UNIVERSITY OF CALIFORNIA SAN DIEGO

The neuron-specific roles of Dual Leucine Zipper Kinase and Leucine Zipper Bearing Kinase in corticospinal axon repair following central nervous system injury

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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2021

The Dissertation of Junmi Mayuree Saikia is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AKT	Protein Kinase B
BDA	Biotinylated dextran amine
CNS	Central nervous system
cKO	Conditional knockout
Cre	Cre recombinase
CSMN	Corticospinal motor neuron
CSPG	Chondroitin sulfate proteoglycan
CST	Corticospinal tract
DH	Dorsal hemisection
DLK	Dual leucine zipper kinase
DRG	Dorsal root ganglion
MAG	Myelin associated glycoprotein
MLK	Mixed lineage kinase
mTOR	Mammalian target of rapamycin
LZK	Leucine zipper-bearing kinase
MAP3K	Mitogen activated protein kinase kinase kinase
n.s.	Not significant
OMgp	Oligodendrocytic myelin glycoprotein
PKCγ	Protein kinase C gamma
PNS	Peripheral nervous system
PTEN	Phosphatase and tensin homolog
RGC	Retinal ganglion cell
SCI	Spinal cord injury

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CONFERENCES, TALKS and PUBLICATIONS

Saikia JM, Kim N, Chavez CM, Allibhoy S, Morka B, Suen J, Roepke D, Muñoz O, Jin Y & Zheng B. Deciphering the roles of LZK and DLK in axonal repair in the mouse spinal cord after injury. Asilomar ISNR, 2020. Conference.

Kim JM, **Saikia JM**, Moore A, Lusk D, Cervantes K, & Zheng B. Single cell transcriptome in understanding the regenerative abilities of CST neurons. Asilomar ISNR, 2020. Conference.

Narang A, Meves JM, **Saikia JM***, Chen M, Kim N, Jin Y, Zheng B. The role of Leucine Zipper-Bearing Kinase in multicellular response to CNS injury. CSHL: Molecular Mechanisms of Neural Connectivity, 2018. Talk. *Presenting author.

Das U, Wang L, Ganguly A, **Saikia JM**, Wagner SL, Koo EH, Roy S. 2016. Visualizing APP and BACE-1 approximation in neurons yields insight into the amyloidogenic pathway. *Nat Neurosci, 19*(1):55-64. doi: 10.1038/nn.4188.

Saikia, JM, Du, J, Tognoni, CM, Koch, LG, Britton, SL, Peace, RM, Jones, LW, Williams, CL. Treadmill exercise training only enhances neurocognitive function if it is accompanied by significant increases in aerobic capacity. Poster session present at Society for Neuroscience; November 12, 2013; San Diego, CA.

Babb EA, Tognoni CM, **Saikia JM**, Fowler E, Britton SL, Koch LG, Williams CL, Jones LW. Moderating effects of high versus low aerobic capacity on adult rat hippocampal neurogenesis following systemic doxorubicin treatment. Poster session present at Society for Neuroscience; October 13-17, 2012; New Orleans, LA.

Pal SK, Yamzon J, Sun V, Carmichael C, **Saikia JM**, Ferrell B, Frankel P, Hsu J, Twardowski P, Stein CA, Margolin K. Paclitaxel-Based High-Dose Chemotherapy with Autologous Stem Cell Rescue for Relapsed Germ Cell Tumor: Clinical Outcome and Quality of Life in Long-Term Survivors. Clin Genitourin Cancer(clgc). DOI: 10.1016/j.clgc.2012.09.007 .

Carmichael C, Yuh BE, Sun V, Lau C, Hsu J, **Saikia JM**, Liu X, Wilson T, Ferrell B, Pal SK. Quality of Life in Patients With Metastatic Renal Cell Carcinoma: Assessment of Long-Term Survivors. DOI:10.1016/j,clgc.2012.09.006.

Pal SK, Jones JO, Carmichael C, **Saikia JM**, Hsu J, Liu X, Figlin RA, Twardowski P, Lau C. Clinical outcome in patients receiving systemic therapy for metastatic sarcomatoid renal cell carcinoma: A retrospective analysis. Urol Oncol. DOI: 10.1016/j.urolonc.2012.04.007.

ABSTRACT OF THE DISSERATION:

The neuron-specific roles of Dual Leucine Zipper Kinase and Leucine Zipper Bearing Kinase in corticospinal axon repair following central nervous system injury

By

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Doctor of Philosophy in Neurosciences

University of California San Diego, 2021

Professor Binhai Zheng, Chair

Damage to the adult central nervous system, including both brain and spinal cord, results in profound long-term functional impairment. While the mammalian CNS is notorious for its inability to repair itself after injury, there are two forms of axonal repair that may contribute to some recovery following CNS injury: regeneration of severed axons and compensatory sprouting of uninjured axons. Of these two mechanisms of repair, sprouting can occur spontaneously, and is believed to contribute at least partially to the clinical manifestations of recovery in individuals with milder forms of CNS trauma, although many continue to show profound lifelong deficits in function. Alternately, regeneration is rarely spontaneous and requires manipulation of neuron-intrinsic and extrinsic mechanisms in order to occur in the laboratory setting. Manipulation of various intrinsic and extrinsic signaling pathways to enhance the efficacy of sprouting or

regenerative repair mechanisms remains an important goal in promoting functional recovery, although the molecular understanding of these processes is still incomplete.

The major goal of this dissertation is to understand the roles of Leucine Zipper-Bearing Kinase (LZK) and Dual Leucine Zipper Kinase (DLK), a pair of evolutionarily conserved mitogen-activated protein kinase kinase kinases (MAP3Ks), in both forms of axonal repair after central nervous system CNS injury, or more specifically after spinal cord injury (SCI). The Jin and Bastiani labs identified *DLK-1* as an injury sensor, and a critical regulator of axon regeneration in *C. elegans*. DLK has been shown to play a role in axon regeneration in the worm motor nerve after laser axotomy (Hammarlund et al., 2009; Nakata et al., 2005). In the mouse, these kinases have been studied in the optic nerve and the sciatic nerve, but the role of DLK in axonal repair in the mammalian spinal cord is not known. Furthermore, nothing is known about neuronal LZK in axonal repair in the mammalian CNS.

My dissertation investigates the roles of these kinases in both sprouting and regeneration using two different surgical models of spinal cord injury. In addition to assessing the roles of these kinases in an unmanipulated genetic background, I leveraged the well described effect of PTEN deletion to enhance basal sprouting and regeneration phenotypes and assessed if further deletion of one or both kinases contributes to any change in repair phenotypes. In these investigations, I found that while deleting both kinases abolishes the effect PTEN-enhanced repair, deletion of LZK alone results in no meaningful changes in either sprouting or regeneration. Furthermore, co-deletion of DLK and LZK in both induced sprouting and regeneration models does not alter the effect of PTEN deletion on AKT/mTOR activity, as evidenced by elevated downstream marker pS6, demonstrating for the first time the failure of axon regeneration in the presence of upregulated AKT/mTOR activity. This implies that regenerative competence, as reflected by pS6 levels, is separate or parallel to the pathway mediated by DLK/LZK. Both pathways must be intact and active in order for regenerative competence to translate to axonal repair.

Chapter 1:

Introduction

1.1 Overview

Central nervous system (CNS) damage in the adult mammalian brain and spinal cord generally results in profound long-term deficits, contributing to economic and social burden. For spinal cord injury patients, clinical therapeutics aimed at enhancing CNS repair are essentially limited to spinal cord stimulation, and these methods provide very limited functional recovery in patients. The key factor contributing to the failure of meaningful recovery boils down to the inability for the central nervous system neurons to exhibit axonal regeneration following injury; as such, there has been a strong effort amongst basic science researchers to elucidate novel molecular mechanisms involved with growth and repair of axons, in the hopes of finding new pathways to target and manipulate.

Within the mammalian CNS, two mechanisms of axon repair are studied extensively: regeneration, or the regrowth of injured/severed axons, or compensatory sprouting from uninjured axons into denervated regions (see Fig. 1.2). Of these two repair mechanisms, regeneration is a harder hurdle to overcome. Even in the laboratory setting, axon regeneration is difficult to achieve, and requires major manipulations in the extremely unfavorable setting in which adult CNS neurons exist. In 2008, a breakthrough study on the AKT/mTOR growth and development pathway revealed that neuronal deletion of PTEN, an upstream suppressor of the AKT/mTOR pathway, can result in robust retinal axon regeneration past a optic nerve crush site (Park et al., 2008). Subsequent to this finding, many scientists have attempted to combine this intrinsically inhibited signaling pathway with other intrinsic and extrinsic manipulations to enhance regenerative axonal repair. Research into the mechanisms of axon regeneration in the laboratory has yet to meaningfully translate to the clinical setting, but these findings are important for developing our general understanding of the molecular systems that play a role in axon repair.

Compared to outright regeneration of axons, sprouting provides a different form of axonal repair that relies more heavily on the plasticity of intact neurons to compensate for the lost function of denervated tissue. In many spinal cord injuries, complete deficits in motor function typically

occur after only partial or incomplete anatomical injuries within the spinal cord. In these injuries, spared descending axonal tracts can undergo post-development plasticity and form new axonal sprouts to synapse onto intact downstream targets below the level of the lesion. Sprouting is thought to contribute to partial recovery of spinal cord function even in the clinical setting, although even this mechanism of repair also leaves much to be desired in the clinical setting. In rodent models, compensatory axonal sprouting can be enhanced through various manipulations of CNS growth regulators, such as extrinsic depots of growth factors, or genetic deletion of tumor suppressor genes like PTEN. These types of manipulations suggest that there may be potential alternative approaches to enhance recovery from CNS injury in humans.

My goal in this dissertation is to explore two specific neuron-intrinsic factors in the form of Leucine Zipper bearing Kinase (LZK) and Dual Leucine Zipper Kinase (DLK), members of the mixed lineage kinase (MLK) family, that may be involved with injury signaling and axonal repair. In my investigations, I seek to probe these kinases in models of both axonal regeneration and sprouting. By leveraging the unilateral pyramidotomy surgical method, I will be able to assess how uninjured axons cross over the spinal cord midline into denervated tissue in a model of axonal sprouting. The second surgical technique I employ, the dorsal hemisection spinal cord injury model, damages both descending tracts of the CST, and allows for assessment of axonal regeneration across and around a glial scar.

1.2 Anatomy of the mouse corticospinal tract

The mammalian CST is the key descending motor tract and is the longest axonal pathway in the CNS. Of the various motor circuits, the CST is the slowest to develop, and its evolutionary development likely was critical for the development of voluntary coordinated motor movements (Figure 1.1). Cell bodies within the sensorimotor cortex in the frontal lobe send axon projections via the cerebral peduncle, passing through the internal capsule, pons and medulla, before roughly the overwhelming majority of these axons cross over to the contralateral side at the level of the

medullary pyramidal decussation, in the caudal most regions of the medullary pons. These axons continue down the spinal cord via the dorsal CST, finally synapsing onto the dorsal horn cell bodies of interneurons. Axons that do not cross over at the level of the medullary decussation make up the ventral corticospinal tract (Welniarz et al., 2015).

The descending CST axons in rodents arise from Layer V neurons in the cerebral cortex, and are called corticospinal motor neurons (CSMNs) or upper motor neurons. In early mouse development, the developing brain sends strong bilateral projections to the spinal cord. Through use-dependent plasticity in later development, the majority of ipsilaterally projecting axons are eliminated, leaving these Layer V CSMNs to have primarily contralaterally projecting axons (Joosten et al., 1992; Martin et al., 2004). The remaining ipsilateral tracts are predominantly involved with innervating axial musculature, while midline crossing axons are more associated with innervation of distal limb muscles (Joosten et al., 1992; Vulliemoz et al., 2005). In this thesis, I will use the terms “CST neurons” and “CSMNs” interchangeably.

1.3 Spinal cord injury

Being a part of the CNS, damage to the spinal cord, like the brain, leads to long term functional deficits, ranging from the more obvious motor paralysis to less conspicuous but equally important clinical complications such as respiratory and urinary problems. These can further result in a spectrum of subsequent problems including ulcer formation, or changes in psychiatric states, such as depression (Khazaeipour et al., 2017). The burden of living with spinal cord injury extend beyond the individual patient, as caretakers often are critical for long-term support for both the physical and mental care of the affected individual.

With regards to the more cellular and molecular aspects of spinal cord injury, a major neuronal class affected is the CSMN or upper motor neuron, with their descending CST axons taking the brunt of the damage. Physical deficits seen in patients occur below the level of injury,

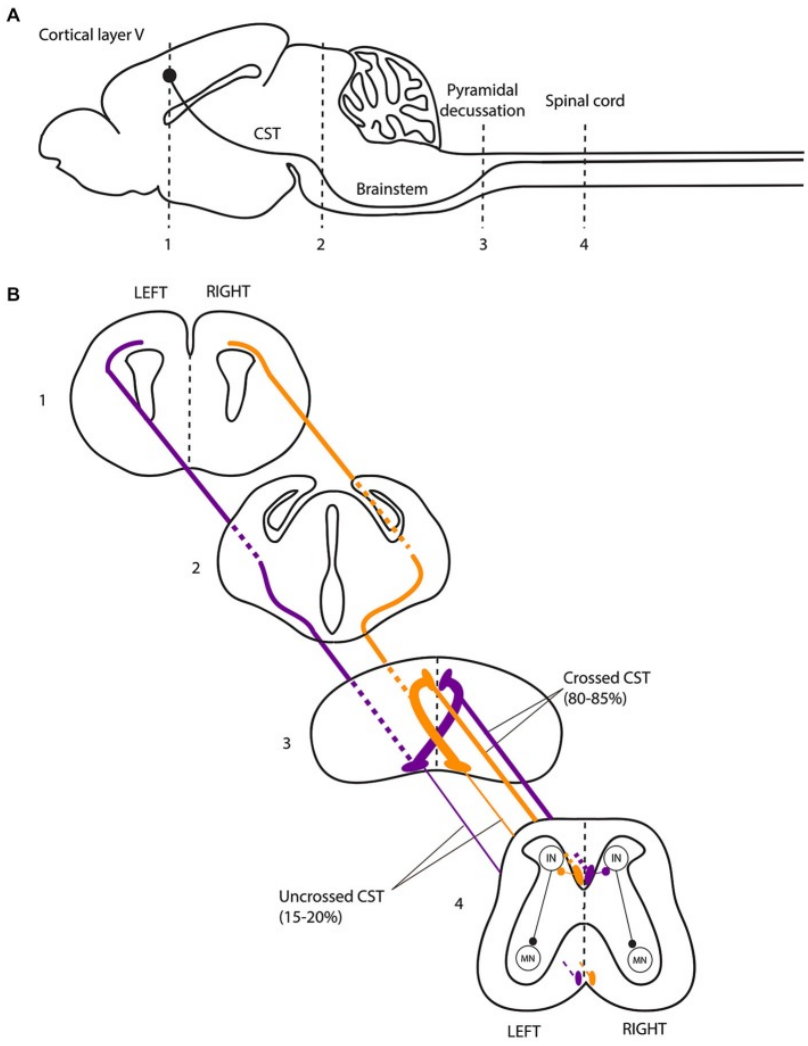

Figure 1.1 Anatomy of the mouse corticospinal tract.
Neuronal cell bodies arise in Layer V motor cortex in the frontal lobe. Descending axons tract ipsilaterally through the pons and into the medullary pyramids. At the caudal most segment of the medullas, the majority of these axons cross past the midline and migrate from the ventral region to the dorsal region of the descending spinal cord. These axons continue to descend via the dorsal CST, before terminating onto interneurons within the dorsal horn of the spinal cord. Dorsal CST axons are primarily involved with innervation of distal limb muscles. Axons that do not cross over make up the ventral CST, which is largely involved with innervation of axial musculature (Welniarz et al., 2015).

meaning that the higher the level of SCI along the cord, the greater the effects are, and the more downstream organs and muscles are affected. However, in most cases, the bodies of the neurons above the injury do not die; rather they become unable to relay signals to downstream targets. Additionally, despite the profound motor and sensory functional deficits seen in SCI patients, the majority of injuries sustained in humans are anatomically incomplete (Angeli et al., 2014). In a subset of these individuals with incomplete spinal cord lesions, a few recover some motor function with intervention, and this is thought to be due to inherent plasticity found within the spared descending axons.

Given the heavy impacts of spinal cord injury on affected individuals and their loved ones, there is a strong push within the medical and research communities to understand and identify novel molecular mechanisms of repair in the CNS, as well to improve rehabilitative therapies and develop targeted pharmaceutical agents to facilitate a patient's recovery of function.

1.4 CST neuron responses to injury

In the adult mammalian CNS, the ability to recover from injury is harshly limited. An injured neuron can either attempt to regenerate, which is a largely non-spontaneous response to injury, or uninjured spared axons can exhibit plasticity, and sprout to form new connections with downstream targets below the site of lesion (Meves et al., 2018). In the laboratory setting, we can study these two main avenues of axonal repair: axonal sprouting and axonal regeneration (Figure 1.2). Of these two mechanisms, it is thought that axonal sprouting, which has been demonstrated to occur spontaneously, may contribute clinically to functional recovery in patients who have incomplete spinal cord lesions (Fouad et al., 2001). In stroke-induced injury, axonal sprouting from uninjured neighboring brain tissue may also serve to compensate for denervation (Carmichael et al., 2017). In clinical populations of spinal cord injury, very few neurologically complete spinal cord lesions result from complete anatomical injury across the spinal cord (Angeli et al., 2014), leaving a surviving population of axons that, with therapy, may be able to provide

some measure of repair following injury via the plasticity in spared circuits (Ueno et al., 2012; Weidner et al., 2001).

Compared to axonal sprouting, axonal regeneration for the most part does not occur spontaneously in the adult mammalian CNS. Finding manipulations to overcome this hurdle has for many years been a major topic of interest. Neuron-extrinsic (i.e. environmental) inhibitory factors surrounding the damaged tissue serve to grossly prevent axonal regeneration: myelin debris, glial scar, and astrocytic secretions of chondroitin sulfate proteoglycans are just a few of the many molecular and cellular components of a lesion site that may hinder axonal regeneration (Ferguson & Son, 2011). With the advent of more recent genetic tools, research has also begun to explore the role of microglia in mediating the immune response and repair pathways involved with axon lesion and repair (Y. Li et al., 2020). In addition to these extrinsic factors (see below, 1.4 Neuron-intrinsic vs. extrinsic regulators of axonal repair), neuron intrinsic molecular signals contribute to limitations in axonal regeneration in major ways. Likely, regeneration requires a pro-growth intrinsic state similar to what is seen in developing neurons (He & Jin, 2016), but this pro-growth state is not typically active in adult post-migratory and post-mitotic neurons. Following maturity to homeostatic adulthood, many of these pro-growth transcription profiles arrest, unless perturbed by injury induced disruptions to physical and metabolic function.

Another key response to injury frequently seen in rodent models of SCI is the retraction of CST axons (Kerschensteiner et al., 2005). In regeneration incompetent neurons, the injured axonal stumps proximal to the lesion site dieback during the first 7-10 days after injury, forming dystrophic end bulbs with disorganized cytoskeletal components, instead of growth cones (He & Jin, 2016; Tom et al., 2004). In a typical wildtype mouse, this type of axon retraction and end bulb formation is seen as rostral as half a millimeter proximal to the site of injury. The retraction of these axons, and their inability to form functional organized growth cones is another crucial hurdle limiting the ability for axons to demonstrate functional recovery after injury. This axonal dieback is seen even in animals that have undergone deletion of intrinsic inhibitors to axon regeneration

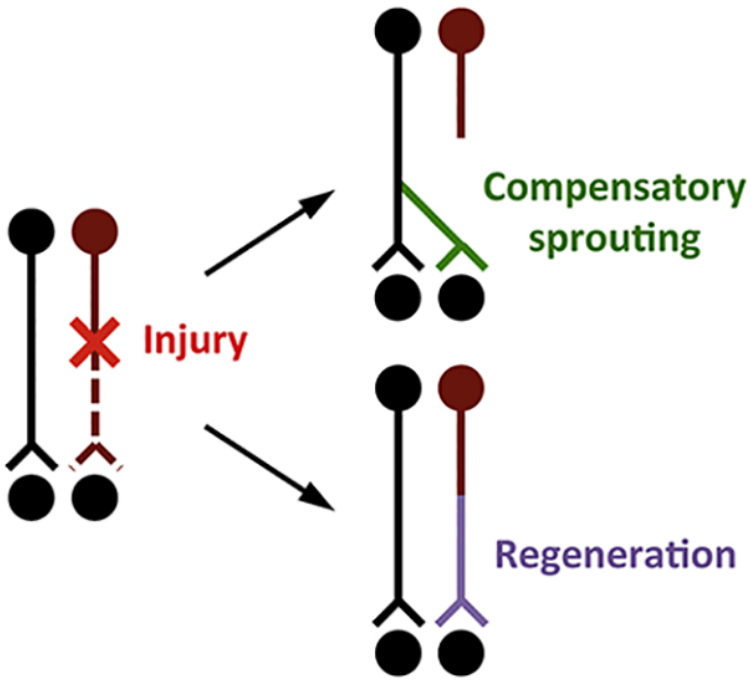

Figure 1.2 Regeneration versus compensatory sprouting
In response to CNS injury (indicated by the red X), neighboring intact axons may be able to grow and attempt to make synaptic connections with otherwise denervated downstream targets spontaneously. Less common than sprouting in the CNS, regeneration occurs when a transected axon regrows following injury. (Meves et al., 2018).

such as PTEN. However, PTEN deletion can overcome this initial phase of retraction, allowing for regeneration towards the site of lesion. A subset of axons that are able to regenerate after axonal dieback are able to continue regenerating past the lesion site into regions caudal to the lesion (Geoffroy et al., 2015; He & Jin, 2016; Liu et al., 2010). Additionally, early work in fly and worm suggest that DLK may function critically as an injury sensor of axon injury, and might be essential for allowing cytoskeletal elements to reorganize to form growth cones (Ghosh-Roy et al., 2012; Valakh et al., 2013).

1.5 Neuron intrinsic and extrinsic inhibitors of axon repair

In order to enhance axonal repair following injury, there has been increased interest in understanding the different neuron intrinsic and extrinsic factors that influence an axon's ability to grow in response to injury. During the early years of studying axon repair, research focused heavily on the effect of extrinsic inhibition on neuronal repair. Although many extrinsic factors are known to inhibit axonal growth after injury, experimental methods involving the selective ablation of these factors have been met with only modest amounts of axonal repair. Indeed, research has focused particularly on the role of glial inhibition of axon regeneration. Myelin associated inhibitors, such as myelin associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp) have been studied extensively especially using in vitro techniques to elucidate their roles at suppressing axon growth (GrandPré et al., 2000; Kottis et al., 2002; Mukhopadhyay et al., 1994). All three molecules signal via a common receptor the glycosylphosphatidylinositol-anchored Nogo-66 receptor (NgR1) expressed on CNS neurons to inhibit growth. Another well studied class of extrinsic glia-associated growth inhibitors are chondroitin sulfate proteoglycans (CSPG), which are secreted by reactive astrocytes in the vicinity of axon injury; enzymatic removal of their inhibitory glycosaminoglycans side chains by chondroitinase ABC has been shown to promote regeneration in sciatic nerve and spinal cord (Bradbury et al., 2002; Zuo et al., 2002).

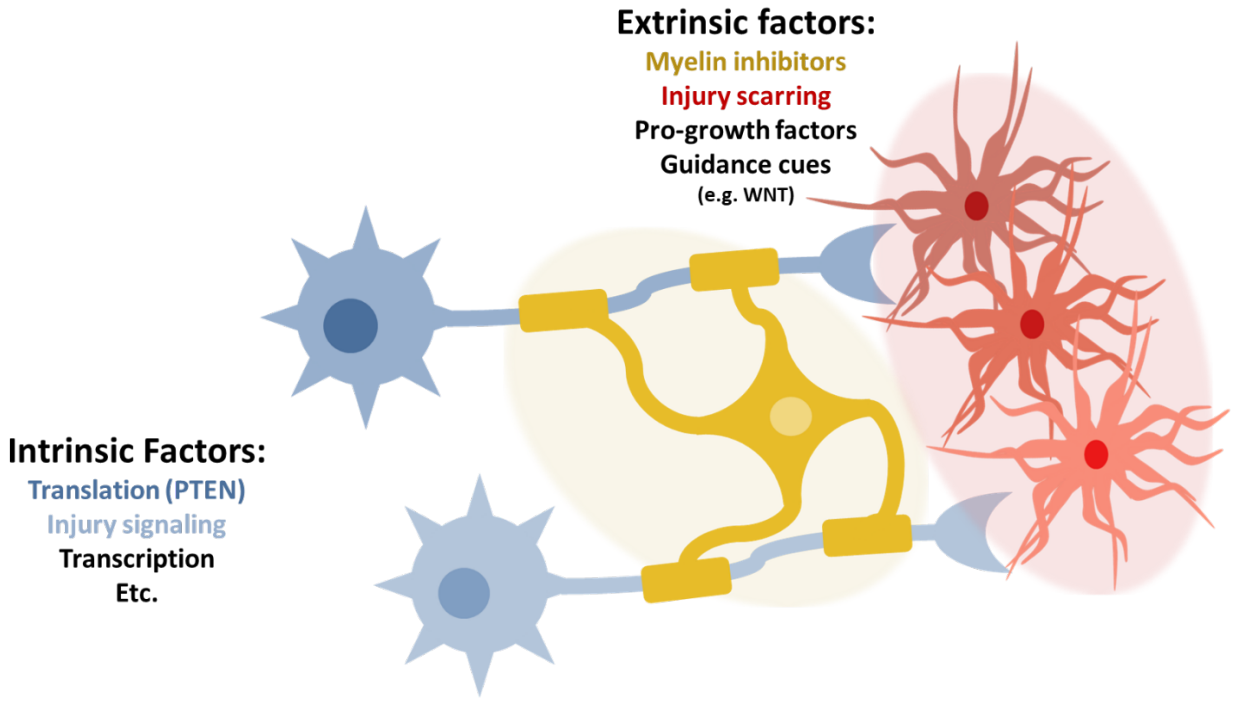

Figure 1.3 In the adult CNS, both intrinsic and extrinsic factors influence the ability for axons to regenerate.
A simplified schematic depicting a few examples of intrinsic and extrinsic factors that affect axonal repair. In an unmanipulated CNS, inhibitory factors, such as intrinsic PTEN suppression of the AKT/mTOR pathway, and extrinsic factors, such as myelin associated debris (e.g. Nogo, MAG, OMgp) and astrocytic CSPGs counter factors that could potentially induce growth, such as injury signaling. Experimental manipulation of both intrinsic and extrinsic factors, for example deletion of PTEN, addition of chondroitinase ABC, or delivery of extrinsic growth factors, can alter the regenerative profile of CNS neurons (adapted from Tedeschi & Bradke, 2017).

