control but were not significantly different from each other. **E**, Western blotting of brainstem and heart lysates from cardiomyocyte-specific Myh6 KO mice show reduced levels of Lis1 protein in heart, but not brainstem compared to no Cre control mice (CON). Dynein intermediate chain (DIC) was used as a loading control. **F**, Whole mount brain (right) and heart (left) from Myh6 KO mouse show recombination (GFP) in heart but not brain. Data in A, C, E, F are representative images from N = 3 mice for each genotype. The RNA quantification in B, D represent mean of data from N = 3 animals of each treatment and genotype +/- SD. Significance in B, D determined by one-way ANOVA; * $p < 0.05$, *** $p < 0.001$ (see Table 3.2 for details). Scale bars: 5 mm (A, C) and 2 mm (F).

CHAPTER 4

REGULATION OF DYNEIN BY APC AND GSK3

4.1 INTRODUCTION

Glycogen synthase kinase 3 (GSK3) is a constitutively active serine/threonine kinase that is a downstream target of the insulin signaling pathway. Patients with metabolic syndrome and diabetes become insensitive to insulin, which can contribute to an increased risk of neurological disorders and cancer (Larsson et al., 2005; Yuhara et al., 2011; Sasazuki et al., 2013; Guraya, 2015; Ramjeesingh et al., 2016). Previously published work from our lab has established a regulatory interaction between GSK3 and dynein where GSK3 phosphorylates and inhibits the motor when cells were serum-starved (Gao et al., 2015). When serum was added back to the cells, or insulin was added to the medium, GSK3 was inhibited and dynein was activated leading to accumulation at microtubule minus-ends (Gao et al., 2015).

APC interacts with GSK3 as part of the β-catenin destruction complex in the Wnt signaling pathway. Mutations in APC that cause colorectal cancer, such as the multiple intestinal neoplasia (MIN) mutation, disrupt this complex. Interestingly, many anti-diabetic drugs have been tested as chemopreventive or therapeutic treatments for colorectal cancer with mixed results (Gupta and Dubois, 2002; Chang et al., 2012; Malek et al., 2013; Park, 2013; Yin et al., 2014; Mendonca et al., 2015; Zhou et al., 2015; He et al., 2016; Ramjeesingh et al.,

2016). APC mutations have been linked to reduced efficacy of diabetes drugs as chemotherapeutic agents, perhaps in part due to the disruption of APC's interaction with GSK3.

APC is also known to interact with microtubules and regulate their dynamics, which can affect dynein function. Importantly, results from our lab show that APC also binds directly to dynein and that the MIN mutation found in many patients with colorectal cancer disrupts this interaction. Additionally, presence of the MIN isoform prevents the regulatory effects of insulin signaling on dynein (Gao et al., 2017). In this chapter, I will discuss my contributions to these studies.

4.2 RESULTS

4.2.1 Insulin Signaling Pathway Remains Functional in Cells with Mutated APC

In this study, we utilized two immortalized mouse colonic epithelial cell lines, one expressing wild-type (WT) APC (YAMC cells) and one expressing the truncated MIN isoform of APC (IMCE cells), to test the effects of mutated APC on insulin signaling and dynein function (Whitehead and Joseph, 1994). To establish if insulin signaling (and specifically GSK3 activity) would have the same effects on dynein in IMCE cells as YAMC cells, we measured the inhibitory phosphorylation of GSK3 on serine-9 (pS9) via western blot. As shown in Figure 4.1A/B, the ratio of pS9-GSK3 to pan-GSK3 increases significantly one hour after treatment in both YAMC and IMCE cells, then falls back to baseline after 6 hours. There was no significant difference in the extent of pS9-GSK3 at 1 h between the

two cell lines (Figure 4.1C). This shows that the insulin signaling pathway has a similar effect on GSK3 activity in cells expressing WT APC or MIN, so the effects seen on dynein activity are either downstream of GSK3 phosphorylation, or due to other effects (e.g., protein-protein interactions).

4.2.2 Analysis of Microtubule Cytoskeleton

Since APC is a known regulator of microtubule organization and dynein distribution could be altered by change in the microtubule cytoskeleton, we analyzed this factor as well. Figure 4.2 shows immunofluorescence images of YAMC and IMCE cells stained for α-tubulin after starvation (Figure 4.2A/B) or 1h insulin treatment (Figure 4.2C/D). All four groups had very similar microtubule morphology at this level, so general microtubule organization does not seem to be the major driver of the effects seen on dynein distribution.

Posttranslational modifications to tubulin can also affect motor function, so we next examined the distribution and levels of three different modified tubulin isoforms (tyrosinated, acetylated, and detyrosinated) via immunofluorescence and western blot, respectively. There did not seem to be a difference in localization or morphology of tyrosinated (Figure 4.3A-D), acetylated (E-H), or detyrosinated (I-J) microtubules between the two cell types or after insulin treatment. Western blot analysis of levels of these tubulin isoforms revealed no significant difference between the two cell types for tyrosinated or acetylated tubulin, but, surprisingly, there was significantly less detyrosinated tubulin (GluTub) in the IMCE cells than YAMC cells (Figure 4.3K-L). Recent studies

have shown that dynein motility is reduced on detyrosinated microtubules (McKenney et al., 2016), so a decrease in detyrosinated tubulin levels likely would not cause the decreased dynein activity seen in our studies.

4.2.3 Dynein Interacts with the C-Terminus of APC

In order to map the region of APC that interacts with dynein, we generated constructs containing two APC fragments. As seen in the schematic in Figure 4.4A, the 746 amino acid N-terminal fragment (nAPC) is similar in size to the MIN isoform. This region contains the oligomerization domain and armadillo repeats, which interact with AMER proteins. The 272 amino acid C-terminal fragment (cAPC) contains the EB1 and PDZ-binding domains, but not the basic region known to interact with microtubules. These two fragments, along with full-length APC (FL-APC) were cloned into an EGFP vector to express EGFP-tagged versions of these proteins in cells. FL-APC was expressed in a small percentage of cells and it colocalized with dynein in some cellular protrusions (Figure 4.4B). nAPC aggregated in cytoplasmic blebs, suggesting the protein aggregates in cells (Figure 4.4C). This fragment did not seem to localize to cellular protrusions or colocalize with dynein as much as FL-APC. cAPC distribution was more diffuse and dynein staining in these cells was also more diffuse, suggesting cAPC might alter dynein distribution (Figure 4.4D). Interestingly, concurrent expression of both nAPC and cAPC greatly reduced cell viability and decreased

the amount of EGFP aggregation compared to nAPC-expressing cells (not shown).

We also performed immunoprecipation (IP) of endogenous dynein to determine if the APC fragments and dynein were interacting directly, as opposed to simply localizing to similar subcellular regions (Figure 4.4E). nAPC was pulled down in the dynein IP, but was also present in the mouse IgG control, so we cannot say that this interaction is specific to dynein. However, cAPC specifically coprecipitated with dynein and not mouse IgG. This indicates that dynein interacts with the C-terminus of APC, which is lost in the truncating MIN mutation.

4.3 Summary and Discussion

Truncating mutations of APC, specifically the MIN mutation, disrupt its interaction with dynein and prevent the regulatory control of insulin signaling over dynein activity. This is likely due to the interaction between dynein and APC occurring via the C-terminus of the APC protein, which is lacking in the MIN isoform. While disruptions in microtubule organization or posttranslational modifications could hypothetically account for changes seen in motor activity, we did not see any dramatic changes in the morphology of the microtubule cytoskeleton. We did find that cells carrying the MIN allele had lower levels of detyrosinated tubulin, but this is unlikely to cause the effects seen, as dynein is less motile on detryosinated microtubles, so lower levels should increase dynein motility in IMCE cells. These results have implications not only in identifying mechanisms of carcinogenesis in APC MIN cancer models, but also in

determining the mechanisms of decreased diabetes drug efficacy in patients carrying the MIN allele. Additionally, this research could aid in identifying potential neurological conditions associated with the mutation. Future studies would identify changes in dynein function and regulation in neurons from APC MIN mice. Specifically, we plan to learn first whether GSK3 inhibition positively regulates motile events (i.e., increased speeds, run lengths, etc.) or if it is important only for the initiation of transport. Next, we could determine whether this regulation is affected in different compartments, such as the AIS or growth cone, of cultured neurons from WT and APC MIN mice.

Additionally, we will examine whether there is crosstalk between the signaling pathways that regulate GSK3 and Lis1/Ndel1. The Lis1 knockout mouse described in Chapter 2 would be a good model for testing this at the cellular and organismal level. Treating Lis1 KO mice, or DRG neurons cultured from them, with GSK3 inhibitors to try to rescue or delay the phenotypes observed would give insights into the regulation of axonal transport.

Finally, it will be interesting to determine the effects of the MIN mutation on the ability of axons to regenerate. This could be done in vivo using a sciatic nerve crush and measuring the number and distance that axons can grow past the injury site. In conjunction, total axon length and the number of neurons extending neurites in culture can be measured in DRG neurons from these mice to determine regenerative capacity. The mechanism underlying any defects seen in regeneration could be due to APC's role as a +TIP in stabilizing microtubules, its role as an mRNA-binding protein, or its role in the β-catenin destruction

complex. Further analysis would be done to determine which of these mechanisms is the cause of any results obtained.