One of the key cell types known to express CSPGs in response to injury is the astrocyte. The role of these cells in response to injury is highly debated; subsequent to injury astrocytes transform into a reactive and proliferative state characterized by elevated expression of glial fibrillary acidic protein (GFAP). This astrocyte gliosis contributes heavily in forming a border surrounding a fibrotic lesion core. The formation of this border may serve to limit the size of the inflammatory lesion core, thereby protecting the uninjured nearby healthy tissue (Sofroniew, 2015). A major counterargument to this “protective” role of astrocytes is the astrocytic expression of inhibitory factors, such as CSPGs. From this perspective, astrogliosis contributes to the development of both a chemical barrier to regeneration, as well as a physical barrier in the form of proliferating astrocytes that tangibly make up the scar border (Cregg et al., 2014).

Aside from the complicated role of astrocytes in axon regeneration, recent work on the role of neonatal microglia in mice suggests that in response to crush injury, neonatal and early post-natal microglia are able to mount an inflammatory response to injury that is rapidly converted back to a homeostatic state. This rapid return to homeostasis allows axons, be they regenerating or late-arriving, to continue to grow beyond the site of lesion, which is characterized as having no major signs of astrocytic scar formation (Y. Li et al., 2020), emphasizing the fact that the extrinsic molecular environment of a post-developmental spinal cord is fundamentally different and largely characterized by inhibition to growth.

In addition to the extrinsically inhibitory environment that interferes with axon regeneration, the CNS also contends with intrinsic inhibitors of axonal regeneration. Manipulations of the extrinsic environment alone fails to produce robust regeneration, strongly lending credence to the role of intrinsic inhibition. The earliest key intrinsic inhibitor to axonal regeneration identified was the tumor suppressor gene PTEN (Park et al., 2008). PTEN is a negative suppressor of the AKT/mTOR growth pathway, which is critically active during early development. With age, the AKT/mTOR pathway becomes selectively downregulated in CNS but not PNS neurons, lending the latter with some regenerative competency even in adulthood (Belin et al., 2015). In the PNS,

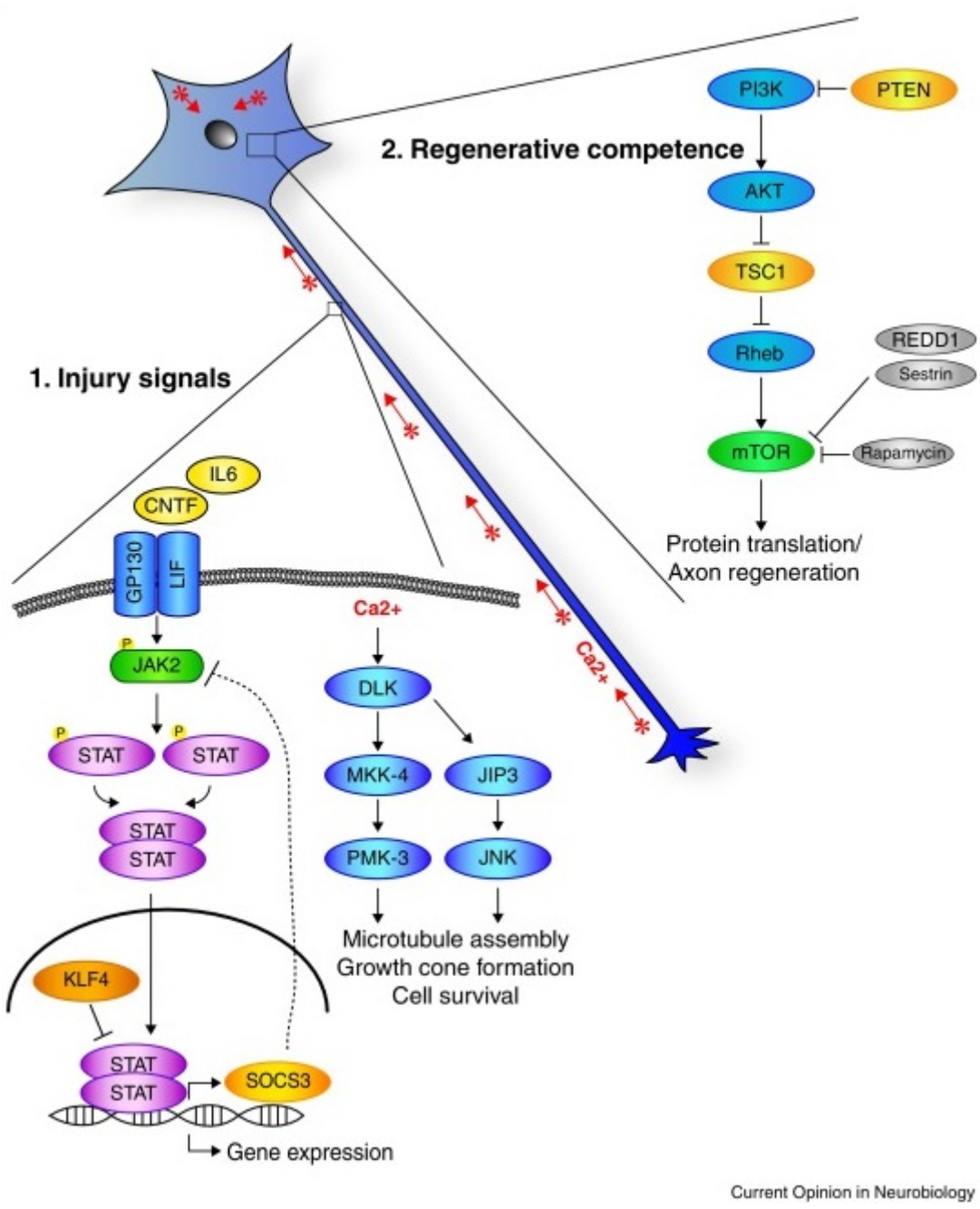

Figure 1.4 Intrinsic factors that influence adult CNS regeneration include injury signaling and regenerative competence.
In order for an axon to regenerate, it must be able to firstly detect a distant injury, and secondly have the regenerative competency to repair the membrane and construct a functional growth cone that can subsequently regrow to its downstream target. The deletion of PTEN enhances the regenerative competency of the neuron, while DLK is among one of the critical injury signals that retrogradely informs the soma of disturbance by sensing calcium signaling and cytoskeletal disruption (Lu et al., 2014).

targeting PTEN enhances DRG regeneration, while inhibiting downstream effector mTOR blocks this effect (Christie et al., 2010), suggesting that PNS neurons retain regenerative capacity in adulthood via AKT/mTOR activity. Deleting or knocking down PTEN in rodent models prior to injury has been shown to enhance both axon regeneration and sprouting, and has even contributed to some functional recovery from experimental spinal cord injury (Lewandowski & Steward, 2014; Liu et al., 2010; Park et al., 2008; Zukor et al., 2013). Regenerative capacity is also influenced by multiple intrinsic cell signaling pathways, including PTEN/AKT/mTOR, SOCS3/STAT3, KLFs, and c-Myc among others (Figure 1.3) (Lu et al., 2014). The identification of PTEN deletion as a robust trigger for axonal repair has led to the design of several experiments aimed at combining the deletion of PTEN with other known inhibitors of axon regeneration to further enhance the phenotype seen with PTEN deletion alone.

In injury, a cell must contend with a damaged membrane, disorganized cytoskeletal elements, and calcium influx; and within this flurry of events, in order to regrow, it must also be able to seal the membrane, reorganize the cytoskeletal elements and prepare a growth cone. In order to trigger the requisite transcriptional events, the soma also must be made aware of a distal injury. Injury sensors are another class of intrinsic molecules that are implicated for a role in axon repair. This class of molecules are thought to be produced in the vicinity of injury, and through retrograde signaling, alert the cell soma and nuclei of distant injuries. Subsequently, the cell can mount a response to injury via upregulation of certain transcriptional profiles aimed at repairing damage to the cell membrane and restructuring of cytoskeletal components. STAT3, for example, is a downstream effector of the Jak/STAT pathway, and is upregulated in response to injury before being retrogradely transported (Ben-Yaakov et al., 2012). Inhibiting the upstream Jak2 kinase via perineural infusion has been shown to limit dorsal column axonal regeneration (Qiu et al., 2005).

In addition to the Jak/STAT cascade, new evidence suggests a novel class of injury signals: DLK, an evolutionarily conserved upstream driver of the MAP3K cascade involving MAP Kinase Kinase 4 (MKK4) and p38, was recently shown to be involved in injury signaling (Figure

1.4). The MAP3K cascade is known to be involved in several cellular processes, including growth, development, response to injury, and apoptosis (Tedeschi & Bradke, 2013, 2017), and more recently, DLK has been studied in neuronal responses to neuronal injury in worm, fly, and mouse models (Hammarlund et al., 2009; Miller et al., 2009; Shin et al., 2012; Watkins et al., 2013; Welsbie et al., 2019; Yan et al., 2009).

1.6 Identification of worm *DLK-1* and mammalian DLK and LZK as injury sensors

The original discovery of DLK as an upstream regulator of MAP3K signaling dates back to the mid-1990s, during a period of extensive kinase discovery (Jin & Zheng, 2019). More recently, worm *DLK-1* was shown to be critical for motor axon regeneration in *C. elegans* (Hammarlund et al., 2009; Yan et al., 2009). *DLK-1* is an evolutionarily conserved upstream regulator of the MAP3K pathway of the mixed-lineage kinase (MLK) family. Within mammals, two homologs to *DLK-1* exist: DLK and LZK (Figure 1.5A). These homologs share >95% sequence homology within their kinase domains, although there is significant divergence at the C-termini. (Yan & Jin, 2012); particularly, mammalian LZK possesses a calcium-sensitive hexapeptide sequence that is identical to the sequence found in worm *DLK-1*, but this sequence is missing in mammalian DLK.

Of the two mammalian homologs, DLK has been studied extensively both in injury signaling, but also in development, during which it plays a critical role in growth cone formation and neuronal migration (Hirai et al., 2006). In fact, DLK null mice fail to survive for more than 2-5 days after birth, with histology revealing disorganized axon growth and cell migration. After this early developmental phase, DLK is subsequently regulated negatively and strictly by Pam/Highwire/RPM-1 (PHR) E3 ubiquitin ligases (Feoktistov & Herman, 2016). While DLK is typically maintained at low levels in the adult CNS, in adult neurons it seems to be strongly connected to injury or cell stressor signaling. In worms, DLK protein levels elevate in response

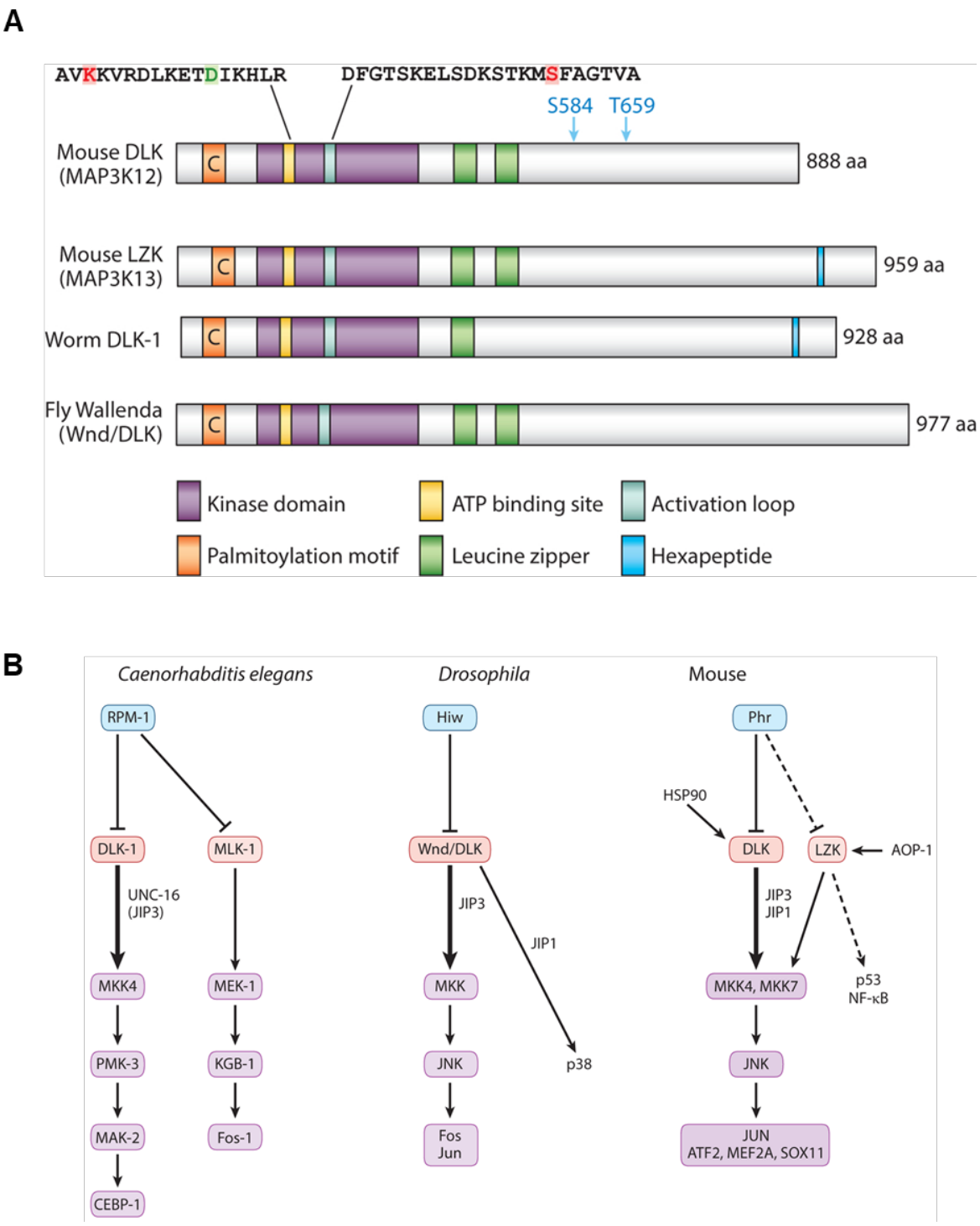

Figure 1.5 Worm, fly and mouse DLK signaling.
Worm *DLK-1* is evolutionarily conserved across worm, fly and mouse. In mammals, two homologs DLK and LZK share >95% homology in kinase domain, and diverge in C-termini. LZK possesses a calcium-sensitive hexapeptide sequence that is shared with worm *DLK-1*, but this sequence is not seen in mammalian DLK. In adult neurons, DLK is maintained at low levels by ubiquitination and degradation via PHR ubiquitin ligases (Figures from Jin & Zheng, 2019).

cellular stressors that alter cytoskeleton structure or dynamics, such as treatment with colchicine, can ramp up DLK expression in neurons (Bounoutas et al., 2009). Additionally, DLK phosphorylation by protein kinase A in response to axon injury also robustly elevates DLK activity (Hao et al., 2016). The act of DLK phosphorylation appears to stabilize the protein, making it resilient to PHR-mediated ubiquitination and degradation. Particularly, c-Jun N-terminal Kinase (JNK)-dependent phosphorylation of DLK may act to amplify neuronal stress signals, and at least in the model of optic nerve crush, this elevation of phosphorylated DLK begins first in the axon and then spreads retrogradely, increasing in the cell bodies, ultimately leading to cell apoptosis (Huntwork-Rodriguez et al., 2013).

With regards to axonal regeneration specifically, DLK has been studied more widely, particularly using models of optic nerve crush for the CNS and sciatic nerve crush/ligation models for PNS. In the sciatic nerve, DLK has been shown to be necessary for retrograde transportation of injury signal phosphorylated STAT3 (pSTAT3) to the cell body. Compared to WT mice, DLK KO mice show reduced regeneration in sciatic nerve after crush (Shin et al., 2012). In the optic nerve crush – a model for CNS injury – DLK KO results increased survival of RGCs. This effect which is enhanced further by double deletion of DLK and LZK in RGCs, although LZK deletion alone does not lend a similar effect (see Figure 2.1, Watkins et al., 2013; Welsbie et al., 2013, 2017). Thus, we see two seemingly opposite results from DLK deletion depending on cell type: peripheral neurons' regeneration is DLK dependent, while RGC survivability is enhanced by DLK deletion. An explanation for these seemingly opposite findings may be due to the cell's intrinsic growth capacity as a response to injury and DLK activation: subsequent to optic nerve crush, DLK activation drives the initiation of several transcriptional programs, including both regenerative and apoptotic processes (Watkins et al., 2013). As RGCs have limited regenerative capacity, secondary to their nature as CNS neurons, these cells typically respond to injury with cell death; oppositely, injured PNS neurons in the sciatic nerve innately are capable of regenerating in response to injury. These findings thus suggest that DLK activation is critical for allowing the cell

to respond to an injury, and whether that response pro-apoptotic or pro-regenerative is both cell-type specific and context dependent (Watkins et al., 2013). The neuronal role of LZK is less well understood; studies in vitro have shown that overexpressing LZK in cerebellar granule neuron cultures enhances neurite outgrowth, similar to DLK overexpression, but co-overexpression does not confer additional benefits to neurite outgrowth (Chen et al., 2016).

Among these, worm *DLK-1* was recently identified as being able to respond to fluctuations in calcium and cytoskeletal dynamics and thus can retrogradely signal via the MAP3K cascade to induce a "response-to-injury" transcriptional program (Ghosh-Roy et al., 2012; He & Jin, 2016; Yan & Jin, 2012). In the sciatic nerve, double ligation studies have revealed the role of DLK in triggering retrograde signaling, including retrograde trafficking of pSTAT3 injury signals, to drive regeneration in the peripheral nervous system (Shin et al., 2012). In fact, knocking out DLK in RGC results in a loss of post injury transcriptional activity driving both pro-growth and pro-apoptotic profiles (Watkins et al., 2013), suggesting that loss of DLK deprives the soma of being able to even recognize the presence of an injury, let alone mount a response. In the optic crush model, DLK deletion results in overall increased cell survival, indicating that while DLK may drive both cell-growth and apoptosis pathways, in this particular model, cell death prevails as the primary response to injury, and that this may be mediated by several MAP3K downstream effectors (Welsbie et al., 2017). A recent publication suggests that in a model of stroke, knockdown of C-C chemokine receptor 5 (CCR5) enhances motor recovery after traumatic brain injury via upregulated DLK signaling that drives increased plasticity in surviving axons (Joy et al., 2019), although this study does not directly draw a connection between DLK signaling and axonal repair.

The role of LZK in injury signaling is considerably less well understood. *In vitro*, the overexpression of LZK in cerebellar granule neuron cultures enhances neurite outgrowth, similar to DLK overexpression, but co-overexpression does not confer additional benefits to neurite outgrowth (Chen et al., 2016) In astrocytes, the overexpression of LZK alone was sufficient to

trigger widespread reactive astrogliosis in the spinal cord, even in the absence of injury. In the presence of spinal cord crush injury, the overexpression of astrocytic LZK resulted in a more compacted lesion core, but the effect of this manipulation on axon regeneration is unknown (Chen et al., 2018). In studies combining DLK and LZK manipulations, deletion of LZK failed to improve RGC survival after optic nerve crush injury, but DLK and LZK double deletion improved RGC cell survival greater than DLK deletion alone (Welsbie et al., 2017), suggesting that, in this model, DLK and LZK may signal cooperatively in response to injury, but the role of DLK is more critical than neuronal LZK. However, in a model of impact acceleration as a model of diffuse traumatic brain injury, only double deletion of both LZK and DLK protected RGCs apoptosis, although DLK disruption alone was sufficient to suppress JUN phosphorylation (Welsbie et al., 2019). Taken together, these findings slowly address the relationship between DLK and LZK in response to multiple models of trauma. Likely, these kinases have a redundant role, and the relative importance of LZK signaling in permitting a cell to mount a response to injury is dependent both on the cell type affected, as well as the type of trauma sustained by the cell.

1.7 Two models of CST injury: the Dorsal Hemisection and Unilateral Pyramidotomy

Given the wide variability of cellular responses to LZK and DLK manipulation, investigating the role of DLK and LZK in responding to CST injury requires the use of multiple models of injury to address the various types of cellular responses to injury. As described earlier, an injured neuron can attempt to mount a regenerative response, although the ability for a CNS neuron to regrow after injury is severely limited, or more commonly it can retract and form a dystrophic end bulb in response to injury. Alternately, the study of spontaneous and induced sprouting allows for probing on how DLK and LZK may be involved in plasticity in uninjured neurons. In order to distinguish all of these different types of cellular responses to injury, multiple models of injury can be leveraged to specifically target a particular cellular response.

For the study of regeneration both rostral and caudal to the injury, the model of dorsal hemisection (DH) spinal cord injury is a well established surgical model to study the injury response of CST axons. The DH model involves lesioning the dorsal half of the spinal cord, e.g., at the level of thoracic spinal cord vertebral segment 8 (T8), completely severing both descending dorsal corticospinal tracts, as well as the descending dorsolateral tract (a minor tract). In this model, virtually all traced regenerating axons suffered axonal injury. To confirm complete lesioning, traced axons are stained in transverse sections of spinal cord collected from well above and below the site of injury. In the caudal sections specifically, a completely lesioned animal should have no signal in the main CST bilaterally nor any signal in the minor dorsolateral tracts (Figure 1.6A). The complete lesioning of both CST tracts limits the possibility of axonal sprouting by spared descending tracts to enable functional recovery. Due to general lack of axonal regeneration observed in wildtype mice in the DH model, we can leverage genetic methods of PTEN deletion to provide an elevated baseline level of regeneration in neurons in order to assess the requirement for DLK/LZK in regeneration. To assess regeneration caudal to the site of injury, we can use GFAP to identify the scar border, and assess whether traced CST axons are able to grow beyond the site of lesion into the caudal spinal cord.

These same sections can also be used to study axonal regeneration rostral to the site of injury following by measuring the decrease in axon density signal in traced axons starting from 1.5 mm anterior to the site of injury. In the subacute phase of injury lasting roughly 7-10 days after initial injury, the vast majority of CST axons exhibit dieback as far as 500-600 μm rostral to the site of injury. Regeneration of these retracted axons can be achieved quite effectively by genetic deletion of PTEN in layer V CSMNs (Liu et al., 2010). Thus, we can also investigate whether deletion of DLK and/or LZK is involved in this rostral side regeneration.

To specifically study both spontaneous and induced sprouting, there are several different types of surgeries that lead to a partial injury of the CST. Among these methods, unilateral

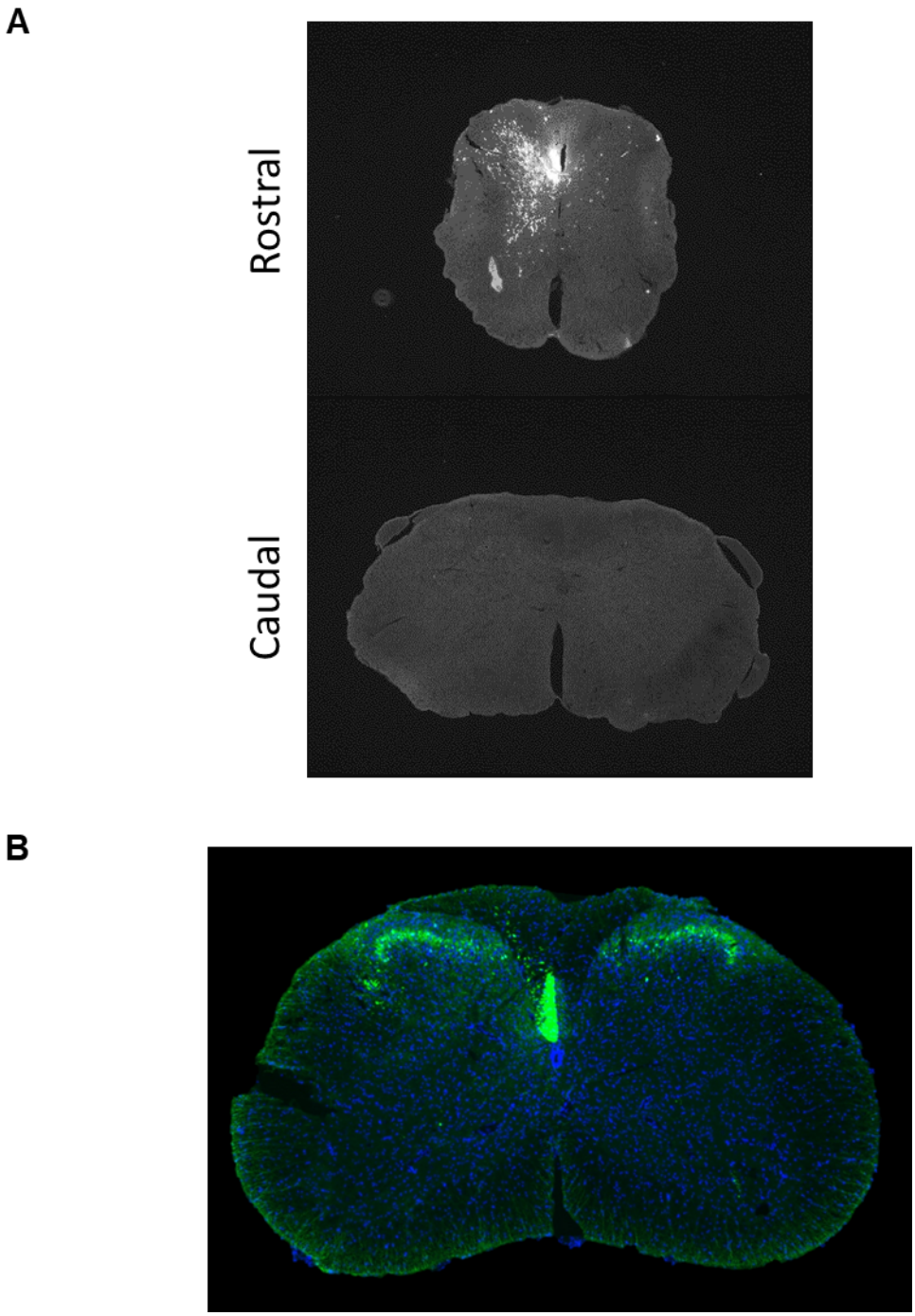

Figure 1.6 Assessing completeness of injury.
A) In DH, complete transection of both CSTs can be confirmed by observing no traced axons in caudal transverse spinal cord sections collected from >2mm away from the lesion site. B) For a pyramidotomy, staining for PKCγ is an effective way to visualize denervation, as PKCγ is produced in CSMN axons at a high rate of turnover, so loss of neural input into downstream axons will quickly deplete the PKCγ signal.

pyramidotomy is a particularly clean form of injury that effectively specifically assess CST. In the unilateral pyramidotomy injury model, the medullary pyramids are exposed ventrally, and one of the two descending tracts is lesioned above the level of the medullary decussation. Thus, a left sided lesion leads to denervation of the right-sided spinal dorsal CST. The anatomical markers of these two descending tracts is easily distinguished by the midline presence of the basilar artery to separate the two tracts (Starkey et al., 2005). Compared to the spinal cord itself, the medullary pyramids is a surgically easier target, and cleanly limiting the injury to just one descending path is significantly less challenging.