4.4 Materials and Methods

Cell lines

The murine young adult mouse colon (YAMC) epithelial cell line was derived from the colonic mucosa of a transgenic mouse generated by the introduction of a temperature-sensitive, interferon-inducible, SV40 T Ag, tsA58 (Immortomouse; (Whitehead et al., 1993)). This line is referred to as “WT”. The Immortomouse colon epithelial cell line (IMCE) was derived from the progeny of a cross between the Immortomouse strain and the Apc (min/+) mouse strain (Whitehead and Joseph, 1994). We refer to these as MIN cells. Both cell lines were first obtained from the Center for Colon Cancer Research at the University of South Carolina and later from a new batch from R. H. Whitehead (Vanderbilt University Medical Center). Cell lines were validated by genotyping and PCR. Cells were maintained at the permissive temperature (33°C) in full RPMI 1640 medium (2 mM glutamine, 10% fetal bovine serum [FBS], 0.5 U/ml penicillin, 100 µg/ml streptomycin, 5 U/ml murine γ-interferon, and 1% ITS [insulin, transferrin, and selenium; Cellgro]). Cos-7 cells were maintained in full DMEM medium (2 mM glutamine, 10% FBS, 0.5 U/ml penicillin, and 100 µg/ml streptomycin). Cell lines

were tested frequently for mycoplasma contamination using a mycoplasma detection kit from Thermo Fisher Scientific.

Expression constructs and transfection

EGFP-nAPC was generated by cloning a BspEI and HindIII fragment of full-length human APC into a pEGFP C1 vector. cAPC was generated by PCR amplification of a 747–base pair fragment of the 3′ end of full-length human APC. An EcoR1 and BamH1 fragment was subcloned into a pET 30 EK/LIC vector (Novagen) for expression in Escherichia coli or the pEGFP-C1 vector for mammalian expression. The rat EGFP-IC1B construct was kindly provided by Kevin Pfister (University of Virginia, Charlottesville, VA). The IC1B mutant was described in Gao et al. (2015). All constructs were verified by sequencing. Cells were transfected using Lipofectamine 2000 or 3000 (Invitrogen) according to the manufacturer's directions.

Antibodies

We used the following antibodies: CDK5RAP2 rabbit polyclonal antibody (pAb; Millipore), DIC mouse monoclonal antibody (mAb; 74.1; Santa Cruz Biotechnology), APC-M2 rabbit pAb (raised against the 15–amino acid repeat region; described in Wang et al., 2009), DIC mouse mAb (74.1; Santa Cruz Biotechnology), GSK-3β mouse mAb (3D10; Cell Signaling Technology), phospho–GSK3β (Ser-9) rabbit pAb (5B3; Cell Signaling Technology), GFP rabbit pAb (Ab290; Abcam), His-probe rabbit pAb (H-3; Santa Cruz Biotechnology), PPARγ1 rabbit pAb (H100; Santa Cruz Biotechnology), detyrosinated α-tubulin rabbit pAb (ab48389; Abcam), tyrosinated tubulin mouse

mAb (TUB1A2; Sigma-Aldrich); acetylated tubulin mouse mAb (Clone 6-11-B1; Sigma-Aldrich); and α-tubulin mAb (T5168; Sigma-Aldrich).

Measuring GSK-3β inhibition

PAGE was performed on WT and MIN cell extracts. Proteins were then transferred to nitrocellulose membrane. Membranes were blocked using Li-Cor Odyssey TBS Blocking Buffer and then probed with a mouse mAb to pan-GSK-3β and rabbit mAb to phospho–GSK-3β (serine 9), both at 1:1000. LiCor IRDye 680LT donkey anti-rabbit and 800CW donkey anti-mouse secondary antibodies were diluted in the Odyssey blocking buffer at 1:10,000. The membrane was then imaged using the Li-Cor Odyssey Sa system, and band intensities were measured using ImageStudio software.

Protein interaction studies in cells Cos-7 cells were used because of the difficulty in transfecting the WT and MIN colon cell lines. Cells were transfected with EGFP, EGFP-nAPC, EGFP-cAPC, or EGFP-IC1B constructs. After 16 h, cells were starved for 4 h, lysed in the lysis buffer on ice for 30 min, and then centrifuged at 50,000 × g for 30 min. Cell lysates were incubated overnight with agarose beads conjugated to DIC antibody or normal mouse IgG (Santa Cruz Biotechnology). Beads were washed extensively in 50 mM Tris, pH 7.5, 0.5% NP-40, 0.1% Triton X-100, 125 mM NaCl, 1 mM MgCl2, and 5 mM EDTA and then in PBST, and then processed for SDS–PAGE and Western blotting. Lysates were prepared 24–36 h after transfection and no starvation step was included. Lysates were sonicated for 10 pulses at level 1 with 10% output three times, incubated on ice for 10 min, and then centrifuged at 17,000 × g for 20 min at 4°C.

Cell lysates were incubated overnight with agarose beads conjugated to DIC antibody or protein A beads alone. After transfection, cells were starved for 12 h and then exposed to 3 μM CT99021 for 12 h in full medium (containing both ITS and FBS).

Statistics

All analyses were carried out using GraphPad Prism, version 5.00 for Mac OSX. In most of the figures, error bars represent ±95% confidence interval (CI). One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests or paired or unpaired, two-tailed Student's t test were used as indicated in the figure legends. Immunofluorescence measurements were made using ImageJ Fiji. For graphs from Western blots, at least three repeats of the experiments were performed.

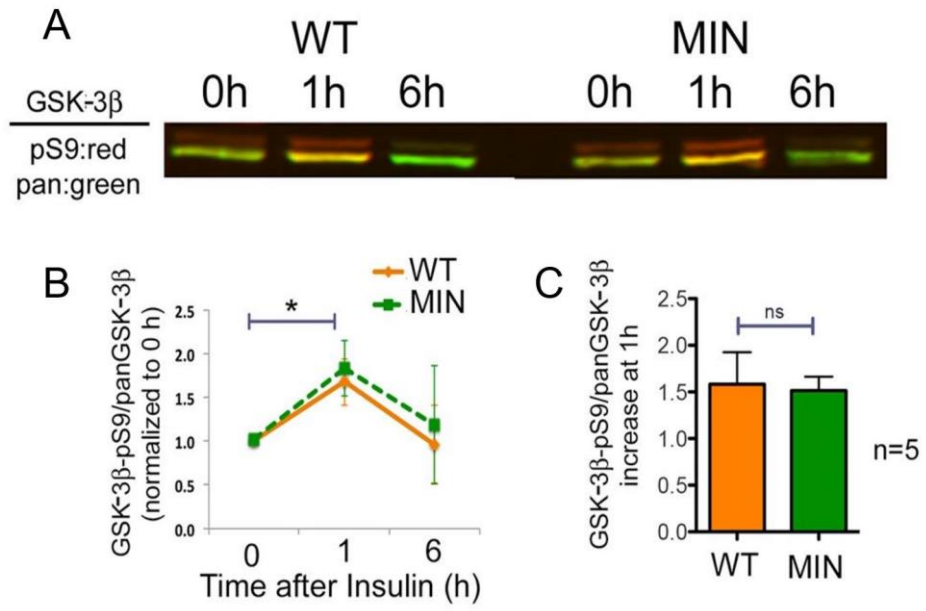

Figure 4.1: Extent of GSK3 inhibition remains the same in WT and MIN cells. A, Representative Western blot of extracts from YAMC and IMCE cells treated with insulin for 0, 1, or 6 hours. pS9 (red); pan-GSK3 (green) **B**, Li-Cor densitometry analysis of three Western blots shows mean ± 95% CI. *$p < 0.05$ by one-way ANOVA. **C**, Average increase in S9/pan-GSK3 after 1 h was determined from five Western blots. Figure adapted from Gao et al., 2017.