Assessing the completeness of injury is critical in the unilateral pyramidotomy, as incomplete lesions can reduce the observed amount of sprouting due to plasticity in the spared axons on the lesioned half of the cord. To confirm complete lesion, cervical spinal cord transverse sections are stained for Protein Kinase C gamma (PKCγ), a protein expressed highly in healthy living CSMN axons. In a properly lesioned animal, we expect to see PKCγ signal only in one of the two CSTs (Figure 1.6B). Sprouting is assessed by quantifying both the number of midline crossing axons, as well as the distances past midline that these axons sprout. While sprouting does occur at a detectable baseline level in wild type mice, this phenomenon can be further induced via genetic deletion of PTEN (Geoffroy et al., 2015).

1.8 Conclusion

The diversity of cellular responses that result from MAP3K signaling make DLK and LZK interesting molecular targets of study in the context of CNS injury and repair. Understanding the fundamental mechanisms of repair is a key step in developing new treatment strategies for improving outcomes for patients with SCIs. A major hurdle in the field of regenerative medicine is understanding the wide array of signaling cascades that play a crucial role in allowing cells to sprout or regenerate.

The goals of this study are to assess the effects of targeting neuronal deletion of DLK and/or LZK in two distinct mechanisms of axonal repair in the spinal cord, sprouting and regeneration, and address how these manipulations interact with PTEN deletion, another well-known signaling pathway that has already been studied extensively in the field of spinal cord injury and repair (Figure 1.7).

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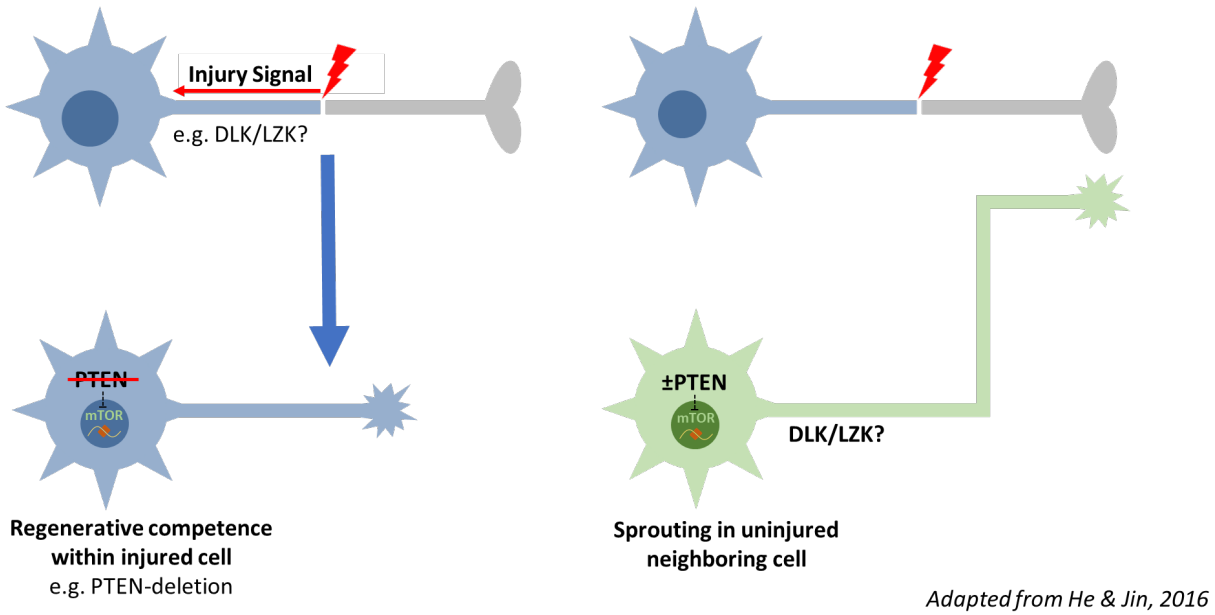

Figure 1.7 Graphical abstract of experiment.
Fundamentally, this series of experiments seeks to interrogate the effect of deleting LZK and DLK in multiple models of CST axonal repair. For regeneration, we will use dorsal hemisection to bilaterally lesion the descending CST. The additional manipulation of PTEN deletion will be leveraged to increase inherent regenerative capacity of these injured axons. in the sprouting model, the unilateral pyramidotomy will target only one of the two descending tracts, and we will investigate both spontaneous and induced sprouting in the uninjured descending tract. As with regeneration, the deletion of PTEN in uninjured axons induces elevated levels of sprouting compared to wildtype, and can help magnify differences in phenotype that may be more difficult to ascertain using just baseline levels of sprouting.

References

Angeli, C. A., Edgerton, V. R., Gerasimenko, Y. P., & Harkema, S. J. (2014). Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. Brain, 137(5), 1394–1409. https://doi.org/10.1093/brain/awu038

Belin, S., Nawabi, H., Wang, C., Tang, S., Latremoliere, A., Warren, P., Schorle, H., Uncu, C., Woolf, C. J., He, Z., & Steen, J. A. (2015). Injury-Induced Decline of Intrinsic Regenerative Ability Revealed by Quantitative Proteomics. Neuron, 86(4), 1000–1014. https://doi.org/10.1016/j.neuron.2015.03.060

Ben-Yaakov, K., Dagan, S. Y., Segal-Ruder, Y., Shalem, O., Vuppalanchi, D., Willis, D. E., Yudin, D., Rishal, I., Rother, F., Bader, M., Blesch, A., Pilpel, Y., Twiss, J. L., & Fainzilber, M. (2012). Axonal transcription factors signal retrogradely in lesioned peripheral nerve. The EMBO Journal, 31(6), 1350–1363. https://doi.org/10.1038/emboj.2011.494

Bounoutas, A., O'Hagan, R., & Chalfie, M. (2009). The Multipurpose 15-Protofilament Microtubules in C. elegans Have Specific Roles in Mechanosensation. Current Biology, 19(16), 1362–1367. https://doi.org/10.1016/j.cub.2009.06.036

Bradbury, E. J., Moon, L. D. F., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., & McMahon, S. B. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature, 416(6881), 636–640. https://doi.org/10.1038/416636a

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2017). Molecular, cellular and functional events in axonal sprouting after stroke. In Experimental Neurology (Vol. 287, Issue Pt 3, pp. 384–394). Academic Press Inc. https://doi.org/10.1016/j.expneurol.2016.02.007

Chen, M., Geoffroy, C. G., Meves, J. M., Narang, A., Li, Y., Nguyen, M. T., Khai, V. S., Kong, X., Steinke, C. L., Carolino, K. I., Elzière, L., Goldberg, M. P., Jin, Y., & Zheng, B. (2018). Leucine Zipper-Bearing Kinase Is a Critical Regulator of Astrocyte Reactivity in the Adult Mammalian CNS. Cell Reports, 22(13), 3587–3597. https://doi.org/10.1016/j.celrep.2018.02.102

Chen, M., Geoffroy, C. G., Wong, H. N., Tress, O., Nguyen, M. T., Holzman, L. B., Jin, Y., & Zheng, B. (2016). Leucine Zipper-bearing Kinase promotes axon growth in mammalian central nervous system neurons. Scientific Reports, 6. https://doi.org/10.1038/srep31482

Christie, K. J., Webber, C. A., Martinez, J. A., Singh, B., & Zochodne, D. W. (2010). PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. Journal of Neuroscience, 30(27), 9306–9315. https://doi.org/10.1523/JNEUROSCI.6271-09.2010

Cregg, J. M., DePaul, M. A., Filous, A. R., Lang, B. T., Tran, A., & Silver, J. (2014). Functional regeneration beyond the glial scar. In Experimental Neurology (Vol. 253, pp. 197–207). NIH Public Access. https://doi.org/10.1016/j.expneurol.2013.12.024

Feoktistov, A. I., & Herman, T. G. (2016). Wallenda/DLK protein levels are temporally downregulated by Tramtrack69 to allow R7 growth cones to become stationary boutons. Development (Cambridge), 143(16), 2983–2993. https://doi.org/10.1242/dev.134403

Ferguson, T. A., & Son, Y. J. (2011). Extrinsic and intrinsic determinants of nerve regeneration. In Journal of Tissue Engineering (Vol. 2, Issue 1, pp. 1–12). J Tissue Eng. https://doi.org/10.1177/2041731411418392

Fouad, K., Pedersen, V., Schwab, M. E., & Brösamle, C. (2001). Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Current Biology, 11(22), 1766–1770. https://doi.org/10.1016/S0960-9822(01)00535-8

Geoffroy, C. G., Lorenzana, A. O., Kwan, J. P., Lin, K., Ghassemi, O., Ma, A., Xu, N., Creger, D., Liu, K., He, Z., & Zheng, B. (2015). Effects of PTEN and Nogo codeletion on corticospinal axon sprouting and regeneration in mice. Journal of Neuroscience, 35(16), 6413–6428. https://doi.org/10.1523/JNEUROSCI.4013-14.2015

Ghosh-Roy, A., Goncharov, A., Jin, Y., & Chisholm, A. D. (2012). Kinesin-13 and Tubulin Posttranslational Modifications Regulate Microtubule Growth in Axon Regeneration. Developmental Cell, 23(4), 716–728. https://doi.org/10.1016/j.devcel.2012.08.010

GrandPré, T., Nakamura, F., Vartanlan, T., & Strittmatter, S. M. (2000). Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature, 403(6768), 439–444. https://doi.org/10.1038/35000226

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E. M., & Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science, 323(5915), 802–806. https://doi.org/10.1126/science.1165527

Hao, Y., Frey, E., Yoon, C., Wong, H., Nestorovski, D., Holzman, L. B., Giger, R. J., DiAntonio, A., & Collins, C. (2016). An evolutionarily conserved mechanism for cAMP elicited axonal regeneration involves direct activation of the dual leucine zipper kinase DLK. ELife, 5(JUN2016). https://doi.org/10.7554/eLife.14048

He, Z., & Jin, Y. (2016). Intrinsic Control of Axon Regeneration. Neuron, 90(3), 437–451. https://doi.org/10.1016/j.neuron.2016.04.022

Hirai, S. I., De, F. C., Miyata, T., Ogawa, M., Kiyonari, H., Suda, Y., Aizawa, S., Banba, Y., & Ohno, S. (2006). The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. Journal of Neuroscience, 26(46), 11992–12002. https://doi.org/10.1523/JNEUROSCI.2272-06.2006

Huntwork-Rodriguez, S., Wang, B., Watkins, T., Ghosh, A. S., Pozniak, C. D., Bustos, D., Newton, K., Kirkpatrick, D. S., & Lewcock, J. W. (2013). JNK-mediated phosphorylation of DLK suppresses its ubiquitination to promote neuronal apoptosis. Journal of Cell Biology, 202(5), 747–763. https://doi.org/10.1083/jcb.201303066

Jin, Y., & Zheng, B. (2019). Annual Review of Cell and Developmental Biology Multitasking: Dual Leucine Zipper-Bearing Kinases in Neuronal Development and Stress Management. https://doi.org/10.1146/annurev-cellbio-100617

Joosten, E. A. J., Schuitman, R. L., Vermelis, M. E. J., & Dederen, P. J. W. C. (1992). Postnatal development of the ipsilateral corticospinal component in rat spinal cord: A light and electron microscopic anterograde HRP study. Journal of Comparative Neurology, 326(1), 133–146. https://doi.org/10.1002/cne.903260112

Joy, M. T., Assayag, B., Shabashov-, D., Silva, A. J., Shohami, E., Thomas, S., & Correspondence, C. (2019). CCR5 Is a Therapeutic Target for Recovery after Stroke and Traumatic Brain Injury In Brief Genetic and small molecule-based perturbation of CCR5 promotes functional recovery from stroke and traumatic brain injury. Cell, 176, 1143-1157.e13. https://doi.org/10.1016/j.cell.2019.01.044

Kerschensteiner, M., Schwab, M. E., Lichtman, J. W., & Misgeld, T. (2005). In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nature Medicine, 11(5), 572–577. https://doi.org/10.1038/nm1229

Khazaeipour, Z., Rezaei-Motlagh, F., Ahmadipour, E., Azarnia-Ghavam, M., Mirzababaei, A., Salimi, N., & Salehi-Nejad, A. (2017). Burden of care in primary caregivers of individuals with spinal cord injury in Iran: Its association with sociodemographic factors. Spinal Cord, 55(6), 595–600. https://doi.org/10.1038/sc.2016.195

Kottis, V., Thibault, P., Mikol, D., Xiao, Z. C., Zhang, R., Dergham, P., & Braun, P. E. (2002). Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. Journal of Neurochemistry, 82(6), 1566–1569. https://doi.org/10.1046/j.1471-4159.2002.01146.x

Lewandowski, G., & Steward, O. (2014). AAVshRNA-mediated suppression of PTEN in adult rats in combination with salmon fibrin administration enables regenerative growth of corticospinal axons and enhances recovery of voluntary motor function after cervical spinal cord injury. Journal of Neuroscience, 34(30), 9951–9962. https://doi.org/10.1523/JNEUROSCI.1996-14.2014

Li, Y., He, X., Kawaguchi, R., Zhang, Y., Wang, Q., Monavarfeshani, A., Yang, Z., Chen, B., Shi, Z., Meng, H., Zhou, S., Zhu, J., Jacobi, A., Swarup, V., Popovich, P. G., Geschwind, D. H., & He, Z. (2020). Microglia-organized scar-free spinal cord repair in neonatal mice. Nature, 587(7835). https://doi.org/10.1038/s41586-020-2795-6

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nature Neuroscience, 13(9), 1075–1081. https://doi.org/10.1038/nn.2603

Lu, Y., Belin, S., & He, Z. (2014). Signaling regulations of neuronal regenerative ability. In Current Opinion in Neurobiology (Vol. 27, pp. 135–142). Elsevier Ltd. https://doi.org/10.1016/j.conb.2014.03.007

Martin, J. H., Choy, M., Pullman, S., & Meng, Z. (2004). Corticospinal System Development Depends on Motor Experience. Journal of Neuroscience, 24(9), 2122–2132. https://doi.org/10.1523/JNEUROSCI.4616-03.2004

Meves, J. M., Geoffroy, C. G., Kim, N. D., Kim, J. J., & Zheng, B. (2018). Oligodendrocytic but not neuronal Nogo restricts corticospinal axon sprouting after CNS injury. Experimental Neurology, 309, 32–43. https://doi.org/10.1016/j.expneurol.2018.07.013

Miller, B. R., Press, C., Daniels, R. W., Sasaki, Y., Milbrandt, J., & Diantonio, A. (2009). A DLK-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci, 12(4), 387–389. https://doi.org/10.1038/nn.2290

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R., & Filbin, M. T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron, 13(3), 757–767. https://doi.org/10.1016/0896-6273(94)90042-6

Park, K. K., Liu, K., Hu, Y., Smith, P. D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., & He, Z. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science, 322(5903), 963–966. https://doi.org/10.1126/science.1161566

Qiu, J., Cafferty, W. B. J., McMahon, S. B., & Thompson, S. W. N. (2005). Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. Journal of Neuroscience, 25(7), 1645–1653. https://doi.org/10.1523/JNEUROSCI.3269-04.2005

Shin, J. E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., & DiAntonio, A. (2012). Dual Leucine Zipper Kinase Is Required for Retrograde Injury Signaling and Axonal Regeneration. Neuron, 74(6), 1015–1022. https://doi.org/10.1016/j.neuron.2012.04.028

Sofroniew, M. V. (2015). Astrocyte barriers to neurotoxic inflammation. In Nature Reviews Neuroscience (Vol. 16, Issue 5, pp. 249–263). Nature Publishing Group. https://doi.org/10.1038/nrn3898

Starkey, M. L., Barritt, A. W., Yip, P. K., Davies, M., Hamers, F. P. T., McMahon, S. B., & Bradbury, E. J. (2005). Assessing behavioural function following a pyramidotomy lesion of the corticospinal tract in adult mice. Experimental Neurology, 195(2), 524–539. https://doi.org/10.1016/j.expneurol.2005.06.017

Tedeschi, A., & Bradke, F. (2013). The DLK signalling pathway - A double-edged sword in neural development and regeneration. In EMBO Reports (Vol. 14, Issue 7, pp. 605–614). European Molecular Biology Organization. https://doi.org/10.1038/embor.2013.64

Tedeschi, A., & Bradke, F. (2017). Spatial and temporal arrangement of neuronal intrinsic and extrinsic mechanisms controlling axon regeneration. In Current Opinion in Neurobiology (Vol. 42, pp. 118–127). Elsevier Ltd. https://doi.org/10.1016/j.conb.2016.12.005

Tom, V. J., Steinmetz, M. P., Miller, J. H., Doller, C. M., & Silver, J. (2004). Development/Plasticity/Repair Studies on the Development and Behavior of the Dystrophic Growth Cone, the Hallmark of Regeneration Failure, in an In Vitro Model of the Glial Scar and after Spinal Cord Injury. https://doi.org/10.1523/JNEUROSCI.0994-04.2004

Ueno, M., Hayano, Y., Nakagawa, H., & Yamashita, T. (2012). Intraspinal rewiring of the corticospinal tract requires target-derived brain-derived neurotrophic factor and compensates lost function after brain injury. Brain, 135(4), 1253–1267. https://doi.org/10.1093/brain/aws053

Valakh, V., Walker, L. J., Skeath, J. B., & DiAntonio, A. (2013). Loss of the spectraplakin short stop activates the DLK injury response pathway in drosophila. Journal of Neuroscience, 33(45), 17863–17873. https://doi.org/10.1523/JNEUROSCI.2196-13.2013

Vulliemoz, S., Raineteau, O., & Jabaudon, D. (2005). Reaching beyond the midline: Why are human brains cross wired? In Lancet Neurology (Vol. 4, Issue 2, pp. 87–99). Lancet Neurol. https://doi.org/10.1016/S1474-4422(05)00990-7

Watkins, T. A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J. S., Tessier-Lavigne, M., & Lewcock, J. W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. Proceedings of the National Academy of Sciences of the United States of America, 110(10), 4039–4044. https://doi.org/10.1073/pnas.1211074110

Weidner, N., Ner, A., Salimi, N., & Tuszynski, M. H. (2001). Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proceedings of the National Academy of Sciences of the United States of America, 98(6), 3513–3518. https://doi.org/10.1073/pnas.051626798

Welniarz, Q., Dusart, I., Gallea, C., & Roze, E. (2015). One hand clapping: Lateralization of motor control. In Frontiers in Neuroanatomy (Vol. 9, Issue June). Frontiers Research Foundation. https://doi.org/10.3389/fnana.2015.00075

Welsbie, D. S., Mitchell, K. L., Jaskula-Ranga, V., Sluch, V. M., Yang, Z., Kim, J., Buehler, E., Patel, A., Martin, S. E., Zhang, P. W., Ge, Y., Duan, Y., Fuller, J., Kim, B. J., Hamed, E., Chamling, X., Lei, L., Fraser, I. D. C., Ronai, Z. A., … Zack, D. J. (2017). Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. Neuron, 94(6), 1142-1154.e6. https://doi.org/10.1016/j.neuron.2017.06.008

Welsbie, D. S., Ziogas, N. K., Xu, L., Kim, B. J., Ge, Y., Patel, A. K., Ryu, J., Lehar, M., Alexandris, A. S., Stewart, N., Zack, D. J., & Koliatsos, V. E. (2019). Targeted disruption of dual leucine zipper kinase and leucine zipper kinase promotes neuronal survival in a model of diffuse traumatic brain injury. Molecular Neurodegeneration, 14(1). https://doi.org/10.1186/s13024-019-0345-1

Yan, D., & Jin, Y. (2012). Regulation of DLK-1 Kinase Activity by Calcium-Mediated Dissociation from an Inhibitory Isoform. Neuron, 76(3), 534–548. https://doi.org/10.1016/j.neuron.2012.08.043

Yan, D., Wu, Z., Chisholm, A. D., & Jin, Y. (2009). The DLK-1 Kinase Promotes mRNA Stability and Local Translation in C. elegans Synapses and Axon Regeneration. Cell, 138(5), 1005–1018. https://doi.org/10.1016/j.cell.2009.06.023

Zukor, K., Belin, S., Wang, C., Keelan, N., Wang, X., & He, Z. (2013). Short hairpin RNA against PTEN enhances regenerative growth of corticospinal tract axons after spinal cord injury. Journal of Neuroscience, 33(39), 15350–15361. https://doi.org/10.1523/JNEUROSCI.2510-13.2013

Zuo, J., Neubauer, D., Graham, J., Krekoski, C. A., Ferguson, T. A., & Muir, D. (2002). Regeneration of axons after nerve transection repair is enhanced by degradation of chondroitin sulfate proteoglycan. Experimental Neurology, 176(1), 221–228. https://doi.org/10.1006/exnr.2002.7922

Chapter 2:

Methods and Materials

2.1 Experimental Animals

Conditional knockout (cKO) mice were generated in which exon 2 of the LZK or DLK gene is flanked by loxP sites. In these mice, exposure to Cre-recombinase would lead to a conditional knockout of the targeted gene. A previous graduate student tested the efficiency of gene deletion by crossing $DLK^{fl/fl}$ mice with a ubiquitously expressed tamoxifen inducible Cre-recombinase mouse line (UbCre) and administering tamoxifen, resulting in near complete suppression of DLK protein expression as assayed by Western blot. To test efficiency of LZK deletion in $LZK^{fl/fl}$ mice, neuronal cultures of $LZK^{fl/fl}$ mice were incubated either in AAV-Cre or a control virus. A PCR targeting the knockout version of the DNA revealed a positive band for cells incubated AAV-Cre, but not AAV-GFP (Figure 2.1). After confirming the efficacy of gene knockout with Cre-recombinase, $LZK^{fl/fl}$ and $DLK^{fl/fl}$ were bred to each other to also generate $DLK/LZK^{fl/fl}$ mice, as well as to $PTEN^{fl/fl}$ to generate $DLK/PTEN^{fl/fl}$, $LZK/PTEN^{fl/fl}$, and $DLK/LZK/PTEN^{fl/fl}$.

In experiments focused on regeneration and induced (e.g. non-spontaneous) sprouting, we leveraged the pro-growth affects of PTEN deletion to either permit neuronal regeneration (which does not occur in wildtype mice) or enhance baseline sprouting into an induced state. $DLK/PTEN^{fl/fl}$, $LZK/PTEN^{fl/fl}$ and $DLK/LZK/PTEN^{fl/fl}$ mice were injected with AAV2-Cag-Cre (AAV-Cre) to target neuronal deletion of DLK, LZK and PTEN genes. These mice were compared against $PTEN^{fl/fl}$ injected with AAV-Cre, and for all genotypes, injection with AAV2-Cag-GFP served as a general wildtype control. These control mice will be referred to as GFP controls in results and figures.

In the series of experiments focusing on spontaneous sprouting, conditional knockout of the LZK or DLK genes or both genes simultaneously were compared to littermate controls injected with AAV-GFP. Mice used in all studies were injected with AAV-Cre or GFP at 6-8 weeks of age to target the effects of gene manipulation in young adult mice, specifically, as the effects of PTEN deletion on neuronal regeneration distal to the injury is limited in older animals (Geoffroy et al., 2016).

2.2 Tissue processing

Mice were administered a lethal dose of Fatal Plus (pentobarbital sodium) and perfused transcardially with ice-cold 4% PFA solution; brain and spinal cord were dissected out and post fixed overnight in 4°C PFA solution. Subsequently, tissues were incubated for 72 hours at 4°C in 30% sucrose solution for cryoprotection before embedding in Tissue-TEK OCT Compound and freezing over dry ice.

For regeneration studies, brain, medulla, and 4 mm thoracic spinal cord (T-spine) segments containing DH injury midway, with additional 1 mm rostral/caudal thoracic segments, were collected an embedded in OCT compound and frozen on dry ice. Tissues were cut on a cryostat to a thickness of 20 μm. For regeneration studies, initially rostral and caudal T-spines sections were transversely sliced and stained for Biotinylated Dextran Amine (BDA); in order to assess completeness of injury, animals containing dorsolateral collateral tract labeling in caudal sections relative to rostral sections were excluded from further processing and analysis. Subsequently, medullas were sliced transversely in order to obtain estimates of total number of CST axons labeled to control for labeling efficiency (see below). Sagittal sections of the 4mm segment of thoracic spinal cord were carefully sliced and stained for both GFAP and BDA. Lastly, brains were cut coronally at 20 μm thickness and stained for pS6, p-c-Jun and p-JNK.