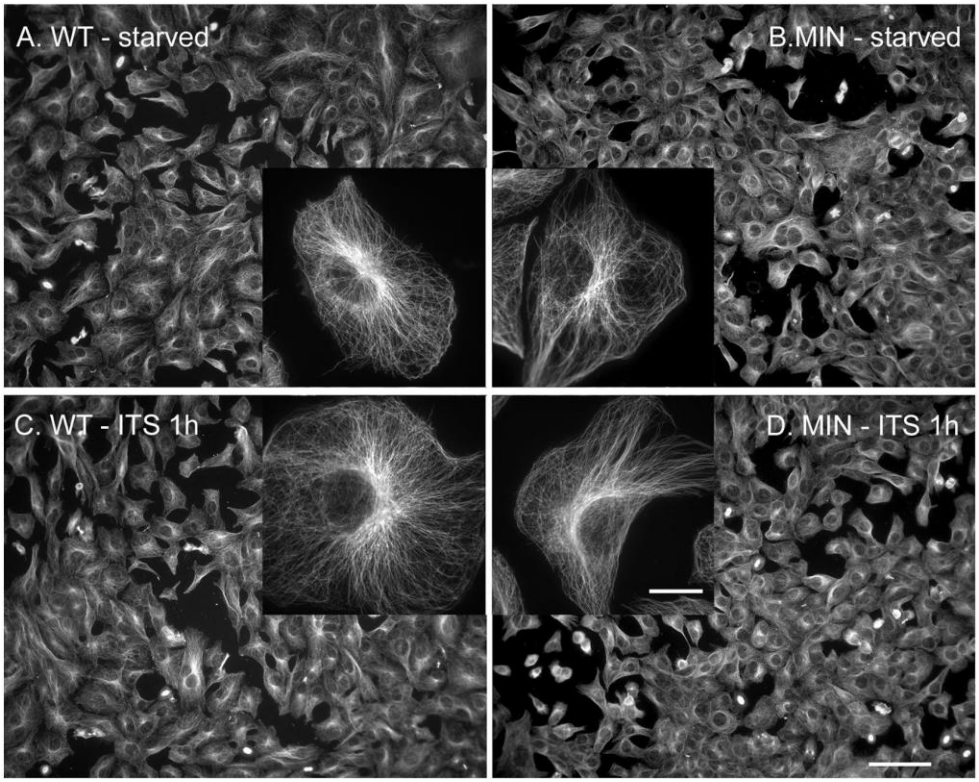

FIGURE 4.2: Microtubule organization is similar in WT and MIN cells with and without 1-h insulin exposure. Normal full culture medium was replaced with serum- and insulin-free medium for 12 h, and then insulin (ITS, 10 µM) was added for 1 h to one set of cultures. **A**, WT cells and **B**, MIN cells with no added insulin or **C**, WT cells and **D**, MIN cells that were exposed to insulin for 1 h were fixed and processed for α-tubulin IF. Insets, individual cells at higher magnification (63X). Scale bars, 50 µm (20X image), 10 µm (inset). Figure adapted from Gao et al., 2017.