For sprouting studies, tissues collected consisted of brain, medulla, and cervical spinal cord ranging from C5-C7. C-spines were sliced in 20 μm transverse sections and immunostained using polyclonal rabbit anti-PKCγ (1:200 dilution, Santa Cruz Biotechnology, in 0.2% NGS and 0.4% PBS-Tx, see below for full protocols). Animals that showed bilateral PKCγ signal were excluded from the study. C-spines were further stained for BDA signal, and analyzed for midline crossing axons. As with regeneration studies, medullas were sliced transversely in 20 μm sections, and processed for BDA staining.

2.3 Immunohistochemistry and BDA staining

Immunohistochemical staining

Cervical spinal cord tissue was stained for PKCγ (1:200 dilution Rabbit anti-PKCγ, Santa Cruz Biologicals), while brain tissue from various experiments were stained for pS6 (200 Rabbit anti-p-c-Jun (Ser73), Cell Signaling), NeuN (1:1000 Guinea pig anti-Neun, Sigma Aldrich), p-c-Jun (1:100, Rabbit anti-p-c-Jun, Cell Signaling), and Ctip2 (1:150 Rat anti-Ctip2, Abcam). Transverse cervical spinal cord and medullas were generally stained free floating, while coronal brains were stained slide mounted. For slide mounted IHC, sections were mounted onto Superfrosted slides in PBS, and then baked onto the slides for a minimum of 30 minutes in a 37°C oven. Prior to the blocking step, mounted tissue was rinsed in PBS on an orbital shaker for 10 minutes to rehydrate the tissue.

For a typical stain, tissue were blocked in 5% Normal Goat Serum (NGS, Vector Labs) in Phosphate Buffered Saline with 0.4% Triton-X (PBS-Tx) for 1-2 hours and incubated overnight at 4°C in primary antibody solution made with 2% NGS in PBS-Tx, using the antibodies described above. The next day, sections were rinsed in PBS-Tx three times and then incubated in a light-protected box with the appropriate secondary. For free floating stains, secondaries were conducted at a 1:500 dilution; for slide-mounted IHC, secondaries were made at a 1:200 dilution. After secondary incubation, sections were rinsed and optionally incubated for five minutes in a 1:2000 dilution of DAPI reagent (Abcam), before drying completely and coverslipping using Fluoromount-G. For staining p-c-Jun, an additional antigen retrieval step using 20-minute incubation in 90ºC sodium citrate (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) followed by two 10 minute washes in PBS-Tx was conducted prior to the initial blocking step.

Traditional BDA staining

For thoracic and lumbar transverse sections in regeneration studies, and for cervical transverse sections in sprouting studies, as well as for all medullas, sections were stained free

floating for BDA using a standard two-day protocol. On the first day, slices were permeabilized in two 30-minute wash steps in Phosphate Buffered Solution with 0.1% Tween-20 (PBS-Tw). These slices were then incubated overnight at 4°C in Vectastain ABC Solution made at 1:1000 dilution of parts A and B (Vector Laboratories). The next day, the slices were washed in two 1 hour steps in 1x PBS, before being mounted onto Superfrost slides (Thermo Fischer). After tissue was sufficiently dried onto the slides, hydrophobic borders were drawn on the edges of the slide using PAP pen, and the BDA signal was amplified using the TSA Plus Cy3 system (10 minutes at room temperature at a 1:200 dilution in PBS, Akoya Bio, formerly PerkinElmer). Slices were subsequently rinsed in PBS, and then allowed to dry before coverslipping using Fluoromount-G.

Combined GFAP and BDA staining

For regeneration studies, combined GFAP and BDA staining of sagittal T-spines was conducted using slight modifications to standard techniques: free-floating sections were initially blocked for 2 hours in 5% Normal Goat Serum and PBS-TritonX (Tx) and then transferred to a primary antibody solution consisting of 2% NGS with 1:200 dilution of monoclonal rat anti-GFAP antibody (Life Sciences) in PBS-Tx for overnight incubation at 4°C. The next day, sections were washed in PBS-Tx and incubated in secondary solution consisting 2% NGS and 1:500 dilution of Alexa-fluor 647 Goat anti-rat antibody (Life Technologies). These sections then were washed in 0.1% PBS-Tween solution in two 30-minute washes, followed by an overnight incubation in Vectastain ABC solution (Vector Laboratories) at 4°C. The next day, sections were washed in two 1-hour steps in PBS, and then mounted onto Superfrost slides (Thermo Fischer). BDA was detected with TSA Plus Cy3 system (10 minutes at room temperature at a 1:200 dilution in PBS, Akoya Bio, formerly PerkinElmer). Sections were rinsed in PBS and incubated with 1:2000 dilution of DAPI before coverslipping with Fluoromount-G.

2.4 Viruses

AAV-Cre and AAV-GFP were acquired from Boston Children's Hospital Viral Core as described previously. Viral titer is confirmed using qPCR to be $0.5x10^{12}$ TU/ml. viruses were injected to the motor cortex via modified 10 µL Hamilton syringe with a fine glass capillary needle affixed at the tip of the syringe using two-part epoxy (details below).

2.5 Surgical procedures

AAV Injections

Injection of AAV2-Cre or AAV2-GFP were performed on 6-week old mice as previously described with minor modifications. Surgeons were blinded to genotype of mice during all days of surgery. Animals were anesthetized with Ketamine and Xylazine injected intraperitoneally (i.p. K/X) dosed at 80-100mg/kg and 10mg/kg, respectively. After anesthesia was sufficiently achieved, mice shaved at the site of surgery and head fixed to a stereotax (Kopf Instruments). The surgical area was sterilized using 70% alcohol and Provadine Iodine swabs. Skin above the skull was cut using a pair scissors or a number 10 surgical scalpel, and the skin was retracted to reveal bregma. A surgical window was drilled carefully to include the coordinates for three sites of injections.

A 10 µL Hamilton syringe with a fine glass capillary needle was loaded with 1.5-2 µL of either AAV-Cre or AAV-GFP and mounted onto the stereotax. Injection coordinates were adapted from previous studies (Geoffroy et al., 2015; Liu et al., 2010). The coordinates relative to bregma are as follows: for sprouting studies or unilateral pyramidotomy cohorts, 1.2mm lateral, 0.5mm anterior, 1.2mm lateral, 0.5mm posterior, 2.2 mm lateral, 0.0mm anterior; for regeneration or dorsal hemisection cohorts, 1.4mm lateral, 0.1mm posterior, 1.4mm lateral, 1.1mm posterior, and 1.0mm lateral, 0.6mm posterior. For each injection site, the needle was lowered to a depth of 0.9mm and then raised by 0.2mm for a final depth of 0.7mm, and the brain was allowed to recover from needle insertion for 2 minutes. Virus was then delivered at a rate of 0.1 µL/min for four

minutes, for a total volume of 0.4 µL per injection site, and then virus was given time to diffuse into the tissue with needle in place for another four minutes.

After the final injection, incision wounds were sealed using Reflex surgical wound 7mm clips. Mice were allowed to recover in their home cages heated over a water bath and were postoperatively injected subcutaneously with 0.5ml saline and buprenorphine dosed at 0.05-0.1 mg/kg for at least 5 days after surgery for pain management. Reflex clips were removed after 7 days.

Dorsal Hemisection

Dorsal hemisection (DH) surgeries were conducted as previously described (Geoffroy et al., 2015). DH surgeries at the level of T8 thoracic spine were performed by a surgeon blinded to both genotype and viral injection condition on mice aged 10 weeks (4 weeks after AAV injection). Mice were anesthetized with i.p. K/X. The spinal cord was exposed at the level of T8 by dorsal laminectomy, followed by bilateral dorsal column transection of roughly 700 µm using marked microfeather scalpel and microdissection scissors. Overlying muscles were sutured together, and the wound was stapled shut using Reflex wound closure systems.

Mice were allowed to recover on heated waterbath and were postoperatively injected subcutaneously with saline and buprenorphine for pain management. For at least the next 5 days up to the next 8 weeks, pain management was achieved using buprenorphine, and infections were treated with Baytril. In mice unable that failed to recover urinary control, manual bladder expression was conducted twice daily for as many days as necessary to prevent urinary tract infections

Unilateral Pyramidotomy

Pyramidotomy surgeries were conducted using previously described methods with slight modifications (Starkey et al., 2005). Surgeries are conducted two weeks after AAV injections in

8-week old mice. For pyramidotomies, mice were anesthetized with i.p. K/X and placed on the stage of a dissection microscope ventral side facing up. An incision is made to expose the esophagus and neighboring muscles of the neck. Blunt dissection is used to expose the medullary pyramids at the base of the skull. Using a pre-marked surgical microfeather scalpel, a 700μm deep incision is made transecting only the animal's left medullary pyramid anterior to the decussation site. Wounds are closed using skin glue (Vetbond, 3M), and mice are allowed to recover with heat support, and are postoperatively injected with saline for volume loss and buprenorphine for pain management.

BDA Tracing

BDA injections are conducted 2 weeks after pyramidotomy surgeries in 10-week old mice or 6 weeks after DH in 16-week old mice. Stock 10% BDA (10,000 MW, Lifetech) prepared in sterile PBS was injected to the same coordinates using the same injection protocols as described above for AAV injections. Wounds were closed with Reflex clips, and mice were treated post-operatively with saline and buprenorphine for pain management. Mice were maintained in their home cages for 2 weeks after BDA injection surgery before ultimately undergoing perfusions at 12 weeks for pyramidotomy cohorts and 18 weeks for DH cohorts.

2.6 Quantification of rostral regeneration analysis

Methods to quantify regeneration rostral to the site of injury were adapted from previously described methods (Liu et al., 2010). To determine regeneration index, sagittal thoracic spinal cord sections containing the labeled descending CST were stained for BDA and GFAP. These were imaged on an upright Zeiss Axio Imager M1 microscope using a 10x objective. All images were acquired as .zvi files and converted to .czi files on using Zeiss software Zen Blue for easier analysis on ImageJ (NIH). For assessment of rostral regeneration, cut site was identified using GFAP, and fifteen 100 μm boxes spanning the dorsal-ventral width of each section were drawn

rostral to the site of injury, covering a total distance of 1.5 mm. Axon density measures were calculated for each box by measuring the average intensity within each box, and subtracting the average intensity of background signal as measured in a 100 μm region with no BDA signal caudal to the site of injury. This value was then normalized to the axon density measure calculated 1.5 mm rostral to the injury site. Axon density indices ± standard error of the means (SEM) are plotted as a function of distance rostral to the site of injury.

2.7 Quantification of caudal regeneration index

Section images acquired for rostral regeneration analysis were used also for quantifying regenerating axons. Using an automated macro wherein a blind quantifier identifies the injury site via GFAP staining, 43 boxes each measuring 50 μm x 2000 μm were aligned to the injury site. The number of axons crossing at each 50 μm increment was counted manually in as many sections containing CST labeling (~5-7 sections per animal). Counts at each distance were averaged for each animal and normalized against total axon count measured in the medulla to obtain a regeneration index, which was plotted as a function of distance caudal from the injury site.

2.8 Quantification of sprouting index

To determine sprouting index, transverse cervical spinal cord sections were sliced and stained for BDA, and normalized to total axon counts measured in medullas. Images were analyzed using FIJI/ImageJ software. A custom FIJI/ImageJ macro was developed to automatically align boxes measuring 50 or 100 μm to a user-indicated midline. The first box measures 50μm in width, and remaining boxes measure 100μm extending up until 1100 μm away from the indicated midline. Axon cross-over events at each of these distances are manually quantified by a blinded individual. For each animal, five sections are quantified, and counts at

every indicated distance are averaged, and then normalized against the medulla total axon count. Axon sprouting index ± SEM is plotted as a function of distance away from the midline.

2.9 Measuring total axon counts in medulla

For total axon counts that are used to normalize regeneration and sprouting indices, 2 medulla sections were analyzed on ImageJ using the "Find Maxima" plugin, which calculates a number . To set a threshold, several thresholds were tested on one section from 5 animals' and were double checked using prior manual counting methods (Lee et al., 2010) to set the threshold for the remaining animals in the cohort. Separate thresholds were used for each separately stained batch of medulla tissue. Axon counts quantified in the medulla were used to normalize sprouting in the unilateral pyramidotomy model and regeneration in the dorsal hemisection model.

2.10 Quantification of pS6 staining in brain tissue

Coronal sections of brains collected from mice that underwent dorsal hemisection and stained for Ctip2 and pS6. For each genotype studied, 3 animals were analyzed, and 3-4 sections were collected from each animal throughout in the region of the injected areas for analysis. Quantifications were conducted in ImageJ by a blinded researcher. To quantify pS6 staining in the injected versus uninjected side, a composite score for intensity signal at the level of Layer 5 neurons was calculated for each hemisphere. To do this, Ctip2 staining was used to identify layer 5 neurons. The injected hemisphere was identified either via GFP signal in the control mice, or gross changes in tissue structure secondary to injection (e.g. altered tissue morphology, reduced DAPI signaling, needle tracks, etc.) in Cre-injected mice. 300x300 μm boxes were drawn over the layer 5 neurons in the Ctip2 channel on both hemispheres equidistant from the central fissure. Within these boxes, in the pS6 channel, background subtraction was used to enhance signal to noise.

Using the “Analyze Particles” plugin on ImageJ, a user-set threshold was established to remove non-real signal from the image. The resultant images were segmented into particles, and these particles’ size and average intensity were calculated for both hemispheres. In order to calculate a ratio of pS6 signaling in the injected versus uninjected hemispheres, the total area within each box above threshold was multiplied to the average intensity of the signal to provide a composite measure of pS6 signal for each hemisphere that reflected the average signal intensity throughout a given image. The final calculated Intensity Index is a ratio of the composite score of the injected hemisphere’s signal divided by the uninjected hemisphere’s signal.

2.11 Quantification of p-c-Jun staining in brain tissue

Coronal sections of brains collected from mice that underwent dorsal hemisection and stained for NeuN and pJNK or p-c-Jun. For each genotype studied, 2-3 animals were analyzed, and images taken at 20x for each of the 2-3 slices stained per animal were collected from each animal throughout in the region of the injected areas for analysis, as well as the equivalent area on the uninjected hemisphere. Total numbers of dense nuclear staining p-c-Jun were manually counted for each image measuring 447 μm by 335 μm. For each genotype, an average number of cells was calculated in both the injured and uninjured hemisphere. Data are presented as a means ± SEM.

2.12 Experimental design and statistical analysis

Axon density, sprouting and regeneration index data were analyzed using 2-Way Repeated Measures ANOVA with Bonferroni post-test corrections. Intensity analysis pS6 and p-c-Jun were analyzed with 1-way ANOVAs, as were total axon counts. For all statistical analyses, GraphPad Prism 6 was used with a $p < 0.05$ set as the threshold for statistical significance. These are denoted in graphs with asterisks, unless noted otherwise. All data are displayed as means

with error bars denoting ±SEM. Specific n values for each study are noted in figures. A mix of male and female mice were used in approximately a 1:1 ratio for all experiments.

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References

Geoffroy, C. G., Hilton, B. J., Tetzlaff, W., & Zheng, B. (2016). Evidence for an Age-Dependent Decline in Axon Regeneration in the Adult Mammalian Central Nervous System. *Cell Reports*, *15*(2), 238–246. https://doi.org/10.1016/j.celrep.2016.03.028

Geoffroy, C. G., Lorenzana, A. O., Kwan, J. P., Lin, K., Ghassemi, O., Ma, A., Xu, N., Creger, D., Liu, K., He, Z., & Zheng, B. (2015). Effects of PTEN and Nogo codeletion on corticospinal axon sprouting and regeneration in mice. *Journal of Neuroscience*, *35*(16), 6413–6428. https://doi.org/10.1523/JNEUROSCI.4013-14.2015

Lee, J. K., Geoffroy, C. G., Chan, A. F., Tolentino, K. E., Crawford, M. J., Leal, M. A., Kang, B., & Zheng, B. (2010). Assessing Spinal Axon Regeneration and Sprouting in Nogo-, MAG-, and OMgp-Deficient Mice. *Neuron*, *66*(5), 663–670. https://doi.org/10.1016/j.neuron.2010.05.002

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature Neuroscience*, *13*(9), 1075–1081. https://doi.org/10.1038/nn.2603

Starkey, M. L., Barritt, A. W., Yip, P. K., Davies, M., Hamers, F. P. T., McMahon, S. B., & Bradbury, E. J. (2005). Assessing behavioural function following a pyramidotomy lesion of the corticospinal tract in adult mice. *Experimental Neurology*, *195*(2), 524–539. https://doi.org/10.1016/j.expneurol.2005.06.017

Chapter 3:

Role of mammalian LZK and DLK in CST axon regeneration

3.1 Significance Statement

In both clinical and mammalian animal models, the ability for severed axons to regrow after trauma is largely non-existent in the adult central nervous system (CNS). Extensive studies have shown that both inhibitory extrinsic environment and limited intrinsic growth capacity contribute to this phenomenon (Ferguson & Son, 2011; Tedeschi & Bradke, 2017). This reduced capacity for CNS axons to undergo regenerative repair may result in long-term or permanent functional impairment following injury, such as what is seen in spinal cord injury patients. Genetic deletion of tumor suppressor gene PTEN has been shown to create a growth-permissive environment that may allow injured axons to regrow via activation of the AKT/mTOR pathway (Liu et al., 2010; Park et al., 2008). Recently, *DLK-1*, a Mitogen-Activated Protein Kinase Kinase Kinase (MAP3K) was identified in *C. elegans* to be critical for axon regeneration following laser axotomy (Hammarlund et al., 2009; Yan et al., 2009). In mammals, the role of *DLK-1* homologs Dual Leucine Zipper Kinase (DLK) and Leucine Zipper Kinase (LZK) in axon regeneration is poorly understood within the CNS. With regards to DLK, there have been several studies focused on these kinases in context of retinal ganglion cell (RGC) survival and regeneration following optic nerve crush (Watkins et al., 2013; Welsbie et al., 2017), as well as in axon regeneration in the peripheral sciatic nerve (Shin et al., 2012). LZK is even less well understood; studies in primary cerebellar granule cell cultures suggests that LZK overexpression enhance neurite outgrowth, but whether this will translate to *in* vivo models is unknown (Chen et al., 2016). Although there appears to be some cooperativity between DLK and LZK in triggering the cell death pathway in various models of RGC injury (Welsbie et al., 2019), these models fundamentally do not address CNS regeneration, and thus the role of these kinases in regeneration in a motor CNS tract such as the CST is unknown.

In this series of experiments, I investigated the roles of DLK and LZK in axon regeneration rostral and caudal to the injury using the dorsal hemisection surgical model of spinal cord injury to lesion both descending dorsal CST and dorsolateral CST, bilaterally. To further enhance

baseline regenerative capacity of these CST axons to undergo regeneration, I leveraged PTEN deletion in combination of either single or double kinase knockout (Geoffroy et al., 2015; He & Jin, 2016).

To our surprise, tracing of regenerating axons caudal to the injury site revealed that while deleting both LZK and DLK in a PTEN deleted background blocks regeneration completely, deletion of just LZK in a PTEN deleted background does not block the effect of PTEN deletion on axon regeneration. Rostral to the lesion, all genotypes exhibited strong BDA labeling 1.5mm anterior to the lesion, but this labeling began to taper off at around 500 μm anterior to the lesion site in both wildtype and DLK/LZK/PTEN^{cKO} mice, with very few axons reaching the GFAP+ scar border. Contrastingly, LZK/PTEN^{cKO} and PTEN^{cKO} mice, while indeed showing a decreased axon density proximal to the scar border, still had a substantial number of axons penetrating into the fibrotic core of the lesion. Another key finding in understanding where DLK and LZK play their role relative to PTEN signaling is that double deletion of these kinases do not prevent the PTEN-deleted neurons from upregulating the expression of pS6, a downstream marker of AKT/mTOR activity, and a crucial player in axon regeneration (Liu et al., 2010; Park et al., 2008). In the present study, it remains unclear whether DLK deletion in isolation is sufficient to block the regenerative effect of PTEN deletion, but as of present findings, it appears as though neuronal LZK is not required for PTEN-deletion driven axon regeneration either rostral or caudal to the injury, suggesting that DLK may play a more critical role of the two kinases, which allows PTEN-deletion to permit axonal regeneration.

3.2 Experimental outline

For a more detailed explanation of methods and materials, please refer to Chapter 2. In summary, the animals studied in this series of experiments compared LZK/PTEN^{cKO} (n = 9) and DLK/LZK/PTEN^{cKO} (n = 17) mice against PTEN^{cKO} (n = 10). For each genetic condition, some

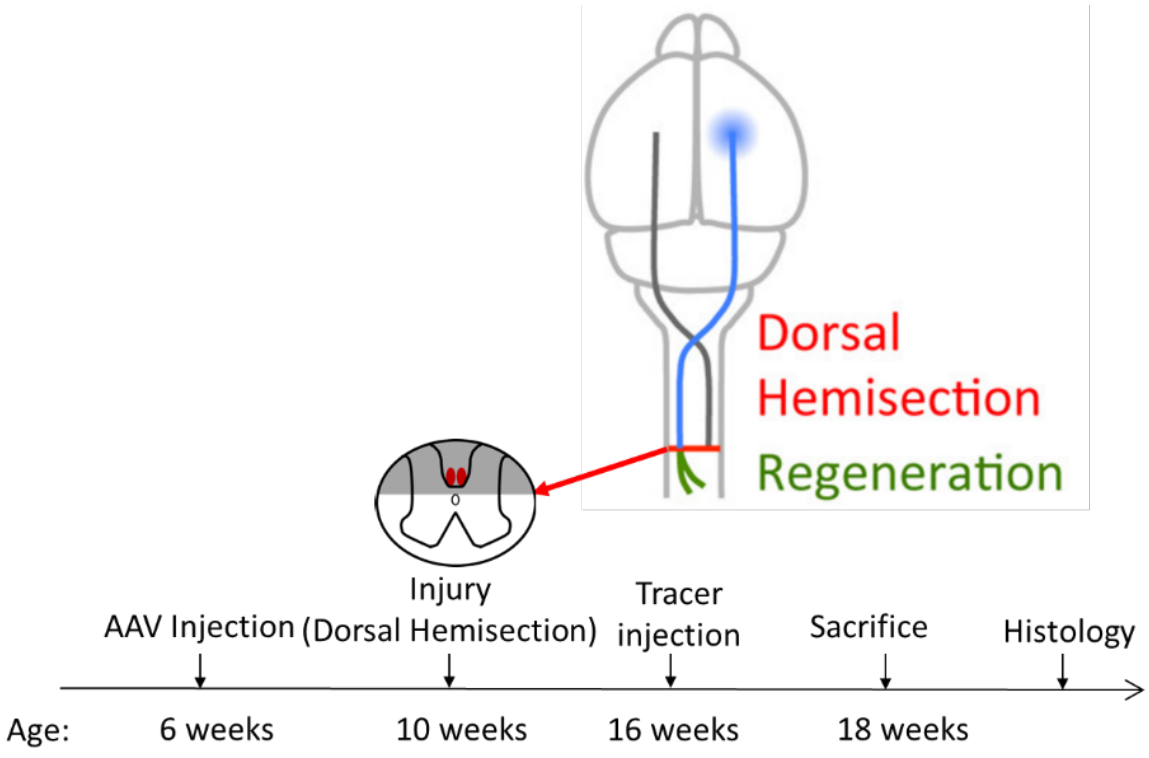


Figure 3.1 Regeneration of CST axons after dorsal hemisection.
Schematic of surgical timeline. Injections of AAV were conducted in 6 week old mice, and injuries were conducted in mice 10 weeks of age. Regenerating axons were traced at 16 weeks, and mice were sacrificed at 18 weeks of age. For full methods and procedures, refer to Chapter 2 (adapted from Geoffroy et al., 2015).

mouse littermates were used as controls, receiving AAV2-GFP in place of AAV2-Cre, and are referred to as wildtype (n = 12) mice. Mice injected with AAV2-Cre will be henceforth distinguished from controls by the notation “cKO.” 6-week old floxed mice of the specific genetic conditions were either injected with AAV-Cre or control AAV-GFP in the layer V region of the right-sided motor cortex targeting hindlimb regions. These axons innervate the left-sided dCST in the spinal cord. Four weeks after injections, the mice underwent the DH spinal cord surgery, which results in a bilateral transection of both descending dorsal main CST and the dorsolateral minor CST. After 6 weeks, regenerating axons were traced by injecting anterograde BDA tracer in the right side motor cortex. The mice were sacrificed two weeks later for processing and analysis (Figure 3.1).

Sagittal thoracic spinal cord tissue was collected and stained for BDA and GFAP for quantifying regeneration both rostral and caudal to the lesion. To quantify rostral regeneration, axon density indices were generated by measuring the average dorsal-ventral intensity in 100 μm intervals anterior to the lesion site, and normalized to average intensity measured in a 100 μm box 1.5mm anterior to the injury site. The rostral regeneration measured is reported as an Axon Density Index at each distance rostral to injury. For caudal regeneration, the number of regenerating axons caudal to injury were counted manually in 50 μm intervals. These were then normalized to total number of axons traced in the medulla (for full methods see Chapter 2). The caudal regeneration measured is reported as a Regeneration Index at each distance caudal to injury.

Brain tissue was stained for pS6 and p-c-Jun for assessment of AKT/mTOR and MAP3K pathway activity, respectively. To quantify relative changes in pS6 signaling in the AAV-injected hemisphere, a composite score for intensity signal in Layer V neurons for both hemispheres was calculated by measuring the average intensity of pS6 signal measured over threshold multiplied by the total area in which signal was measured as being greater than threshold. The relative change in signal was calculated by dividing the composite index in the injected hemisphere by

the composite index for the uninjected hemisphere. The resulting measurement is reported as an Intensity Index.

3.3 Results

3.3.1 LZK is dispensable for axonal regeneration rostral to injury in PTEN-deleted mice.

In the early response to axonal injury, CST axons undergo axonal dieback, characterized by the formation of dystrophic endbulbs. This process occurs within the first seven to ten days after injury. Both wild type and PTEN mice are known to have similar levels of axonal dieback, but only PTEN-deleted axons rostral to the lesion are able to overcome this initial period of dieback and instead regenerate towards the lesion (Liu et al., 2010, see Fig. 3.2). To test whether LZK or DLK is critical for regeneration of axons rostral to injury in PTEN-deleted mice, I compared axonal density rostral to a dorsal hemisection lesion in LZK/PTEN^{cKO} and DLK/LZK/PTEN^{cKO} mice, and compared these mice to PTEN^{cKO} and GFP-injected wildtype mice.

As previously described in the literature, the majority of GFP-injected wildtype animals exhibited the dense BDA labeling in the main CST tract roughly 1 millimeter anterior to the lesion. Beginning around 500 μm anterior to the lesion, this dense BDA labeling tapered off drastically, and a few individual axons were visualized to be terminating in retraction bulbs several hundred micrometers anterior to the proximal edge of the GFAP+ scar border. In PTEN^{cKO} animals, the main CST was also densely labeled with BDA, with a far slighter drop in axon density starting around 500 μm rostral to injury. The majority of labeled axons appeared to reach the proximal edge of the GFAP+ scar border favoring the dorsal side of the spinal cord, penetrating into the lesion core. A few individual axons did appear to trace towards the ventral edge of the lesion. DLK/LZK/PTEN^{cKO} showed axon densities that were comparable to wildtype GFP-injected mice, while LZK/PTEN^{cKO}-mice appeared phenotypically similar to PTEN^{cKO}mice (See Figure 3.2 A,B). To quantify rostral regeneration, 2-way Repeated Measures ANOVA revealed an interaction

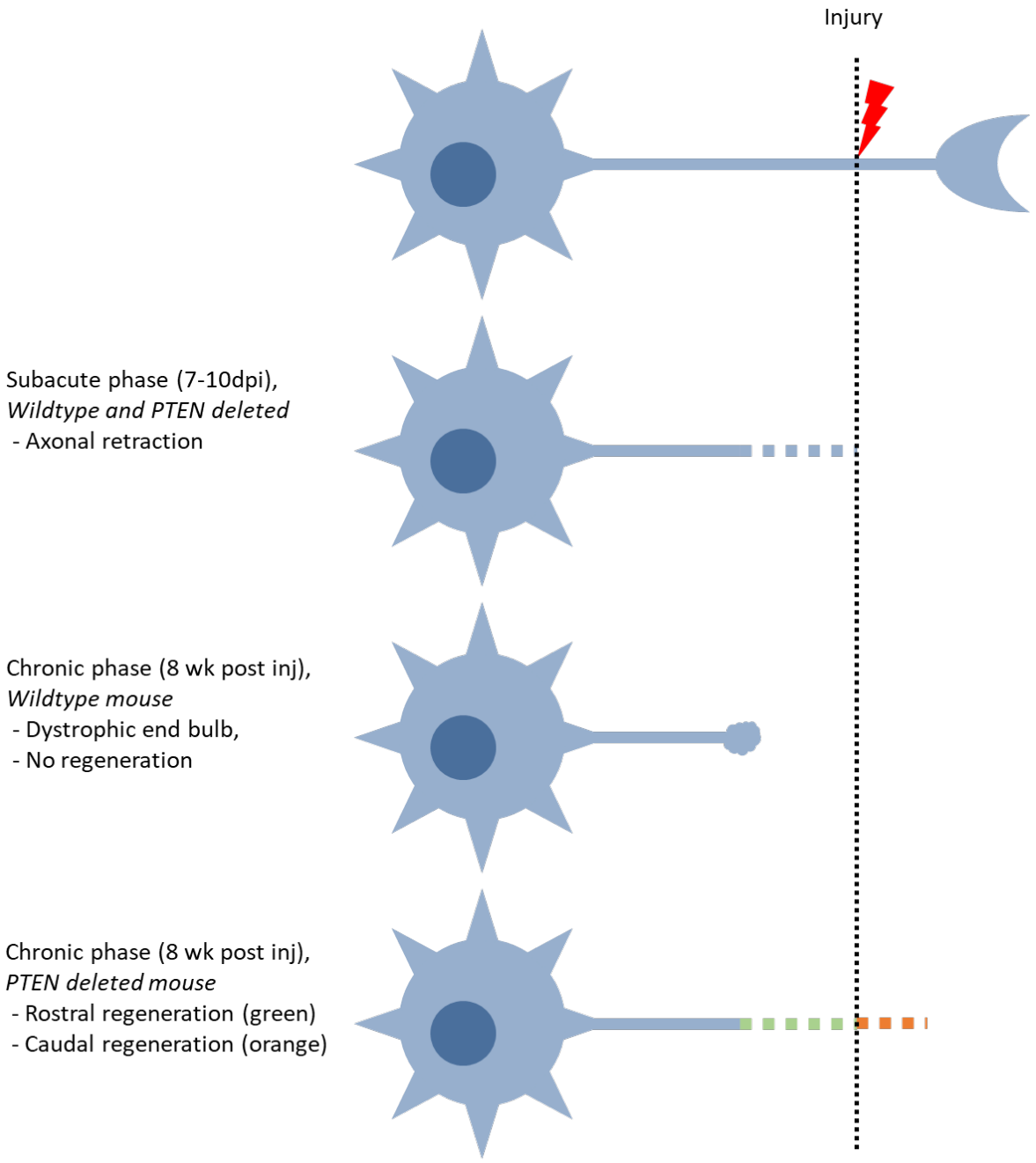


Figure 3.2 Rostral versus caudal regeneration.
In the initial 7-10 days after axonal injury, in both wildtype and PTEN-deleted animals, CST neurons exhibit axonal dieback or retraction away from the lesion site. In PTEN-deleted mice, however, it was shown that axons can overcome this initial period of dieback and regenerate towards the lesion site, and beyond into the caudal tissue (Liu et al., 2010).

between genetic condition and distance caudal to the injury ($p < 0.0001$, $F(42, 616) = 5.16$). Multiple comparisons with Bonferroni's correction revealed that $PTEN^{cKO}$ and $LZK/PTEN^{cKO}$ mice showed significant differences in axon density indices compared to GFP injected controls at distances500-100 μm rostral to the site of injury (at 500 μm, $PTEN^{cKO}$ $p = 0.0014$, $LZK/PTEN^{cKO}$ $p = 0.0153$; at 400 μm, $PTEN^{cKO}$ $p = 0.0005$, $LZK/PTEN^{cKO}$ $p = 0.0019$; at 300-200 μm, $PTEN^{cKO}$ and $LZK/PTEN^{cKO}$ $p < 0.0001$; at 100 μm, $PTEN^{cKO}$ $p = 0.0003$, $LZK/PTEN^{cKO}$ $p = 0.0007$).

3.3.2 LZK is dispensable for axon regeneration caudal to injury in PTEN-deleted mice.

The mice described above to study rostral regeneration were also used to investigate caudal regeneration. BDA positive axons were counted at 50 μm intervals caudal to the GFAP+ border of the scar. As previously shown, none of the GFP-injected mice exhibited regeneration. $DLK/LZK/PTEN^{cKO}$ mice similarly exhibited a near complete loss of axon regeneration, producing an immunohistological phenotype similar to GFP-injected control mice; in fact, only one mouse from this genotype had BDA labeling below the site of injury, with a single axon extending barely past 100μm caudal to the lesion. However, the $LZK/PTEN^{cKO}$ mice demonstrated robust regenerating axons, and were phenotypically indistinguishable from $PTEN^{cKO}$ mice. In both genotypes, the majority of traced axons appeared to regenerate across the dorsal aspect of the lesion. A small fraction of more ventrally-traced axons appear to exhibit regenerative sprouting, around the ventral aspect of the lesion into the caudal spinal cord. While the bulk of labeled axons seem to measure around 400-600 μm, a small fraction of axons continued to show BDA labeling well past a millimeter caudal to the injury.

A 2-way repeated measures ANOVA with Bonferroni post-test confirmed the visual assessment that $LZK/PTEN^{cKO}$ and $PTEN^{cKO}$ had significantly more regeneration than either $DLK/LZK/PTEN^{cKO}$ or GFP Control mice, as well as the observation regeneration in $LZK/ PTEN^{cKO}$ and $PTEN^{cKO}$ are not significantly different from one another. A multiple comparisons assessment demonstrated that both $LZK/PTEN^{cKO}$ and $PTEN^{cKO}$ continues to have significantly more

regeneration than controls for up to 450 μm away from the site of injury (see Figure 3.2 A, C; from 50-350 μm, PTEN^{cKO} and LZK/PTEN^{cKO} $p < 0.0001$; at 400 μm, PTEN^{cKO} $p < 0.0001$ and LZK/PTEN^{cKO} $p = 0.0004$; at 450 μm, PTEN^{cKO} $p = 0.0006$, LZK/PTEN^{cKO} $p = 0.0044$). There was no statistical difference between GFP-injected mice and DLK/LZK/PTEN^{cKO} mice.

Given these results, LZK is dispensable for PTEN-deletion driven axonal regeneration both rostrally and caudally to the site of injury. Interestingly, the double deletion of LZK and DLK suppresses this same form of axon regeneration, suggesting that DLK may be a more critical injury signal that triggers the cell to respond by initiating and injury response, however this has yet to be confirmed. The alternative explanation is that LZK and DLK are redundant, and that knockout of one kinase results in compensatory signaling by the remaining kinase. A follow-up study in DLK/PTEN^{cKO} mice would help elucidate this mystery.

3.3.3 Deletion of DLK and LZK does not affect pS6 signaling in PTEN-deleted mice

In the adult CNS, the AKT/mTOR pathway, a critical signaling cascade for development and growth, is usually inhibited by PTEN. However, in mice that have undergone neuron-specific PTEN deletion, pS6 immunostaining increases as a result of the reduced inhibition on the AKT/mTOR pathway. Given that DLK and LZK double deletion prevents axons from regenerating even in a PTEN-deleted background, we wondered whether deletion of these MAP3Ks significantly altered downstream AKT/mTOR activity. More specifically, we wondered whether deletion of DLK and LZK prevented the activation of the AKT/mTOR pathway in spite of PTEN deletion, or if regeneration in these animals failed despite increased AKT/mTOR activity. The prior hypothesis would suggest that perhaps DLK/LZK signaling exhibits some upstream regulation on the AKT/mTOR pathway, while the latter would instead imply that these are two parallel pathways that must both be functional and activated in order to trigger regeneration. The literature has suggested that DLK and LZK act as injury sensors within the axon, and are critical for retrograde

Figure 3.3: Rostral and caudal regeneration of CST axons after DH.
6wk old mice were injected with either AAV-Cre or AAV-GFP 4 weeks prior to DH. 6 weeks after DH, regenerating axons were traced using BDA. A: Representative images of GFAP staining (green) and BDA tracing (white) in GFP controls (n=12), $PTEN^{cKO}$ (n=10), $LZK/PTEN^{cKO}$ (n=9), and $DLK/LZK/PTEN^{cKO}$ (n=17) mice. B: Quantification of rostral regeneration. Axon density index indicates average intensity of region minus background intensity, normalized to axon density at 1.5 mm rostral to injury (mean±SEM). 2-way RM Anova, main effect of genetic condition at each distance, $p < 0.05$, multiple comparisons with Bonferroni corrections showed significant differences compared to GFP mice from 500-100 µm: *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$ C: Quantification of regeneration. Regeneration index indicates the ratio of the average number of axons counted at each distance caudal to injury site normalized to total axon counts labeled in medulla (mean±SEM). 2-way RM Anova, main effect of genetic condition at each distance, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$. D: Total axon counts from medullas; 1-way ANOVA, *n.s.*

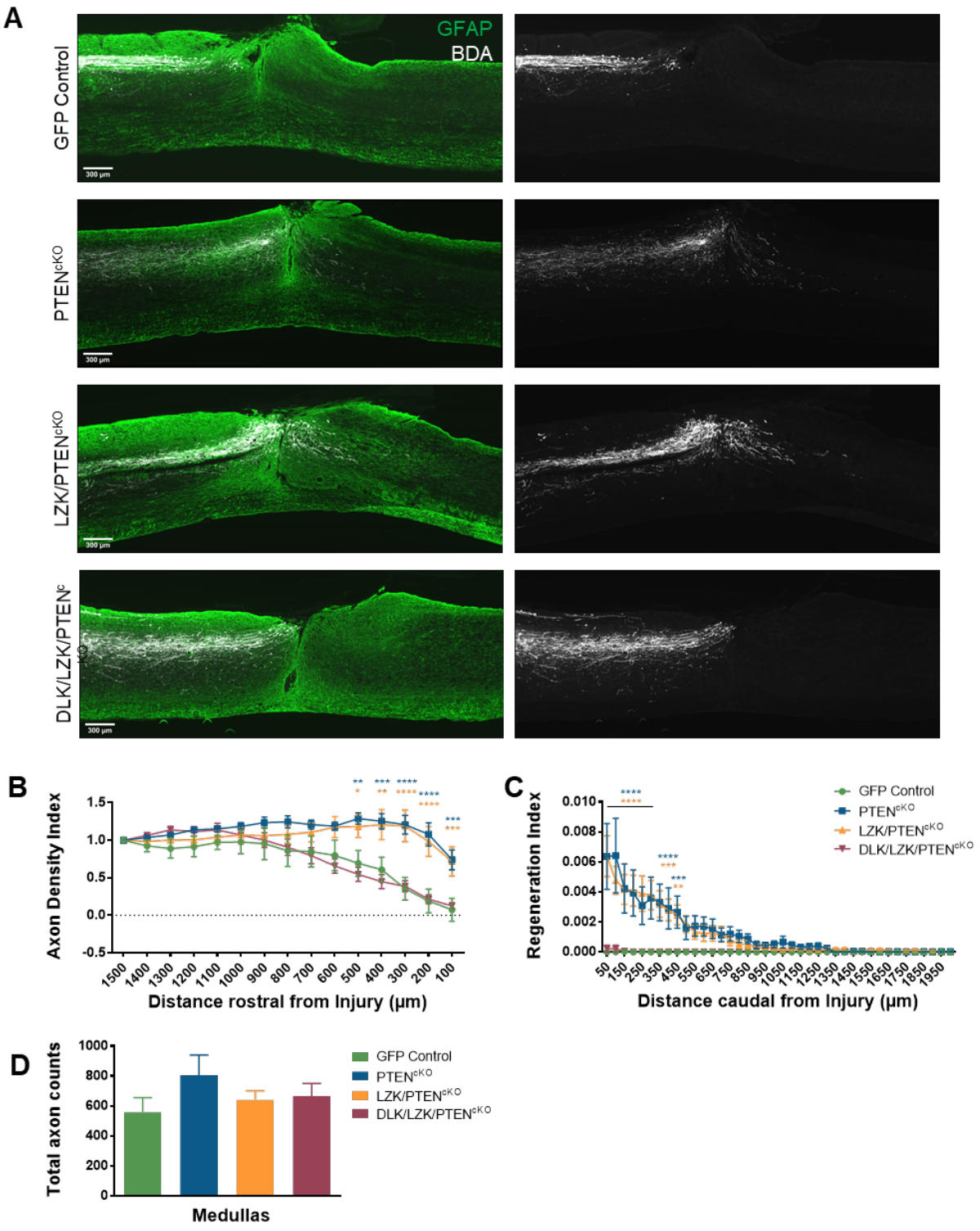
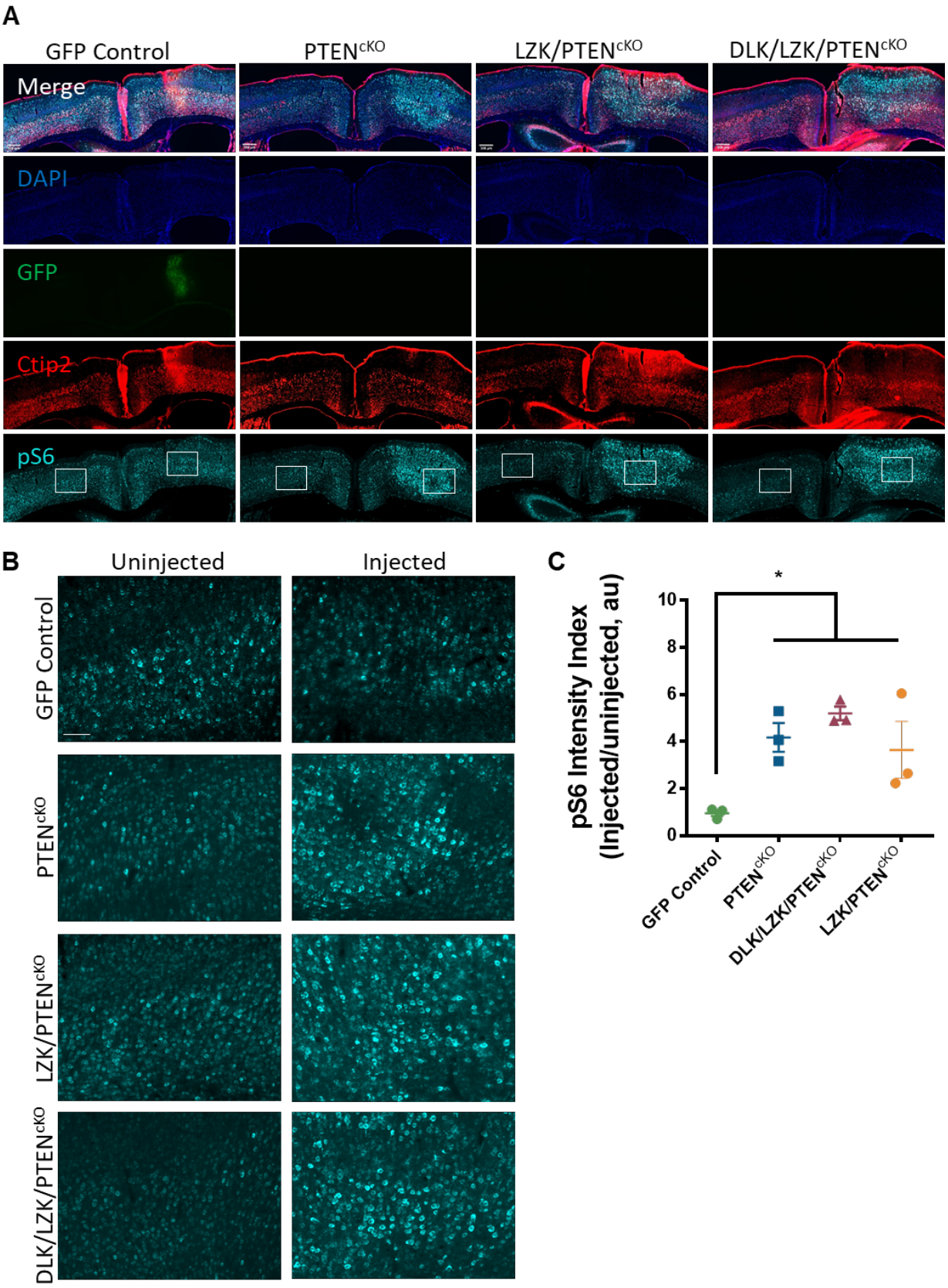

A
GFP Control
PTEN cKO
LZK/PTEN cKO
DLK/LZK/PTEN c
KO
GFAP
BDA
300 µm
B
Axon Density Index
Distance rostral from Injury (µm)
C
Regeneration Index
Distance caudal from Injury (µm)
GFP Control
PTEN cKO
LZK/PTEN cKO
DLK/LZK/PTEN cKO
D
Total axon counts
Medullas

Figure 3.4: DLK and LZK double deletion does not alter pS6 signaling downstream of PTEN deletion

A: Coronal sections of GFP injected, $PTEN^{cKO}$, $LZK/PTEN^{cKO}$, $DLK/LZK/PTEN^{cKO}$ mice stained for pS6 scale bar 300μm. B: 20x images of pS6 staining in layers 5-6 neurons, scale bar 100μm. C: Quantification of pS6 signal. Results displayed as a ratio of composite pS6 signal in injected hemisphere divided by uninjected hemisphere. 1-way ANOVA revealed main effect of genetic condition on pS6 staining, $*p < 0.05$.


A
GFP Control
PTEN cKO
LZK/PTEN cKO
DLK/LZK/PTEN cKO
Merge
DAPI
GFP
Ctip2
pS6
B
Uninjected
Injected
GFP Control
PTEN cKO
LZK/PTEN cKO
DLK/LZK/PTEN cKO
C
pS6 Intensity Index
(Injected/uninjected, au)
*
10
8
6
4
2
0
GFP Control
PTEN cKO
DLK/LZK/PTEN cKO
LZK/PTEN cKO

signaling in order for the nucleus to turn on response-to-injury transcriptional profiles (Welsbie et al., 2017). We therefore considered that perhaps, co-deletion of DLK and LZK leads to reduced AKT/mTOR signaling and thus reduced pS6 signaling. To answer this question, we looked at immunostaining of pS6 in brain tissue in DLK/LZK/PTEN^{cKO}, LZK/PTEN^{cKO}, PTEN^{cKO}, and GFP control mice.

To assess whether deletion of DLK and LZK alters the downstream effects of PTEN signaling in injured cells, brain cortices containing the injection sites were sliced and stained for pS6, a downstream marker of AKT/mTOR activity. We compared pS6 signal in the AAV-injected hemisphere relative to the uninjected hemisphere using an Intensity Index, which is described in more detail in Chapter 2. Briefly, coronal brain sections were stained for Ctip2 and pS6. Ctip2 staining was used to identify layer 5 neurons. The injected hemisphere was identified using gross changes in tissue morphology (e.g. enlarged nuclei, needle track, etc). After setting a threshold, we used the "Analyze Particles" plugin on ImageJ, to segment above-threshold signal into particles. The total area within each box above threshold was multiplied to the average intensity of the measured signal to provide a composite score of pS6 intensity. Lastly, the signal measured in the injected hemisphere was divided by that of the uninjected hemisphere to calculate the fold change in pS6 in the genetically manipulated hemisphere.

As expected, in GFP control mice there was no significant elevation of pS6 signal in the injected hemisphere compared to the uninjected hemisphere, while in PTEN^{cKO} mice elevated pS6 signal was easily ascertained (Figure 3.3A); to our surprise, though, both LZK/PTEN^{cKO} and DLK/LZK/PTEN^{cKO} mice exhibited pS6 elevation in the injected region in a pattern that was indistinguishable from PTEN^{cKO}-mice (1 way ANOVA $F(3, 8) = 7.834$, $p = 0.0091$, see Figure 3.3 A, B). Visually speaking, there was no obvious differences between the PTEN, LZK/PTEN or DLK/LZK/PTEN deleted mice, all of which demonstrated obvious increases in pS6 in the injected hemisphere, unlike GFP controls which had no such change in pS6 signal.

Given these results, it is evident that deletion of DLK and LZK do not alter the signaling cascade triggered by PTEN deletion. Thus, we conclude that kinases do not regulate AKT/mTOR upstream of PTEN, but rather that these pathways are parallel to one another. The present data do not confirm whether or not PTEN deletion affects DLK/LZK signaling, but from the present dataset, we believe that these pathways are separate, and both are required for axonal regeneration to occur.

3.4 Discussion

While DLK and to a lesser extent LZK have been studied in various model of neuronal injury in both optic and sciatic nerves, the series of experiments conducted in the present study are the first look at how DLK and LZK deletion affects axon regeneration in CST neurons after axotomy. As axon regeneration is already an uncommon and very limited cellular response in CNS neurons, we leveraged the effects of PTEN-deletion to induce a pro-growth state in our genetic models. In the present studies, we have shown that while deletion LZK alone does not block the pro-regenerative effects of PTEN deletion, doubly deleting both DLK and LZK in a PTEN-deleted background blocks the effects of PTEN deletion on regeneration both rostral and caudal to the lesion. Furthermore, co-deletion of LZK and DLK do not seem to significantly change the effect of PTEN deletion on AKT/mTOR signaling, as seen via the elevation in pS6 signal in both DLK/LZK/PTEN^{cKO} and LZK/PTEN^{cKO} mice. Thus, while it appears that pS6 elevation subsequent to PTEN deletion is independent of DLK and LZK signaling, the pro-growth capacity this deletion supposed to confer is substantially blocked by the combined deletion of DLK and LZK. This effect of DLK and LZK double deletion in a PTEN-deleted background on both regeneration and dieback is not seen in LZK/PTEN^{cKO} mice, suggesting that LZK on its own is dispensable for both of these cellular responses to injury.

As of these present findings, it is unclear whether DLK deletion in a PTEN deleted background would result in negligible, partial, or complete loss of regeneration compared to PTEN

deletion alone. Without the DLK results, it is difficult to draw any clear conclusions. However, we might be able to draw some comparison to studies leveraging the optic nerve crush model. In the optic nerve crush, deletion of LZK alone does not confer any improvement in cell survival, while DLK deletion enhanced cell survival after crush. However, double deletion of LZK and DLK results in cell survival greater than just DLK deletion alone (Welsbie et al., 2017), suggesting that LZK may cooperates with DLK to activate downstream injury signaling cascades.

In our studies, we show definitively that deletion of both MAP3Ks completely abolishes the regenerative phenotype of PTEN-deletion, despite the intact upregulation of the AKT/mTOR pathway secondary to PTEN-deletion. Previous literature has posited that DLK and LZK may function as injury sensors that allow the cell body to recognize a distal injury. These kinases are critical to alert the cell body via retrograde transportation of relevant injury signals such as STAT3 to the cell body from the site of distal injury, thereby initiating the post-injury transcriptional profile (Shin et al., 2019; Watkins et al., 2013; Xiong et al., 2010). In a WT mouse, these CNS neurons generally lack regenerative competence (Bradke et al., 2012), so perhaps in a cell that did have enhanced growth capacity, DLK and LZK signaling may be critical for triggering a pro-growth response leading to regeneration. In leveraging PTEN-deletion, regenerative competence is enhanced to increase basal regeneration. Our current data reaffirms previous studies that have shown that PTEN deletion alone is sufficient to induce a regenerative phenotype, but in the absence of both DLK and LZK, this elevated growth capacity fails to translate to meaningful regenerative responses, although co-deletion of LZK and PTEN appears phenotypically indistinguishable from PTEN deleted mice.