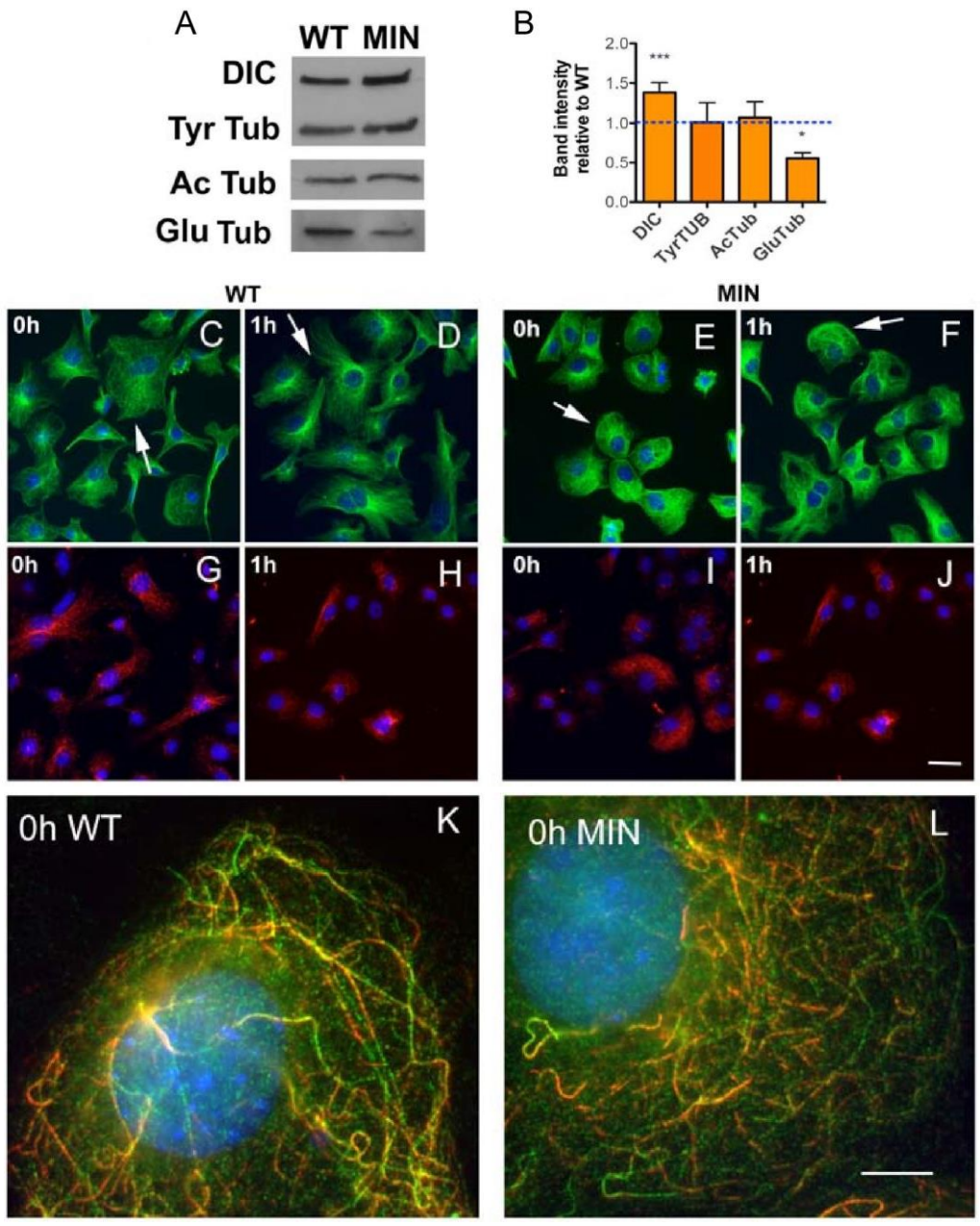

Figure 4.3: Comparison of tubulin modifications in WT vs. MIN cells. A, Western blot of extracts from WT and MIN cells probed for DIC, tyrosinated tubulin (Tyr Tub), acetylated tubulin (Ac Tub) and detyrosinated tubulin (Glu Tub). **B**, Band intensity was measured for 3 separate experiments. The graph shows the ratio of MIN to WT levels for each antibody in A. The data are shown as the average +/- 95% CI, and statistical differences determine by ANOVA of three separate experiments. *=p<0.05, ***=p<0.001. **C-F**. WT (left) and MIN cells (right) stained for tyrosinated tubulin at 0 and 1 hour after insulin. **G-J**. WT (left) and MIN cells (right) stained for acetylated tubulin at 0 and 1 hour after insulin. **K-L**. WT (left) and MIN cells (right) stained costained for acetylated and detyrosinated tubulin. Scale bar for C-J = 25µm. Scale bar for K and L = 2.5µm. Figure adapted from Gao et al., 2017.