This finding initially led us to wonder: if DLK and LZK are injury sensors, then are cells lacking DLK and LZK unable to recognize distal injury and thus fail to exhibit strong pro-growth transcriptional activity even in a PTEN deleted background? By exploring the activity of AKT/mTOR by proxy of pS6 staining, we concluded that even in cells lacking DLK and LZK, the deletion of PTEN is sufficient to trigger AKT/mTOR signaling, suggesting that the loss of DLK and

LZK do not affect the upregulation of this pathway after PTEN deletion. Yet, these injured neurons that have enhanced growth capacity and are actively producing proteins strongly affiliated with repair and growth still fail to regenerate in DLK/LZK/PTEN^{cKO} mice. The mechanism by which DLK and LZK co-deletion blocks the effects of PTEN-deletion on regeneration is currently unknown, and remains an open question moving forward.

There is a large body of literature documenting the critical role DLK plays during CNS development. We know that across multiple species DLK is involved with growth cone formation in growing axons and migratory cells, and that as development terminates, the activity of DLK is drastically downregulated by PHR E3 ubiquitin ligase activity (Borgen et al., 2017; Feoktistov & Herman, 2016; Nakata et al., 2005). Particularly in developing mammalian neurons, DLK proteins are expressed more in the axons and growth cones compared to soma and dendrites (Hirai et al., 2005; Lewcock et al., 2007). Even in postmitotic neurons, DLK continues to be elevated in migratory neurons, and subsequently downregulated postnatally (Suenaga et al., 2006) This critical role of DLK in development is further implied by perinatal lethality in germline DLK knockouts, exhibiting defective axon growth and neuronal migration (Hirai et al., 2006). Additionally, downstream targets of DLK such as JNK have been studied in growth cone dynamics in zebrafish: deletion of PHR resulted in elevated JNK phosphorylation in mutant growth cones exhibiting growth cone pausing in development (Hendricks & Jesuthasan, 2009) . Taken together, these results suggest that DLK and its downstream targets are involved with cytoskeletal organization during development and may reprise this role in organizing the cytoskeleton during regeneration as well.

We know that during development, the DLK-JNK relationship is critical for axon growth and neuronal migration. On the other hand, in the adult nervous system, DLK and LZK's role in neurodegeneration is understood to rely on downstream signaling via JNK and p38 MAPK pathways (Tedeschi & Bradke, 2013). Given the broad role of DLK and LZK signaling and its interconnectedness with other MAPK pathways, it would not be surprising for DLK/LZK to be

critically involved with organizing cell-type specific responses to injury then acts as a trigger to launch a wide class of responses that are unique to both injury context and cell type. In the sciatic nerve, we see regeneration as cells in the PNS inherently possess elevated regenerative capacity (Shin et al., 2012), while in optic nerve, we see cell apoptosis, as these cells are relatively non-regenerative by default (Welsbie et al., 2017), and subsequently allows adult injured axons to build growth cones that are necessary for regeneration. In the CST, which consists of cells that largely lack regenerative competence, DLK/LZK signaling may generally be involved in cell responses that do not promote regrowth, but in the background of PTEN deletion, the presence of DLK at least one of the two kinases seems necessary for the regenerative phenotype to exist. The global picture we see regarding DLK's role in axon regeneration may require both the act of triggering a cell response to injury and also mediating the mechanical changes in the cytoskeleton that are necessary for carrying out that response.

3.5 Future Directions

This study represents the first demonstration of the role of DLK and LZK signaling in axonal regeneration in the CST and spinal cord in general. However, the results gathered from these experiments open the door to many new questions, the foremost of which is how essential is DLK for PTEN-deletion mediated axonal regeneration? We see, definitively, that knocking out both DLK and LZK abolishes the regenerative phenotype usually seen in PTEN deletion, but seemingly deletion of LZK and PTEN appears no different from PTEN deletion alone. Interestingly, deletion of either LZK or both of the MAP3Ks does not seem to affect changes in pS6 subsequent to PTEN deletion, suggesting that the activity of AKT/mTOR is independent of DLK/LZK signaling. However, the failure of DLK/LZK/PTEN^{cKO} mice to promote regeneration implies two possibilities: either DLK is critical for PTEN-deletion to induce regeneration, or these kinases share redundant function, and compensation by either kinase allows for the effects of PTEN deletion to occur. If this latter hypothesis turns out to be correct, it will further lead to the

question of how much functional redundancy is shared between these two kinases. To address all possibilities, the natural follow-up experiment would be to conduct dorsal hemisection surgeries in DLK/PTEN^{cKO} mice.

A second question worthy of note is to ask how loss of DLK/LZK signaling blocks the pro-growth effects of PTEN deletion. To the best of my knowledge, the present study is a first display of neurons that exhibit elevated pS6 failing to demonstrate pro-regenerative phenotypes, suggesting that DLK/LZK signaling may function independently of PTEN to deletion to trigger a cellular response to injury (such as those found in non-PTEN deleted background). Even with elevated pro-growth signaling as evidenced by elevated pS6, we see a failure to sustain regeneration in DLK/LZK/PTEN^{cKO} mice. The key takeaway here that we can conclude is that the AKT/mTOR pathway and DLK/LZK signaling pathway are parallel pathways; while DLK/LZK may not affect pS6 expression after PTEN deletion, this unfettered activity of this pathway is necessary for PTEN deletion to result in axonal regeneration.

In general, it would be interesting to see if DLK and LZK are critical in reorganizing the cytoskeleton in preparation for axon regrowth, similar to what is seen in axon growth and neuronal migration during development. Additionally, it would be interesting to see if deletion of DLK and LZK in a PTEN-deleted background result in strikingly different JNK/p38 signaling, as DLK and LZK are critical upstream regulators of this pathway, which are fundamentally involved in several cell processes, including growth, development, degeneration, and apoptosis.

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Chapter 3, in part, is currently being prepared for submission for publication of the material. Saikia, Junmi M.; Chavez Martinez, Carmine; Kim, Noah; Allibhoy, Sahar; Kim, Hugo; Simonyan, Lidiya; Smadi, Samraa; Romaus Sanjurjo, Daniel; Jin, Yishi; Zheng, Binhai. The dissertation author was the primary investigator and author of this material.

References

Borgen, M. A., Wang, D., & Grill, B. (2017). RPM-1 regulates axon termination by affecting growth cone collapse and microtubule stability. *Development (Cambridge)*, *144*(24), 4658–4672. https://doi.org/10.1242/dev.154187

Bradke, F., Fawcett, J. W., & Spira, M. E. (2012). Assembly of a new growth cone after axotomy: The precursor to axon regeneration. In *Nature Reviews Neuroscience* (Vol. 13, Issue 3, pp. 183–193). Nat Rev Neurosci. https://doi.org/10.1038/nrn3176

Chen, M., Geoffroy, C. G., Wong, H. N., Tress, O., Nguyen, M. T., Holzman, L. B., Jin, Y., & Zheng, B. (2016). Leucine Zipper-bearing Kinase promotes axon growth in mammalian central nervous system neurons. *Scientific Reports*, *6*. https://doi.org/10.1038/srep31482

Feoktistov, A. I., & Herman, T. G. (2016). Wallenda/DLK protein levels are temporally downregulated by Tramtrack69 to allow R7 growth cones to become stationary boutons. *Development (Cambridge)*, *143*(16), 2983–2993. https://doi.org/10.1242/dev.134403

Ferguson, T. A., & Son, Y. J. (2011). Extrinsic and intrinsic determinants of nerve regeneration. In *Journal of Tissue Engineering* (Vol. 2, Issue 1, pp. 1–12). J Tissue Eng. https://doi.org/10.1177/2041731411418392

Geoffroy, C. G., Lorenzana, A. O., Kwan, J. P., Lin, K., Ghassemi, O., Ma, A., Xu, N., Creger, D., Liu, K., He, Z., & Zheng, B. (2015). Effects of PTEN and Nogo codeletion on corticospinal axon sprouting and regeneration in mice. *Journal of Neuroscience*, *35*(16), 6413–6428. https://doi.org/10.1523/JNEUROSCI.4013-14.2015

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E. M., & Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. *Science*, *323*(5915), 802–806. https://doi.org/10.1126/science.1165527

He, Z., & Jin, Y. (2016). Intrinsic Control of Axon Regeneration. *Neuron*, *90*(3), 437–451. https://doi.org/10.1016/j.neuron.2016.04.022

Hendricks, M., & Jesuthasan, S. (2009). PHR regulates growth cone pausing at intermediate targets through microtubule disassembly. *Journal of Neuroscience*, *29*(20), 6593–6598. https://doi.org/10.1523/JNEUROSCI.1115-09.2009

Hirai, S. I., De, F. C., Miyata, T., Ogawa, M., Kiyonari, H., Suda, Y., Aizawa, S., Banba, Y., & Ohno, S. (2006). The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. *Journal of Neuroscience*, *26*(46), 11992–12002. https://doi.org/10.1523/JNEUROSCI.2272-06.2006

Hirai, S. I., Kawaguchi, A., Suenaga, J., Ono, M., Cui, D. F., & Ohno, S. (2005). Expression of MUK/DLK/ZPK, an activator of the JNK pathway, in the nervous systems of the developing mouse embryo. *Gene Expression Patterns*, *5*(4), 517–523. https://doi.org/10.1016/j.modgep.2004.12.002

Lewcock, J. W., Genoud, N., Lettieri, K., & Pfaff, S. L. (2007). The Ubiquitin Ligase Phr1 Regulates Axon Outgrowth through Modulation of Microtubule Dynamics. *Neuron*, *56*(4), 604–620. https://doi.org/10.1016/j.neuron.2007.09.009

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature*

Neuroscience, *13*(9), 1075–1081. https://doi.org/10.1038/nn.2603

Nakata, K., Abrams, B., Grill, B., Goncharov, A., Huang, X., Chisholm, A. D., & Jin, Y. (2005). Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell*, *120*(3), 407–420. https://doi.org/10.1016/j.cell.2004.12.017

Park, K. K., Liu, K., Hu, Y., Smith, P. D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., & He, Z. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science*, *322*(5903), 963–966. https://doi.org/10.1126/science.1161566

Shin, J. E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., & DiAntonio, A. (2012). Dual Leucine Zipper Kinase Is Required for Retrograde Injury Signaling and Axonal Regeneration. *Neuron*, *74*(6), 1015–1022. https://doi.org/10.1016/j.neuron.2012.04.028

Shin, J. E., Ha, H., Kim, Y. K., Cho, Y., & DiAntonio, A. (2019). DLK regulates a distinctive transcriptional regeneration program after peripheral nerve injury. *Neurobiology of Disease*, *127*, 178–192. https://doi.org/10.1016/j.nbd.2019.02.001

Suenaga, J., Cui, D. F., Yamamoto, I., Ohno, S., & Hirai, S. I. (2006). Developmental changes in the expression pattern of the JNK activator kinase MUK/DLK/ZPK and active JNK in the mouse cerebellum. *Cell and Tissue Research*, *325*(1), 189–195. https://doi.org/10.1007/s00441-006-0164-x

Tedeschi, A., & Bradke, F. (2013). The DLK signalling pathway - A double-edged sword in neural development and regeneration. In *EMBO Reports* (Vol. 14, Issue 7, pp. 605–614). European Molecular Biology Organization. https://doi.org/10.1038/embor.2013.64

Tedeschi, A., & Bradke, F. (2017). Spatial and temporal arrangement of neuronal intrinsic and extrinsic mechanisms controlling axon regeneration. In *Current Opinion in Neurobiology* (Vol. 42, pp. 118–127). Elsevier Ltd. https://doi.org/10.1016/j.conb.2016.12.005

Watkins, T. A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J. S., Tessier-Lavigne, M., & Lewcock, J. W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(10), 4039–4044. https://doi.org/10.1073/pnas.1211074110

Welsbie, D. S., Mitchell, K. L., Jaskula-Ranga, V., Sluch, V. M., Yang, Z., Kim, J., Buehler, E., Patel, A., Martin, S. E., Zhang, P. W., Ge, Y., Duan, Y., Fuller, J., Kim, B. J., Hamed, E., Chamling, X., Lei, L., Fraser, I. D. C., Ronai, Z. A., … Zack, D. J. (2017). Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. *Neuron*, *94*(6), 1142-1154.e6. https://doi.org/10.1016/j.neuron.2017.06.008

Welsbie, D. S., Ziogas, N. K., Xu, L., Kim, B. J., Ge, Y., Patel, A. K., Ryu, J., Lehar, M., Alexandris, A. S., Stewart, N., Zack, D. J., & Koliatsos, V. E. (2019). Targeted disruption of dual leucine zipper kinase and leucine zipper kinase promotes neuronal survival in a model of diffuse traumatic brain injury. *Molecular Neurodegeneration*, *14*(1). https://doi.org/10.1186/s13024-019-0345-1

Xiong, X., Wang, X., Ewanek, R., Bhat, P., DiAntonio, A., & Collins, C. A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. *Journal of Cell Biology*, *191*(1), 211–223. https://doi.org/10.1083/jcb.201006039

Yan, D., Wu, Z., Chisholm, A. D., & Jin, Y. (2009). The DLK-1 Kinase Promotes mRNA Stability and Local Translation in C. elegans Synapses and Axon Regeneration. *Cell*, *138*(5), 1005–1018. https://doi.org/10.1016/j.cell.2009.06.023

Chapter 4:

Roles of DLK and LZK signaling in spontaneous compensatory sprouting

4.1 Significance Statement

Where axon regeneration is the study of the regeneration of injured axons, compensatory sprouting is a second mechanism of CNS repair, by which spared uninjured axons exhibit plasticity to and sprout into denervated regions to compensate for the lost function of injured axons. While both of these two repair mechanisms are of academic interest for their potential translation to a clinical population, sprouting can occur spontaneously, and may contribute to some physical recovery of function seen in clinical populations. As such, like with regeneration, there is strong interest in understanding the molecular mechanisms underlying spontaneous compensatory sprouting. So far, all key regulators of axon regeneration have also been shown to play a role in axon sprouting, so there may be some role for DLK and LZK signaling in compensatory sprouting as well.

Of course, where DLK and LZK fit into the sprouting side of CNS repair is particularly unclear. Unlike with regeneration, sprouting adds a level of complexity in that uninjured axons are exhibiting plasticity, and so it is unclear how or even if DLK and LZK are involved in injury signaling in this mechanism. There is reason, however, to be interested in how intrinsic signaling mechanisms may play a role in sprouting; deletion of PTEN, an inhibitor of the AKT/mTOR pathway, enhances the growth of uninjured axons into denervated regions, suggesting that while uninjured axons may not be directly responding to the molecular cascades resulting from injury (e.g. calcium influx), they may use similar injury response mechanisms to initiate a pro-growth transcriptional profile (Liu et al., 2010). However, DLK and LZK are likely not completely unimportant for CNS sprouting. A recent study showed that knockdown of C-C chemokine receptor type 5, a molecule canonically studied for its role in HIV infection, enhanced sprouting from contralateral motor cortex after a model of stroke. In neurons that had CCR5 knockdown, DLK was found to be upregulated (Joy et al., 2019). While this finding does not directly tie DLK function to sprouting, it is the first study demonstrating a connection between increased DLK signaling after injury and sprouting in the mammalian motor system. Additionally, we know that

DLK activity can increase in a cell in response to non-injury cell stressors, such as modulation in microtubule activity after colcichine treatment (Bounoutas et al., 2009). These studies imply that DLK signaling can be activated in CNS neurons even in absence of a direct axonal lesion. However, the exact roles of DLK and LZK in sprouting within the spinal cord is essentially unchartered territory. In this series of experiments, I explore the role of DLK and LZK in spontaneous sprouting using the unilateral pyramidotomy injury model, in which only one of the descending motor CST tracts is severed. The benefit of leveraging the unilateral pyramidotomy over other forms of partial lesions is due to the anatomical location of the medullary pyramids. Located anterior to the decussation of these fibers, the medullary pyramids contain all descending CST axons. At this level, each hemisphere's descending CST is distinctly bounded by the basilar artery, allowing for easy identification and isolated injury of the targeted tract (Starkey et al., 2005). Initial findings suggest that while DLK and LZK deletion in isolation does not alter sprouting significantly as compared with GFP-injected wildtype control mice; however, co-deletion of both kinases suppresses sprouting to below wild type measures, suggesting that perhaps these kinases together are involved in initiating a sprouting response to injury.

4.2 Experimental outline

For a more detailed explanation of methods and materials, please refer to Chapter 2. In summary, the animals studied in this series of experiments compared DLK^{cKO} (n = 11), LZK^{cKO} (n = 8), and DLK/LZK^{cKO} mice (n = 6) against GFP-injected littermate controls (n = 12). At 6 weeks of age, mice floxxed for the specified genes were injected with AAV-Cre or AAV-GFP in the layer V region of the right-sided motor cortex targeting the forelimb region. These axons innervate the left-sided dCST in the spinal cord. After two weeks, they underwent the pyramidotomy surgery, which denervates the left cerebral cortex's descending motor tract in the right half of the spinal cord. After another two weeks, sprouting axons were traced by injecting anterograde BDA tracer

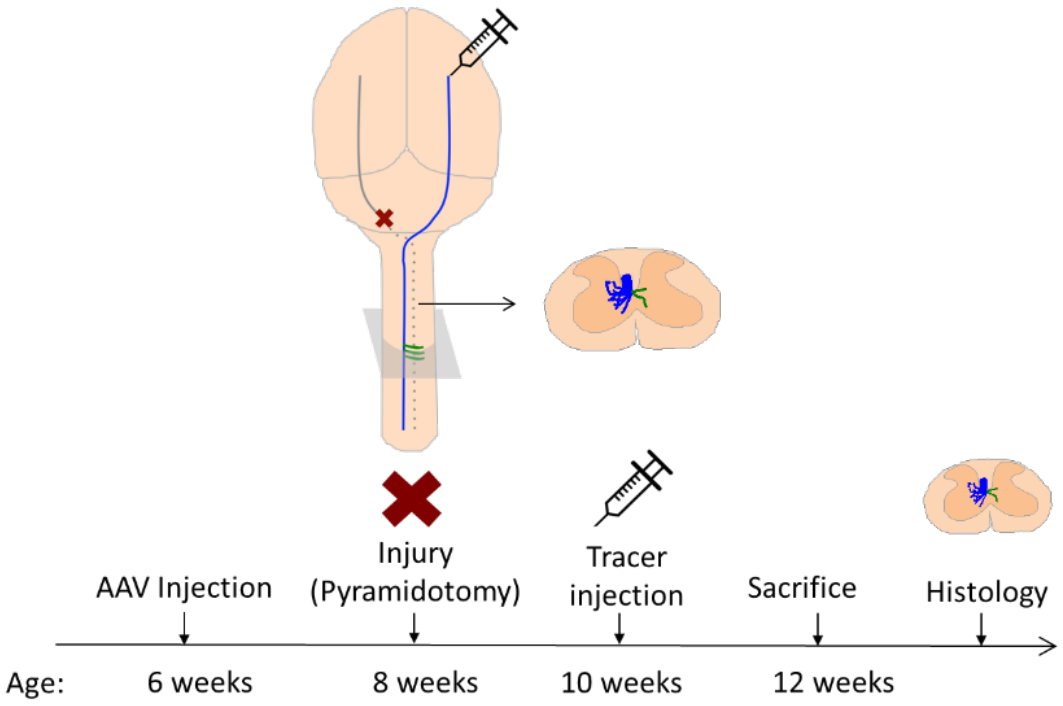

Figure 4.1 Overview of the pyramidotomy method.
Experimental timeline for unilateral pyramidotomy studies. 6wk old mice were injected with either AAV-Cre or AAV-GFP two weeks prior to pyramidotomy (PYR). Two weeks after PYR, regenerating axons were traced using BDA (Meves et al., 2018).

in the right side motor cortex. The mice were sacrificed two weeks later for processing and analysis (Figure 4.1).

Completeness of injury was assessed using immunohistochemistry for PKCγ, which is expressed highly in the axonal tracts of dCST neurons. Upon pyramidotomy, expression of this protein drops in the injured descending tract, allowing for assessment of injury. Transverse sections of cervical spinal cord sections were stained for tracer BDA, and midline crossing axons within the grey matter of the cord were identified as sprouting axons. These midline crossing axons were counted manually at pre-determined distances away from midline and then normalized to total axon counts measured in the medulla (for full methods, refer to Chapter 2). Quantification of axonal sprouting is presented as Axon Sprouting Indices measured at each reported distance away from midline.

4.3 Results

4.3.1 Neither LZK nor DLK deletion alters sprouting of CST axons after pyramidotomy.

We investigated whether deletion of DLK or LZK in uninjured neurons altered spontaneous sprouting in response to unilateral pyramidotomy in contralateral CST. To assess the roles of DLK and LZK in compensatory sprouting of CST axons, $LZK^{fl/fl}$ and $DLK^{fl/fl}$ mice were injected with AAV2-Cre in the right hemisphere to generate conditional knockout LZK^{cKO} and DLK^{cKO} mice. These were compared to GFP injected littermate controls. Two weeks after AAV injection, mice underwent unilateral pyramidotomies. Two weeks subsequent to injury, BDA anterograde tracer was injected to the same gene-deleted cortical coordinates used for viral injections in the right hemisphere. this model allowed for tracing of uninjured right CST motor neurons' sprouting phenotype into the left hemisphere's descending CST.

In transverse sections of cervical spinal cord, we manually quantified the number of midline crossing axons at specified distances. Wildtype mice are naturally able to exhibit low levels of sprouting axons. On the traced half of the cerival section, we see strong labeling in dCST,

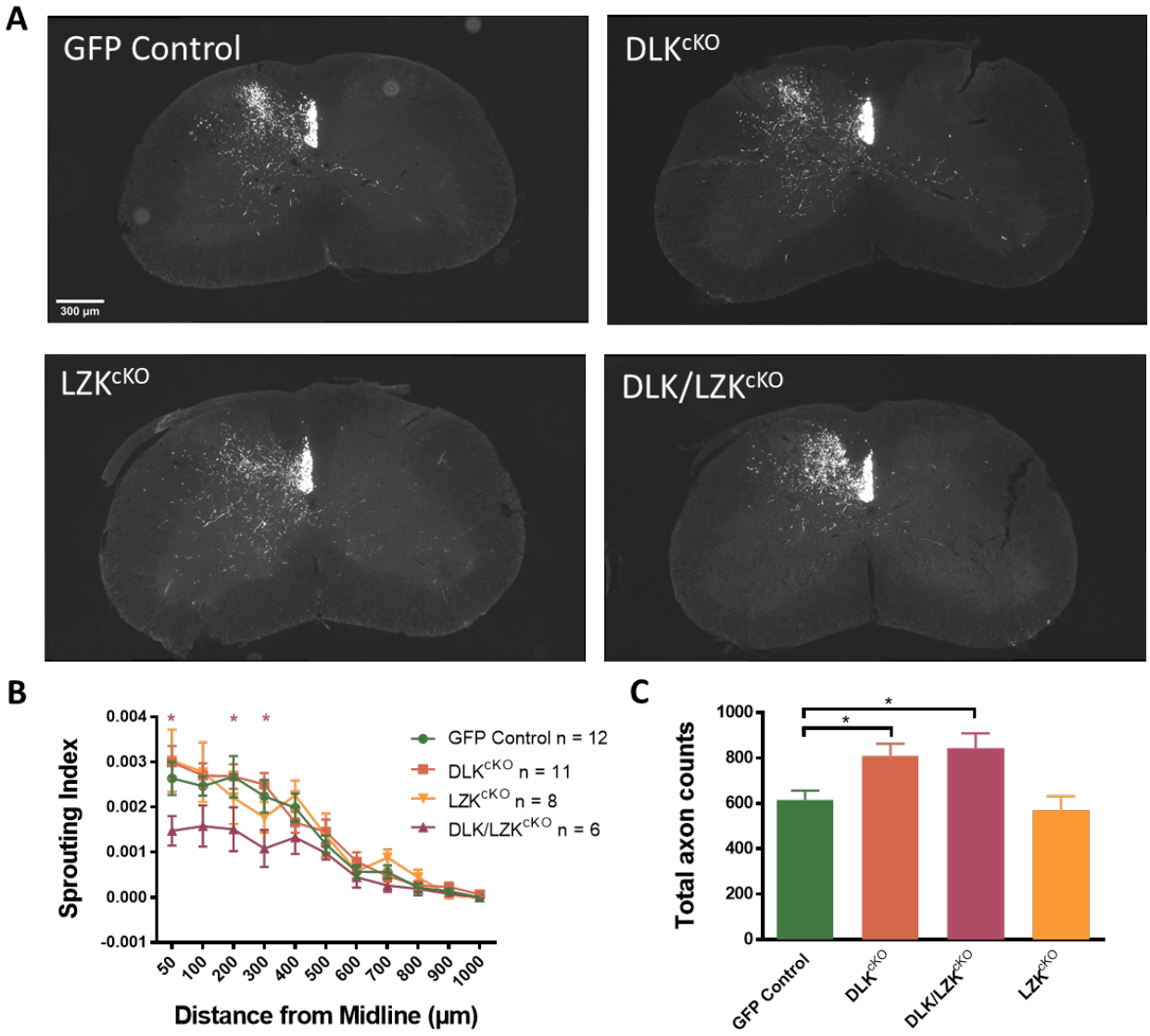

Figure 4.2 Deletion of DLK and LZK in isolation does not impact spontaneous compensatory sprouting.