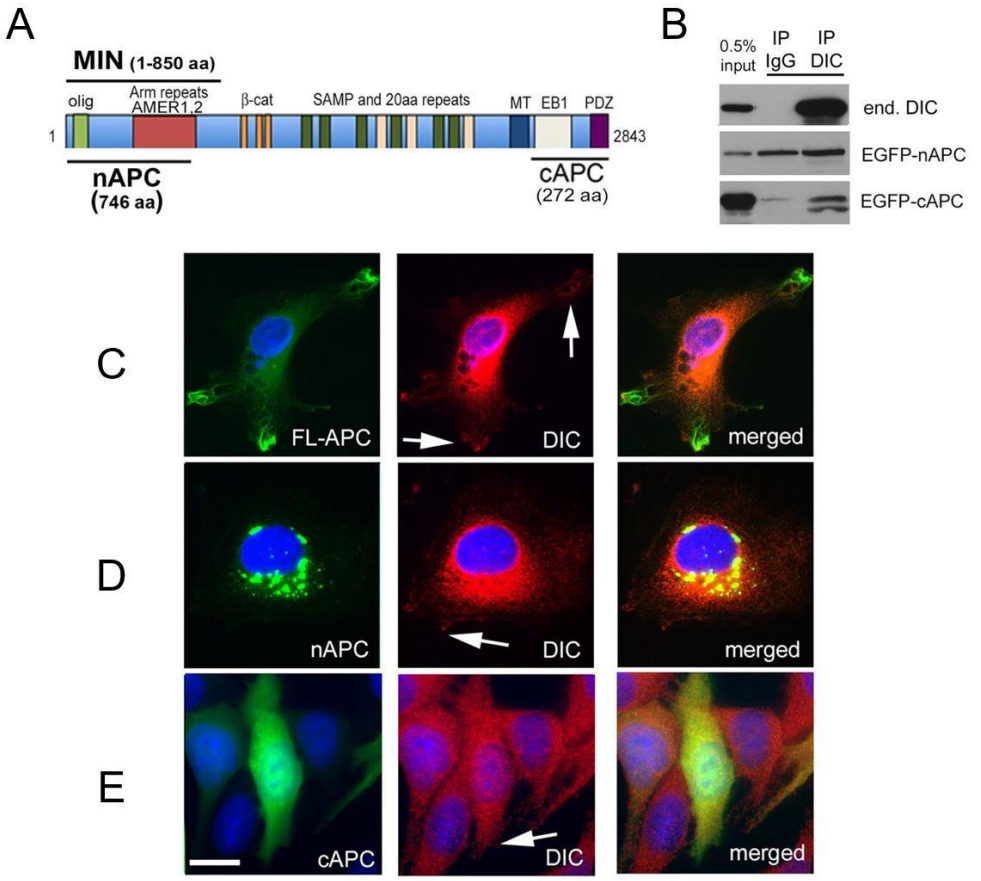

FIGURE 4.4: A C-terminal APC fragment expressed in Cos-7 cells coimmunoprecipitates with endogenous dynein. A, Location of an N-terminal 746–amino acid APC fragment (nAPC) and a C-terminal 272–amino acid APC (cAPC) fragment used in several experiments. Also shown is the 850–amino acid MIN isoform. **B**, Western blot of proteins in a dynein immunoprecipitate. Top, endogenous dynein (end. DIC) is precipitated by a dynein antibody but not by nonspecific mouse IgG. Middle, EGFPnAPC is present in both the IgG and the DIC immunoprecipitate. Bottom, EGFP-cAPC coprecipitated specifically with dynein, not IgG. The lower anti-GFP–labeled band may be a proteolytic fragment or a modified peptide that is enriched in the DIC immunoprecipitate. **C**, Full-length APC fused to EGFP (green) transiently expressed in Cos-7 cells localizes along MTs in cellular protrusions. DIC (red) is concentrated in the cytoplasm but present near the peripheral regions of protrusions (arrows). **D**, EGFP-nAPC (green) is enriched in large aggregates in the cytoplasm. Very small puncta of EGFP-nAPC associate with DIC (red) in cellular protrusions at the cell periphery (arrow). **E**, EGFP-cAPC (green) is diffuse throughout the cytoplasm and is present in the nucleus. Scale bar, 10 μm. Figure adapted from (Gao et al., 2017).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Cytoplasmic dynein is a critical mediator of a diverse array of cellular functions, such as nuclear migration, multiple events during mitosis, and microtubule-based intracellular transport. These processes all must be tightly regulated to avoid potential disease states, such as neurodegeneration and cancer. This is can be done via protein-protein interactions with Lis1/Ndel1, dynactin, and APC amongst others, and posttranslational modifications of the motor itself or its regulators. Disrupting these regulatory entities can impair axon regeneration after injury, cause neurological diseases, and possibly play a role in cancer and diabetes. Specifically, haploinsufficiency of Lis1 during development causes lissencephaly in humans while postdevelopmental knockout of Lis1 in mice can cause pathology consistent with neurodegeneration, and mutations in APC can cause colorectal cancer. In the studies described in this dissertation, we further investigated the potential consequences of these disruptions on dynein-dependent transport.

The function of Lis1 during neurodevelopment has been well characterized due to mutations in this gene causing lissencephaly in humans. Since Lis1 is still highly expressed in the adult mouse nervous system, we wanted to investigate its role postdevelopmentally, focusing particularly on

axonal transport. To do this, we used an inducible knockout system to circumvent the effects of Lis1 depletion on developmental process like neuronal migration, spindle orientation and nuclear envelope breakdown (at least in post-mitotic cells, such as neurons), and interkinetic nuclear migration. This revealed an important postdevelopmental role for Lis1 in mice. Specifically, we saw a rapid decline in health, sensorimotor defects, and death. At the same time, we saw disruption of axonal transport, which was likely the cause of pathological changes in cultured DRG neurons (axonal swellings and altered neurofilament distribution) and in cardiorespiratory neurons in brainstem sections (chromatolysis). This suggests that Lis1 plays a critical role in neuronal maintenance, at least in part through axonal transport, postdevelopmentally. There were also defects in axon growth in cultured adult DRG neurons, which indicates Lis1 may play a role in axon regeneration.

In more broad terms, these experiments show that disruption of axonal transport (and potentially other Lis1-related cellular processes) can lead to a degenerative phenotype that primarily affects brainstem neurons and sensorimotor systems in mice. This is interesting because several neurodegenerative diseases are associated with defects in axonal transport, but it is not known whether this is a byproduct of other dysfunctional processes or one of the causes of degeneration itself. Our studies show that it is quite possible that impaired axonal transport is sufficient to cause axonal pathology and a similar behavioral phenotype to other neurodegenerative disease models, such as ALS.

The differences seen between Lis1 deficient mouse models and human patients with lissencephaly were a little surprising at first, but may make sense in the context of long-distance axonal transport. That is, human neurons must extend their axonal processes over a much further distance than even the longest mouse axons. If Lis1 is responsible for regulating long-distance transport, then it makes sense that a 50% reduction in Lis1 protein levels would have a more noticeable effect on longer human axons than the shorter mouse axons. If we also consider the possibility that any defects in transport can lead to accumulation of effects over time and distance, then it is easy to see how reduced Lis1 levels in humans produce a much more severe phenotype than seen in mice.

A similar principle may also partially why different parts of the nervous system are affected in the two models (cortex in humans, brainstem/motor neurons in our mouse model). First, in humans, Lis1 haploinsufficiency causes mitotic and migrational defects, which would preferentially affect cortical neurons because they require more cell divisions to become neurons than those in the brainstem. Additionally, these cortical neurons have to migrate much further in humans than mice, which may make this process more sensitive to reduced Lis1 levels. This may be why Lis1 heterozygous mice, where Lis1 levels are reduced during development, don't exhibit the drastic cortical defects observed in humans.