A: Representative images of BDA tracing at the level of cervical spinal cord in GFP injected controls (top left), DLK^{cKO} (top right), LZK^{cKO} (bottom left) and DLK/LZK^{cKO} (bottom right). B: quantification of sprouting across all four genotypes, scale bar is 300 µm; 2-way RM ANOVA for DLK: F(1,23) = 0.2702, p = 0.6038; LZK: F(1,20) = 0.05835, p = 0.8116; DLK/LZK^{cKO}: F(1,18) = 3.968, p = 0.0618. Bonferroni test for multiple comparisons revealed DLK/LZK^{cKO} mice have significantly fewer axons at 50, 200, and 300 µm p = 0.0284, 0.0277 and 0.0283, respectively.

in either DLK or LZK knockout condition we did not observe significant change in sprouting compared to control mice (See Figures 4.2 A & B, 2-way RM ANOVA for DLK: $F(1,23) = 0.2702$, $p = 0.6038$; LZK: $F(1,20) = 0.05835$, $p = 0.8116$). An unexpected complication in this study was that, for the DLK^{cKO} study in particular, GFP control mice appeared to have fewer axons labeled relative to DLK^{cKO} and DLK/LZK^{cKO} (Figure 4.2C, $p < 0.05$ by 1-way ANOVA); as such, variability in sprouting exhibited in GFP control mice may be influenced somewhat by the lower labeling efficiency of the CST axons. Nevertheless, despite having fewer axons labeled, it appears as though following normalization, GFP control mice had sprouting similar to DLK^{cKO} mice. Indeed, excluding "highly labeled" mice from the study (e.g. mice who had total axon counts above 2 standard deviations above the mean) did not alter the overall result that deleting DLK from uninjured axons in this model does not enhance or hamper sprouting relative to wildtype GFP-control mice (data not shown).

4.3.3 Double deletion of DLK and LZK appears to suppress spontaneous sprouting.

In addition to studying DLK and LZK deletion in compensatory sprouting, we also investigated how double deletion of DLK and LZK could alter sprouting in otherwise wildtype mice. Literature in the field strongly implies that LZK and DLK signal cooperatively in response to injury. For example, in a model of optic nerve crush injury, DLK^{cKO} mice had fewer retinal ganglion cell (RGC) deaths than wildtype mice, and this effect was enhanced in DLK/LZK^{cKO} mice, although LZK deletion alone had no major effect (Welsbie et al., 2017). Additionally, in a model of impact acceleration to induce diffuse traumatic brain injury, DLK deletion alone suppressed JUN phosphorylation in the MAP3K cascade, but only double deletion of DLK and LZK was sufficient to actually prevent RGC death (Welsbie et al., 2019). Between these two studies conducted by the same research group, the redundancy or cooperativity between these two kinases in response to injury appears to be largely dependent on the model of injury. Although both the optic nerve crush and the impact acceleration models are used to induce RGC death, we see from these

studies that the protection against cell death via DLK deletion is context dependent, and that LZK deletion may either enhance survival (in the crush model) or may be required for survival (impact acceleration model).

In comparing DLK and LZK in terms of their structure and sequence, this is not surprising. DLK and LZK have over 95% sequence identity in their kinase domain, suggesting there may be redundancy in these MAP3Ks kinase activity. However, other structures such as the C-termini diverge significantly: LZK, but not DLK, contains a calcium-sensitive hexapeptide sequence in its C-terminus, identical to the hexapeptide sequence found in worm *DLK-1* (Yan & Jin, 2012).

Thus, we sought to ask if DLK and LZK co-deletion may reveal changes in sprouting phenotype that isolated DLK and LZK deletion may not highlight. By qualitative visualization, DLK/LZK double knockouts appeared to have in general fewer midline crossing axons at 50 μm, and generally appeared to have fewer sprouts throughout the grey matter, in spite of strong labeling of the traced dCST. Statistically, while isolated deletion of DLK and LZK did not appear to affect compensatory sprouting, co-deletion of DLK and LZK exhibited a trend towards sprouting to below wildtype levels, but did not reach statistical significance (see Fig. 4.2, 2-way RM ANOVA for DLK/LZK^{cKO}: $F(1,18) = 3.968$, $p = 0.0618$). Bonferroni's test for multiple comparisons revealed significantly fewer sprouting axons at 50, 200 and 300 μm away from the midline ($p = 0.0284$, 0.0277 and 0.0283, respectively) with trending suppression at 100 μm ($p = 0.1396$).

As with the DLK^{cKO} cohort, BDA axon tracing in DLK/LZK^{cKO} mice appeared to be significantly higher than GFP-injected controls (1-way ANOVA, $p < 0.05$). Nevertheless, despite improved labeling in DLK/LZK^{cKO} mice, very few axons were found to cross midline, suggesting that there may be a stronger effect of suppression on sprouting than the data here show.

Taken together, these findings suggest that DLK and LZK have redundant roles in mediating sprouting as a response to CNS injury, such that double deletion of these kinases results in a more pronounced suppression of sprouting, in contrast to just DLK or LZK deletion alone which has no impact on sprouting.

4.4 Discussion

In the present study, we show that deletion of DLK and LZK in isolation seems to have no significant impact on spontaneous sprouting in mice that have undergone unilateral pyramidotomy. However, co-deletion of the two kinases appears to cause some level of suppression in sprouting. One possible explanation for these results is that, at least for sprouting, DLK and LZK have redundancy and are able to compensate for each other when only one kinase is deleted, although this explanation requires further validation of gene knockout to confirm this conclusion.

The study of DLK and LZK signaling in compensatory sprouting, as compared to regeneration, is a little less straightforward, in that DLK and LZK are canonically described as injury sensors after their initial role in development and neuronal migration. As sprouting leverages plasticity in uninjured neurons to respond and compensate for injured axons, it was unclear whether or not DLK and LZK would even be relevant in this response-to-injury response. Indeed, the initial findings that implied that neither DLK nor LZK deletion altered sprouting strongly seemed to imply that these kinases serve no purpose in axonal sprouting. However, in the double knockout mice, we see trend towards suppression of sprouting below wildtype mice, although this phenotype is not statistically significant overall in our present study. This instead reveals that for compensatory spontaneous sprouting, DLK and LZK may have partially redundant function, and the presence of one kinase is enough to maintain wildtype levels of sprouting.

Compared to the effects of DLK and LZK deletion in axonal regeneration, these effects seem less striking, although the comparison is somewhat clouded by the effects of PTEN inducing a pro-growth state in the regeneration model. Ultimately, it is hard to draw a direct comparison between these two sets of studies due both the surgical model and the genetic manipulation both being different. Given what we know about DLK activation in adult neurons, it is likely that spared axons, although uninjured, may experience cellular stressors due to the extrinsic environmental changes induced by the lesion in neighboring axons, and the subsequent cellular response relies

on DLK/LZK signaling to properly trigger a sprouting response. In particular, it is known that DLK is very sensitive to changes in cytoskeletal dynamics. In worms, for example, treatment with colchicine can lead to loss of mechanosensation, a feature that can be blocked by DLK inhibition (Bounoutas et al., 2009). This suggests that DLK activity in response to the colchicine treatment contributed to impaired neuronal function, even in the absence of a direct neuronal lesion. Thus, DLK may be able to respond generally to cell stressors that result from CNS injury as a form of indirect injury signaling. In the injured CNS, inflammatory cross-talk between the injured neurons and non-neuronal cells would not be limited to only the region of injury. These signals are likely able to communicate with neighboring healthy neurons. Perhaps, DLK and LZK then can be activated in response to this inflammatory CNS environment, then acting as a signal for the spared axons to exhibit the requisite plasticity necessary for compensatory axonal sprouting.

4.5 Future Directions

The present study explored whether or not DLK and LZK are involved in compensatory sprouting in uninjured axons following unilateral pyramidotomy. A key hope in exploring the potential roles of these kinases in intact spared neurons is to understand the molecular mechanisms involved with post-injury plasticity, and developing strategies aimed at enhancing plasticity in uninjured axons.

The key finding from this study was that while single deletion of either MLK failed to alter sprouting significantly, the co-deletion of both kinases leads to a trend towards suppression of sprouting in uninjured axons, implying that these two kinases may have some functional redundancy in promoting the plasticity required of these cells to induce the sprouting phenotype. To confirm functional redundancy between these two kinases, a follow-up of these studies focusing on gene expression or transcriptional assays would further clarify the effect of single gene deletion versus double knockout and establish a more concrete interpretation of the results presented here. Additionally, looking at distinct downstream markers of DLK/LZK signaling

cascade, such as p-c-Jun or p-JNK, in both injured and uninjured cells may be helpful in understanding key differences in how DLK or LZK single mutants compare to DLK/LZK double deleted mice.

Sprouting, as an assay, interrogates the inherent plasticity in uninjured axons and their ability to respond to neighboring sites of injury. So, another angle to consider exploring is how DLK and LZK, rather than acting as injury signals, promote plasticity subsequent to injury. A recent finding suggested that CCR5, a receptor heavily involved in HIV infection, is a strong suppressor of plasticity in the hippocampus. Decreasing CCR5 expression in hippocampal in turn elevates CREB/MAPK signaling, long term potentiation and hippocampus-dependent memory function in mice (Zhou et al., 2016). This surprising finding led to the subsequent discovery that CCR5 knockdown improved motor recovery following stroke, and that this recovery was mediated specifically through DLK signaling. In CCR5 knockdown mice, DLK expression increased after stroke and mice showed enhanced motor recovery. Furthermore, if both CCR5 and DLK are knocked down following stroke, animals showed reduced motor recovery (Joy et al., 2019). These findings together portray a potential role for DLK activity as a requirement for plasticity to occur following injury. Given these findings and the inherent relationship between sprouting and plasticity, it would be interesting to further investigate whether DLK/LZK signaling is a requirement for plasticity to occur in our model of CST sprouting.

Another point to consider is the role of DLK in organizing cytoskeletal elements. In the present study, our genetic models do not investigate cellular mechanisms directly. Yet, there is a large body of literature in the field of axon guidance and nervous system development that show that DLK is strongly involved with directly organizing cytoskeletal elements in the growth cone (Borgen et al., 2017; Valakh et al., 2013). Currently, much of literature studying the role of DLK in axonal repair focuses heavily on axonal regeneration, specifically, and these findings do not distinguish differences between axonal elongation and growth cone development, which is a critical step that is required for axon elongation to even occur (Bradke et al., 2012).

Particularly in sprouting, the mechanism by which uninjured and otherwise quiescent axons suddenly undergo branching to form sprouting collaterals is unknown. The kinds of signals that are in the external environment, and how these healthy uninjured cells are able to recognize nearby injury to trigger a sprouting phenotype is a largely unanswered question. DLK and LZK are uniquely situated; as injury signals, they may be critical for uninjured cells to recognize nearby injury, triggering axonal repair in the form of sprouting in these uninjured neurons. The findings in this present study may give reason to consider such an expanded view for the role of injury signaling molecules as being global markers for nerve injury, in addition to their role of injury signaling within the lesioned cell, itself.

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References

Borgen, M. A., Wang, D., & Grill, B. (2017). RPM-1 regulates axon termination by affecting growth cone collapse and microtubule stability. *Development (Cambridge)*, *144*(24), 4658–4672. https://doi.org/10.1242/dev.154187

Bounoutas, A., O'Hagan, R., & Chalfie, M. (2009). The Multipurpose 15-Protofilament Microtubules in C. elegans Have Specific Roles in Mechanosensation. *Current Biology*, *19*(16), 1362–1367. https://doi.org/10.1016/j.cub.2009.06.036

Bradke, F., Fawcett, J. W., & Spira, M. E. (2012). Assembly of a new growth cone after axotomy: The precursor to axon regeneration. In *Nature Reviews Neuroscience* (Vol. 13, Issue 3, pp. 183–193). Nat Rev Neurosci. https://doi.org/10.1038/nrn3176

Joy, M. T., Assayag, B., Shabashov-, D., Silva, A. J., Shohami, E., Thomas, S., & Correspondence, C. (2019). CCR5 Is a Therapeutic Target for Recovery after Stroke and Traumatic Brain Injury In Brief Genetic and small molecule-based perturbation of CCR5 promotes functional recovery from stroke and traumatic brain injury. *Cell*, *176*, 1143-1157.e13. https://doi.org/10.1016/j.cell.2019.01.044

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature Neuroscience*, *13*(9), 1075–1081. https://doi.org/10.1038/nn.2603

Meves, J. M., Geoffroy, C. G., Kim, N. D., Kim, J. J., & Zheng, B. (2018). Oligodendrocytic but not neuronal Nogo restricts corticospinal axon sprouting after CNS injury. *Experimental Neurology*, *309*, 32–43. https://doi.org/10.1016/j.expneurol.2018.07.013

Starkey, M. L., Barritt, A. W., Yip, P. K., Davies, M., Hamers, F. P. T., McMahon, S. B., & Bradbury, E. J. (2005). Assessing behavioural function following a pyramidotomy lesion of the corticospinal tract in adult mice. *Experimental Neurology*, *195*(2), 524–539. https://doi.org/10.1016/j.expneurol.2005.06.017

Valakh, V., Walker, L. J., Skeath, J. B., & DiAntonio, A. (2013). Loss of the spectraplakin short stop activates the DLK injury response pathway in drosophila. *Journal of Neuroscience*, *33*(45), 17863–17873. https://doi.org/10.1523/JNEUROSCI.2196-13.2013

Welsbie, D. S., Mitchell, K. L., Jaskula-Ranga, V., Sluch, V. M., Yang, Z., Kim, J., Buehler, E., Patel, A., Martin, S. E., Zhang, P. W., Ge, Y., Duan, Y., Fuller, J., Kim, B. J., Hamed, E., Chamling, X., Lei, L., Fraser, I. D. C., Ronai, Z. A., … Zack, D. J. (2017). Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. *Neuron*, *94*(6), 1142-1154.e6. https://doi.org/10.1016/j.neuron.2017.06.008

Welsbie, D. S., Ziogas, N. K., Xu, L., Kim, B. J., Ge, Y., Patel, A. K., Ryu, J., Lehar, M., Alexandris, A. S., Stewart, N., Zack, D. J., & Koliatsos, V. E. (2019). Targeted disruption of dual leucine zipper kinase and leucine zipper kinase promotes neuronal survival in a model of diffuse traumatic brain injury. *Molecular Neurodegeneration*, *14*(1). https://doi.org/10.1186/s13024-019-0345-1

Yan, D., & Jin, Y. (2012). Regulation of DLK-1 Kinase Activity by Calcium-Mediated Dissociation from an Inhibitory Isoform. *Neuron*, *76*(3), 534–548. https://doi.org/10.1016/j.neuron.2012.08.043

Zhou, M., Greenhill, S., Huang, S., Silva, T. K., Sano, Y., Wu, S., Cai, Y., Nagaoka, Y., Sehgal, M., Cai, D. J., Lee, Y.-S., Fox, K., & Silva, A. J. (2016). CCR5 is a suppressor for cortical plasticity and hippocampal learning and memory. *ELife*, *5*. https://doi.org/10.7554/eLife.20985

Chapter 5:

Roles of DLK and LZK signaling in PTEN-induced CST axon sprouting

5.1 Significance Statement

While sprouting is a spontaneous cellular response to injury in the CNS, there are well-known genetic tools that can be leveraged to enhance CST plasticity in spared descending pathways. Deletion of intrinsic tumor suppressor gene PTEN triggers robust increases in axonal sprouting (Geoffroy et al., 2015; Liu et al., 2010). The combinatorial manipulation of multiple signaling pathways has been leveraged experimentally as a potential avenue to identify synergistic combinations that significantly bolster the sprouting phenotype. Presently, the MAP3K pathway is known to be involved with several cellular processes including cell development, mitosis, apoptosis, as well as response to injury. In the previous aim, we found that co-deletion of DLK and LZK can suppress sprouting to below baseline wildtype levels, revealing that this MAP3K pathway contributes to plasticity seen in spontaneous sprouting.

While we did not see any significant differences in sprouting between DLK or LZK knockouts and wildtype mice, is possible that the effects of DLK and LZK deletion on sprouting in wild type mice was difficult to pinpoint due to the naturally low amount of sprouting seen in wildtype mice. PTEN deletion is known to elevate sprouting to above baseline levels through activation of the AKT/mTOR pathway. Leveraging this genetic tool to induce sprouting allows us to assess the effects of DLK and LZK deletion in a setting where CST sprouting is enhanced. The key difference between induced sprouting and compensatory sprouting is the enhanced growth capacity of neurons seen in the induced scenario. As previously demonstrated, we showed that mice that underwent PTEN deletion had considerably more sprouting than wildtype mice. By comparison, we found that triple deletion DLK/LZK/PTEN^{cKO} results in wildtype levels of sprouting, but double deletion of LZK/PTEN results in sprouting similar to PTEN deletion. Similar to what was seen in the regeneration studies, we found that DLK/LZK/PTEN^{cKO} and LZK/PTEN^{cKO} mice exhibited elevations of pS6 in the injected hemisphere comparable to what is seen in PTEN^{cKO} mice. Additionally, we found that across all genetic conditions, the pyramidotomy model led to an increase in p-c-Jun expression in the nuclei of Layer V CSMNs ipsilateral to the side of injury,

confirming that these injury models do result in expression of downstream markers of the DLK/LZK signaling cascade.

5.2 Experimental outline

DLK and LZK floxxed lines were bred to a PTEN floxxed mouse line to generate LZK/PTEN$^{fl/fl}$, and DLK/LZK/PTEN$^{fl/fl}$ mouse lines. In the presence of AAV-Cre, recombinase activity leads to the conditional knockout of targeted genes. LZK/PTEN^{cKO} and DLK/LZK/PTEN^{cKO} were compared to PTEN^{cKO} and GFP-injected littermate control mice. Right-sided cortical AAV injections described previously in chapter 2, were conducted in 6-week old mice. These mice were then subjected to unilateral pyramidotomy surgeries targeting the left medullary pyramid at 8 weeks. Then at 10 weeks of age, the right side forelimb motor cortex was injected with anterograde tracer BDA to trace midline-crossing axons at the level of the cervical spinal cord.

Mice were sacrificed at 12 weeks, and transverse sections of cervical spinal cord tissue was processed for PKCγ immunohistochemistry to assess completeness of injury. In animals were the lesion was complete, BDA amplification was used to identify midline crossing axonal sprouts. Midline crossing axons were manually quantified at the level of the C5-C7 spinal cord at specified distances away from the midline. These measurements were normalized to total axon counts in acquired from sections collected within the rostral medulla, and presented here as Sprouting Indices as a function of distance away from the midline.

Brain tissue was stained using slide-mounted IHC for p-c-Jun, pS6, NeuN and Ctip2. pS6 signal was measured by calculating a composite Intensity Index for each hemisphere, in which average signal intensity above threshold was multiplied by the percent of area above threshold for a given region of analyzed. This index was calculated for both hemispheres, and a ratio was calculated by dividing the intensity index of the AAV-injected hemisphere by the uninjected hemisphere. For full methods, see chapter 2. For these studies, p-c-Jun was qualitatively assessed in injured versus uninjured CST tracts.

5.3 Results

5.3.1 Double deletion of DLK and LZK in a PTEN-deleted background blocks the effect of PTEN-deletion on axonal sprouting

In the previous chapter, I investigated whether DLK and LZK deletion altered sprouting phenotypes in otherwise wildtype mice. The findings from that series of experiments suggested that DLK and LZK have redundant functions in axonal sprouting, and that double deletion of both kinases trends towards reduced sprouting to below wildtype measures. In asking what happens to sprouting in DLK/LZK deleted mice when PTEN is also deleted, we are addressing two main ideas: 1) co-deletion of DLK/LZK suppresses spontaneous sprouting, but are these kinases required for induced sprouting? And 2) If the effect of deletion of either DLK or LZK is to reduce sprouting, this effect may be easier to discern under conditions of elevated basal sprouting, such as in PTEN-deletion.

As expected, deletion of PTEN enhanced sprouting relative to wildtype mice. Midline crossing axons fanned out across the grey matter butterfly of the transversely sectioned c-spine, reaching to both the dorsal and ventral horns. The majority of these sprouting axons reached roughly 300-400 μm away from the midline, but a handful of sprouting axons did extend as far as 700-800 μm away from midline. In both DLK/LZK/PTEN^{cKO} and wildtype mice, fewer far-reaching midline crossing axons were visualized in the vast majority of sections analyzed, although some sections appeared to exhibit sprouting levels comparable to PTEN-deleted mice, making it difficult to identify genotype by looking at individual sections.

Quantification of sprouting in this study revealed that the deletion of DLK and LZK blocked the increased sprouting phenotype commonly seen with PTEN deletion. Although sprouting was not below baseline as was seen in DLK/LZK^{cKO} mice, sprouting in DLK/LZK/PTEN^{cKO} mice was indistinguishable from wildtype mice. PTEN^{cKO} mice, as expected, showed significantly elevated sprouting at 50-500 μm away from midline (Fig. 5.1 A,B: 2-way RM ANOVA $F(2, 33) = 5.422$, p=0.0092, Bonferroni test for multiple comparisons: $p = 0.0014$ at 50 μm, $p = 0.0160$ at 100 μm,

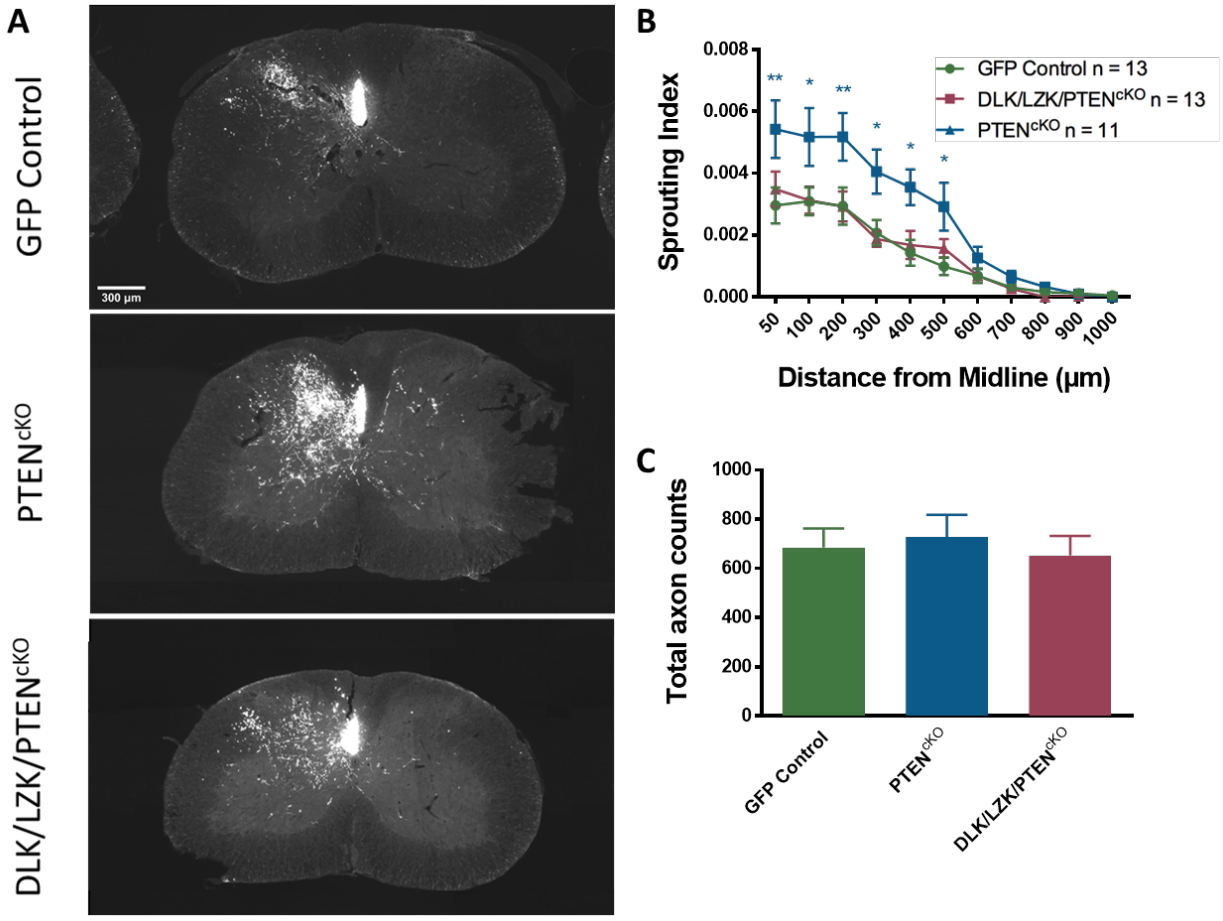


Figure 5.1 Sprouting of CST axons in DLK/LZK/PTEN^{cKO} after unilateral pyramidotomy.
A: Representative images of BDA labeling staining (white) in GFP controls, PTEN^{cKO}, and DLK/LZK/PTEN^{cKO} mice. B: Quantification of sprouting. Sprouting index indicates the ratio of the average number of axons counted at each distance past midline relative to the number of fibers labeled in the medulla (mean±SEM). 2-way RM ANOVA with Bonferroni post-test corrections, $F(2, 33) = 5.422$, $p = 0.0092$ main effect of genetic condition, $^{*}p < 0.05$, $^{**}p < 0.01$. C: Quantification of total axon counts in medullas; 1-way ANOVA, *n.s.*

p = 0.062 at 200 µm, p = 0.0292 at 300 µm, p = 0.0130 at 400 µm, p = 0.0369 at 500 µm). Analysis of total axons traced at the level of the medulla revealed no significant differences in labeling (Fig. 5.1C).

5.3.2 LZK is dispensable for PTEN-deletion induced sprouting

To further probe the specific roles of DLK and LZK in induced sprouting, we compared sprouting in LZK/PTEN^{cKO} mice relative to PTEN^{cKO} mice. Per Chapter 3, deletion of LZK and PTEN permitted robust axon regeneration past the lesion site, which was not seen in the DLK/LKZ/PTEN^{cKO} mice. This finding led to the hypothesis that DLK, and not LZK, plays a more critical role in axonal regeneration, although this claim must be further confirmed in DLK/PTEN^{cKO} mice.