In our inducible Lis1 knockout mice, these neurodevelopmental processes are bypassed, allowing us to investigate the effects loss of Lis1 may have in

addition to mitotic and migrational defects seen in lissencephaly patients. The fact that we observe minimal recombination and Lis1 loss in the cortex indicates that the phenotype observed is not due to cortical dysfunction. In fact, we see reduced Lis1 mRNA and protein levels, as well as signs of axonal pathology, in the brainstem of our Lis1 knockout mice. Sensorimotor defects are also seen in the hindlimbs of these mice. The susceptibility of these neurons to reduced Lis1 expression may be due to their relatively long axon length.

These results raise many questions that are potential avenues for future research. First, since the phenotype developed in the Lis1 knockout mice has many parallels with neurodegenerative diseases linked to defective axonal transport, such as ALS, Charcot-Marie-Tooth, and SMA-LED (Harms et al., 2012; Ikenaka et al., 2012; Sabblah et al., 2018), it is possible that mild perturbation of this interaction could produce a less severe phenotype than that caused by haploinsufficiency in humans. One way the interaction could be altered is if Lis1 mutations exist that produce a milder effect than those that cause lissencephaly. Hypothetically, these mutations would have minimal impact on dynein-dependent developmental processes while potentially impairing transport-related processes postdevelopmentally. Our lab is currently researching published sequencing data, as well as working with the Greenwood Genetics Center, to determine if there are any such mutations known in the Lis1 gene that can be linked to other neurological diseases.

It would also be interesting to determine what role Lis1 plays in axon regeneration. As described in Chapter 4, there are injury-induced signals that are

retrogradely transported by dynein, which bolster regenerative capacity (Hanz and Fainzilber, 2006; Ben-Yaakov et al., 2012). These signals may also require Lis1 for efficient transport to promote the switch from arborizing axon growth to elongating axon growth. Additionally, Lis1 and dynein are required to advance microtubules in the growth cone during fast axonal outgrowth and downregulation of Lis1 during development limits axonal outgrowth (Grabham et al., 2007; Kumamoto et al., 2017). Given the axon growth defects seen in DRG cultures from Lis1 KO mice, it will be interesting to determine the mechanism through which this is occurring. This is something we have already begun investigating by looking at the effect of Lis1 depletion on retrograde injury signaling via accumulation of the transcription factor, phospho-STAT3, in the nucleus of DRG neurons following sciatic nerve injury.

To better understand the role of APC in dynein-dependent transport, we took advantage of a well-characterized cancer-causing mutation in the APC gene, the MIN mutation, to explore how disruption of APC function leads to aberrant dynein regulation. Our results show that the MIN mutation disrupts the normal dynein-APC interaction at microtubule plus ends. This also abrogates the regulatory effects of insulin signaling on dynein-dependent transport. In cells expressing only full-length APC, insulin signaling decreased GSK3-dependent phosphorylation of dynein, thus increasing minus-end dependent transport, shown by increased accumulation of dynein at the centrosome. However, in cells with the MIN mutation (IMCE cells), this regulatory effect was diminished and accumulation of dynein at the centrosome was not seen, despite similar levels of

insulin-dependent GSK3 inhibition. This could be an effect of decreased efficacy of insulin-sensitizing drugs as chemopreventive or therapeutic agents in patients with colorectal cancer. Further investigation is required to determine the precise implications of this dysregulation, but some potential effects include disruption of transport-dependent degradative processes, such as mitophagy and the endo-lysosomal pathway, and altered mitochondrial distribution. If this effect extends to other dynein-dependent processes, then this could influence mitotic events such as nuclear envelope breakdown, microtubule capture at kinetochores, or spindle formation and orientation in APC MIN cells. Alterations in these processes could provide mechanistic insights into the initiation of MIN-induced colorectal cancer.

One other project would determine the mechanistic role of APC in axon regeneration. Since APC serves many functions in neurons, including microtubule stabilization, RNA-binding, β-catenin destruction, and dynein regulation, it seems likely that the truncating mutation seen in patients with colorectal cancer could influence axon growth after injury. If defects in regeneration are seen, then we would investigate which of APC's functions are the cause of this phenotype. There is evidence that APC plays a role in axon growth and arborization during development, as well as axon sorting in the PNS (Chen et al., 2011; Elbaz et al., 2016).

While the studies conducted in this dissertation focus on two dynein regulatory pathways, there are a number of other proteins and signaling cascades that impact microtubule-based transport (e.g., dynactin, Ndel1, BicD2, etc). It is currently unclear how all of these elements coordinate to regulate

axonal transport. Having a comprehensive view of how axonal transport is regulated will help us understand how and why transport is perturbed in many degenerative diseases and how this contributes to the progression of the disease. This is an area of ongoing research, as it may be possible to manipulate different regulatory components to alleviate the effects of defective transport seen in many neurological diseases, such as Alzheimer's, schizophrenia, and ALS.

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