Qualitatively, LZK/PTEN^{cKO} mice almost appeared to have more sprouting than even PTEN^{cKO} mice at distances farther away from the midline. Visually, it appeared as though while fewer midline crossing axons were identifiable within the first 50-100 µm, the axons that did cross over seemed to branch widely to deeply penetrate into the grey matter of the denervated region. Quantitatively, we see here in the present study that LZK/PTEN^{cKO} mice show elevated levels of sprouting over GFP controls, comparable to PTEN^{cKO} mice (Fig. 5.2 A,B). A 2-way RM ANOVA revealed a significant main effect of genetic condition ($F(2, 29) = 10.46$, $p = 0.0004$), and Bonferroni's correction for multiple corrections revealed significantly elevated sprouting in both PTEN^{cKO} and LZK/PTEN^{cKO} mice at distances 50-500 µm away from the midline (at 50 µm, PTEN^{cKO} p = 0.0001, LZK/PTEN^{cKO} p = 0.0040; at 100 µm, PTEN^{cKO} p = 0.0003, LZK/PTEN^{cKO} p < 0.0001; at 200 µm, PTEN^{cKO} and LZK/PTEN^{cKO} p < 0.0001; at 300 µm, PTEN^{cKO} p = 0.0029, LZK/PTEN^{cKO} p < 0.0001; at 400 µm, PTEN^{cKO} and LZK/PTEN^{cKO} p < 0.0001; at 500 µm, PTEN^{cKO} p = 0.0021, LZK/PTEN^{cKO} p < 0.0063). 1 way ANOVA of total axons traced at the level of medullas revealed no significant differences in labeling (Fig. 5.2 C).

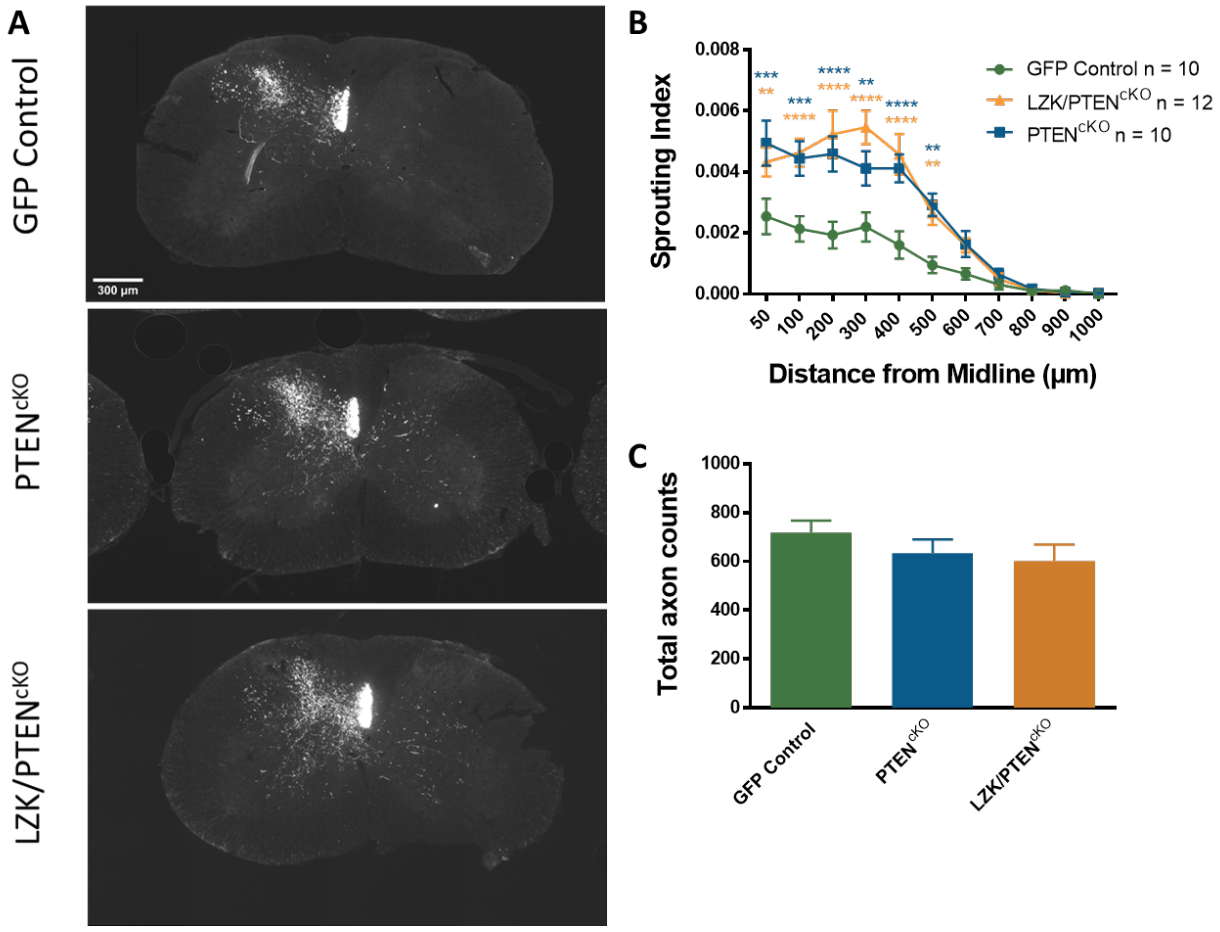


Figure 5.2 Sprouting of CST axons in LZK/PTEN^{cKO} after unilateral pyramidotomy.
A: Representative images of BDA labeling staining (white) in GFP controls, PTEN^{cKO}, and LZK/PTEN^{cKO} mice. B: Quantification of sprouting. Sprouting index indicates the ratio of the average number of axons counted at each distance past midline relative to the number of fibers labeled in the medulla (mean±SEM). 2-way RM ANOVA with Bonferroni post-test corrections, $F(2, 28) = 9.729$, $p = 0.0006$ main effect of genetic condition, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. C: Quantification of total axon counts in medullas; 1-way ANOVA, *n.s.*

5.3.3 Deletion of DLK and LZK does not block the elevation of pS6 in response to PTEN-deletion

Deletion of PTEN is known elevate pS6 via disinhibition of the AKT/mTOR pathway. Presence of pS6 elevation implies a state of enhanced growth. In Chapter 3, we showed that injured neurons that have undergone PTEN deletion show elevation in pS6 signaling regardless of DLK/LZK knockout. In the present study, we were curious if this phenotype is consistent in uninjured neurons, as the injected hemisphere in this study that undergo genetic manipulation are uninjured sprouting neurons. The pattern of pS6 staining in the present sprouting cohort was comparable to what was seen in the regeneration studies. $PTEN^{cKO}$ mice demonstrated a characteristic elevation of pS6 signal broadly throughout the injected regions, stretching from Layers 2/3 to Layer 6 neurons, which has been previously reported (Geoffroy et al., 2015). $LZK/PTEN^{cKO}$ and $DLK/LZK/PTEN^{cKO}$ mice were indistinguishable from $PTEN^{cKO}$ mice, as well. We further saw similar elevations of pS6 signal in $DLK/LZK/PTEN^{cKO}$ mice, implying that deletion of these upstream MAP3K molecules has no impact on AKT/mTOR signaling subsequent to PTEN deletion (see Fig. 5.3). 1-way ANOVA revealed main effect of genetic condition ($F(3, 15) = 4.275$, $p = 0.0228$). Taken together, these findings mirror the findings from the regeneration cohort: deletion of DLK and LZK do not alter the effect of PTEN-deletion on AKT/mTOR signaling in uninjured neurons, as measured by proxy of elevated pS6 staining.

5.3.4 Unilateral pyramidotomy injury induces p-c-Jun expression in the CSMNs in injured tract

It is known that, in response to injury, DLK/LZK signaling activates a series of MAPKs, which can eventually lead to upregulation of downstream phosphorylated c-Jun (p-c-Jun) (Jin & Zheng, 2019; Yuan et al., 2010, 2014). We were curious whether the pyramidotomized mice would exhibit such elevations in p-c-Jun in either the injured or uninjured tracts across all three genotypes, as this would lend credence to the possibility that non-injured cells are able leverage

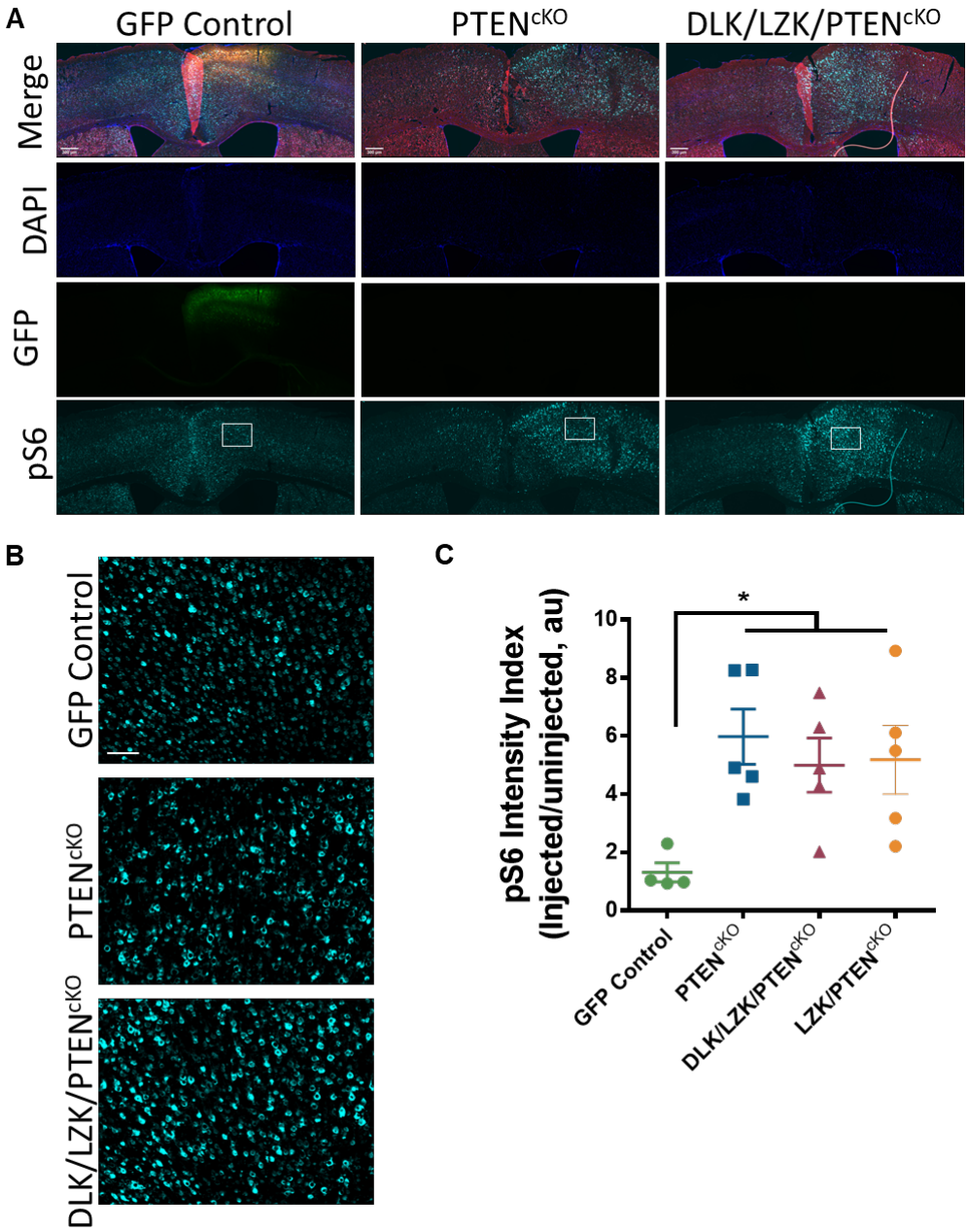


Figure 5.3 PTEN-deletion induces elevated pS6 in DLK/LZK knockout.

A: Representative images of pS6 stained in cortex in GFP controls, PTEN^{cKO} and DLK/LZK/PTEN^{cKO} mice, scale bar 300 μm. B: Representative images taken at 20x magnification of pS6 signal within white-boxed regions in A, scale bar 100 μm. C: Quantification of pS6 signal for GFP controls, PTEN^{cKO}, DLK/LZK/PTEN^{cKO} and LZK/PTEN^{cKO}.

the same signaling pathway as injured neurons to elicit a cellular response to injury, and if so, whether genetic manipulations can alter this p-c-Jun readout. p-c-Jun is elevated maximally shortly after injury in neurons, with the signal disappearing over time (Repici et al., 2007; Yuan et al., 2010). Given the relatively long (four-week) time frame between injury and tissue histology, it was not entirely clear a priori if we would even be able to visualize changes in p-c-Jun in response to the pyramidotomy lesion in the injured CST. By co-staining for NeuN and p-c-Jun, we were able to assess neuronal expression of p-c-Jun specifically. It should be noted that in this analysis, the uninjured side of the CST had underwent genetic manipulation via AAV injection, while the injured side did not.

Across all three genotypes, we visualized dense, nuclear staining of p-c-Jun in the injured but genetically unmanipulated hemisphere that co-labeled with NeuN among Layer V neurons in sections where cell bodies would have axons projecting to the forelimb. Interestingly, in some wildtype mice, we also were able to visualize the same pattern of staining in the GFP-injected uninjured hemisphere, although the total number of p-c-Jun labeled cells were lower than the injured hemisphere (see Fig. 5.4 & 5.5, white arrowhead indicates p-c-Jun positive nuclei). In $PTEN^{cKO}$ and DLK/LZK/$PTEN^{cKO}$ mice, the presence of these dense staining nuclei was rarer although not nonexistent, and in their place emerged a more dim, diffuse staining pattern (Fig. 5.5). Quantification of neurons with high signal intensity for p-c-Jun revealed that DLK/LZK/$PTEN^{cKO}$ had significantly fewer high p-c-Jun expressors in the uninjured tract compared to wildtype, which was expected given the deletion of upstream kinases. To our surprise, $PTEN^{cKO}$ mice appeared more similar to DLK/LZK/$PTEN^{cKO}$ mice than wildtype mice. While further investigation of this effect is required, if this phenotype holds, it would imply that PTEN-deletion may impact the expression of p-c-Jun. As of now, we cannot exclude the possibility that this pattern of staining is reflective of the PTEN deletion present in both genotypes. Across multiple models and using various Cre-promoters, PTEN deletion is known to result in increased

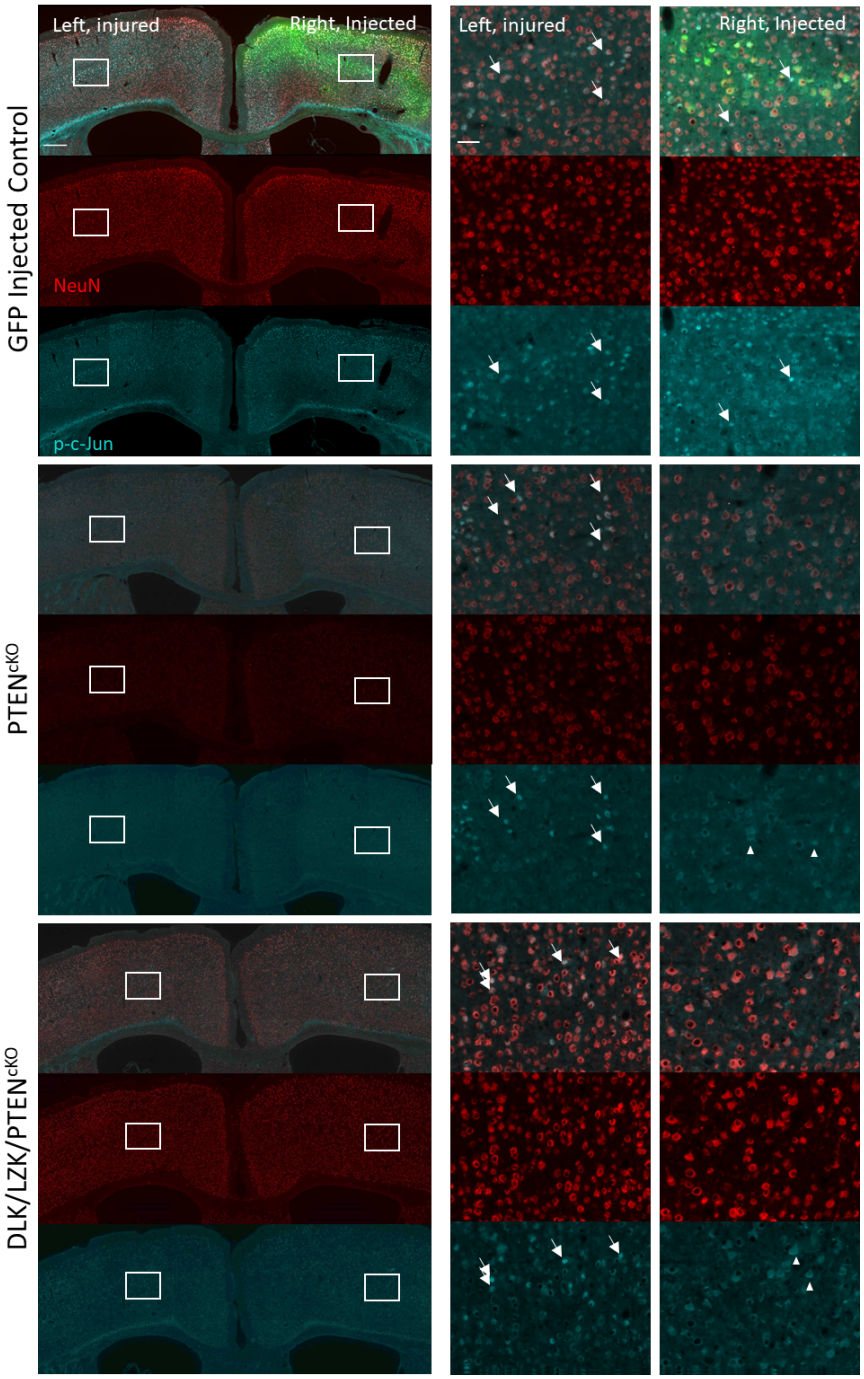
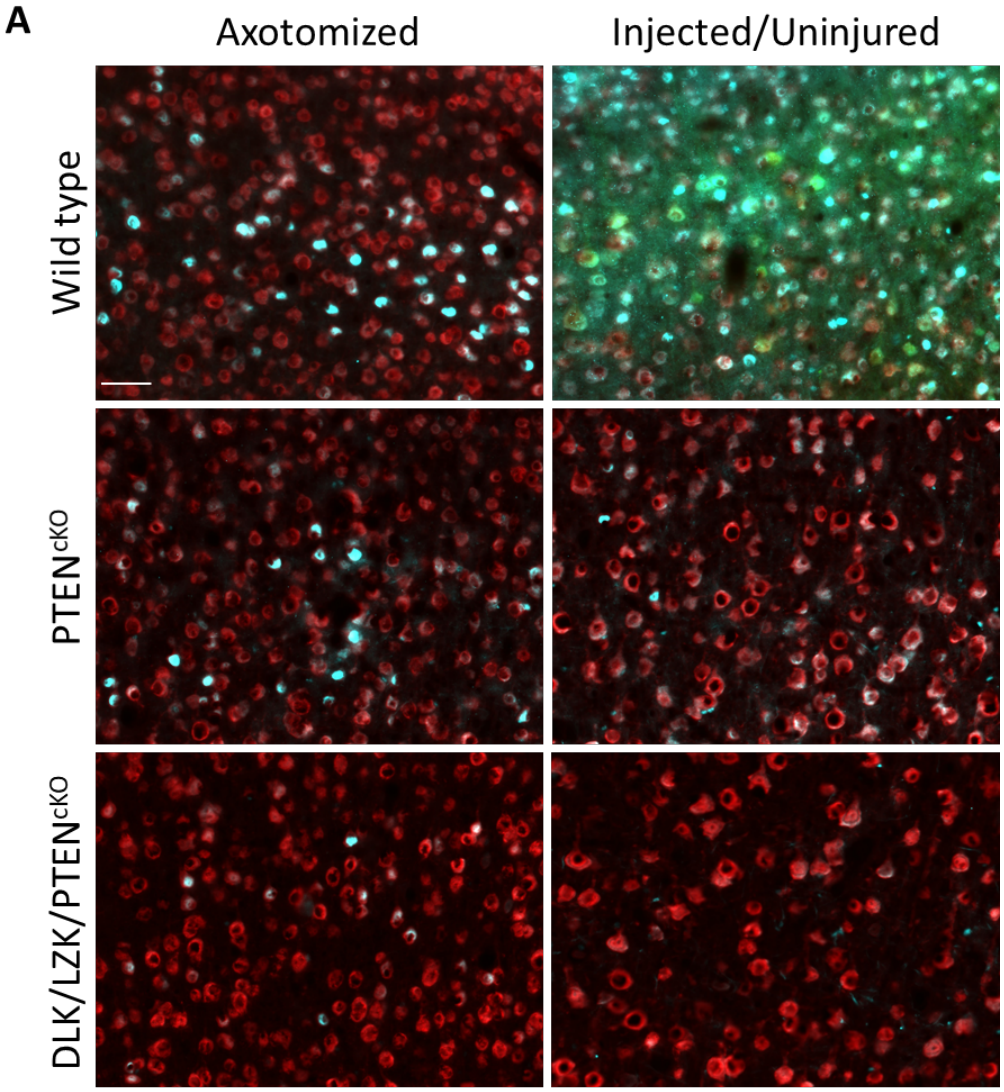
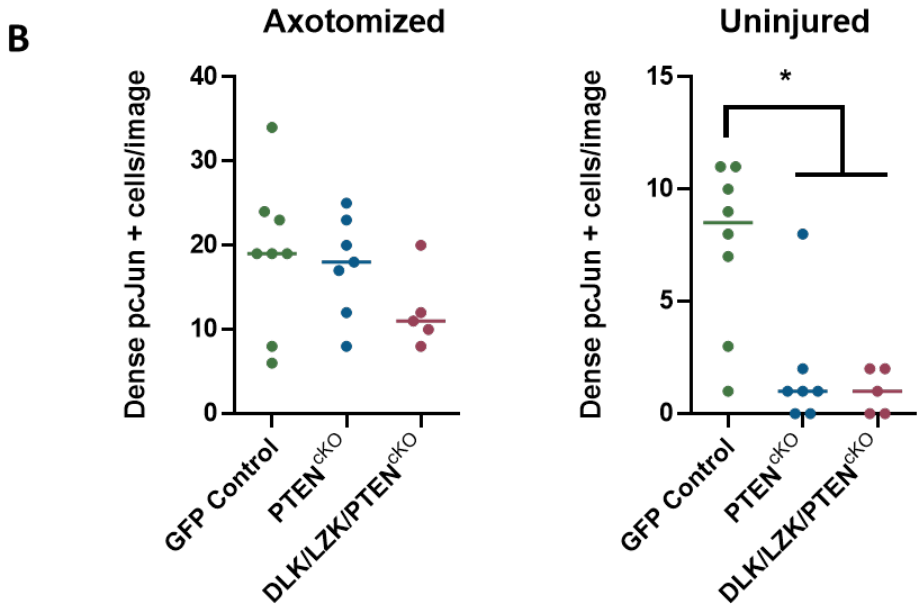


Figure 5.4 Unilateral pyramidotomy induces p-c-Jun expression in injured CSMNs. Across GFP-injected controls, PTEN^{cKO} and DLK/LZK/PTEN^{cKO} mice, we are able to visualize clear expression of p-c-Jun in the injured hemisphere (left, scale bar 300 μm), but expression of p-c-Jun is not as consistent in the AAV-injected hemisphere. This implies demonstrates that injury at the level of the medulla is able to trigger a MAP3K signaling cascade in the CSMNs that is restricted to the injured hemisphere. White boxes on indicate zoomed in regions, depicted on the right (scale bar 50 μm). Arrowheads indicate dense nuclear-localized p-c-Jun+ cells, while triangles indicate more diffuse-staining p-c-Jun.

Figure 5.5 Dense nuclear p-c-Jun staining in wild type, PTEN^{cKO} and DLK/LZK/PTEN^{cKO} mice

A: Images taken at 20x of both injured and uninjured CSMNs in layer 5. Across GFP-injected controls, PTEN^{cKO} and DLK/LZK/PTEN^{cKO} mice, we are able to visualize clear expression of p-c-Jun in the axotomized hemisphere (left), but expression of p-c-Jun is not as consistent in the AAV-injected hemisphere. In general, wildtype GFP-injected uninjured neurons expressed brighter, denser p-c-Jun signal compared what is observed in PTEN^{cKO} and DLK/LZK/PTEN^{cKO}. However, PTEN^{cKO} and DLK/LZK/PTEN^{cKO} uninjured CSMNs appeared to have elevated levels of weakly positive CSMNs. B: Quantification of number of brightly labeled nuclear p-c-Jun signal in either injured (left) or uninjured (right) hemisphere. 1-way ANOVA revealed significant differences between groups (p val = 0.0038); Dunn's test for multiple comparisons revealed significant differences between PTEN^{cKO} and wildtype (p-val = 0.0172) as well as between DLK/LZK/PTEN^{cKO} and wildtype (p-val = 0.0188).


A
Axotomized
Injected/Uninjured
Wild type
PTEN cKO
DLK/LZK/PTEN cKO


B
Axotomized
Uninjured
Dense pcJun + cells/image
40
30
20
10
0
15
10
5
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GFP Control
PTEN cKO
DLK/LZK/PTEN cKO

cell size (L. Li et al., 2003). The lack of stereotypically dense p-c-Jun staining may be an effect of signal dilution resulting from enlarged cells following PTEN deletion; accordingly, p-c-Jun may be more diffusely expressed, leading to an overall lower signal. However, from this preliminary assessment of p-c-Jun signaling, it is clear that the pyramidotomized CST neurons particularly are able to strongly express p-c-Jun four weeks after injury, leaving this surgical model as a potential tool by which changes in downstream mediators of DLK and LZK signaling can be more rigorously tested, e.g without the complication resulting from PTEN deletion. Due to the rather ambiguous p-c-Jun signal in the injected hemispheres of PTENcKO and DLK/LZK/PTENcKO mice, it is hard to draw any clear conclusion about changes in p-c-Jun signal as a consequence of these genetic manipulations.

5.4 Discussion and future directions

The study of DLK and LZK signaling in sprouting, as compared to regeneration, is a little less straightforward, in that DLK and LZK are canonically described as injury sensors after their initial role in development and neuronal migration. As sprouting leverages plasticity in uninjured neurons to respond and compensate for injured neurons, it was unclear whether or not DLK and LZK would even be relevant in this response-to-injury response. From the DLK/LZK co-deletion study described in the previous chapter, it seems that DLK and LZK are possibly fully redundant in their roles for compensatory sprouting.

In the present study, we show that double deletion of DLK and LZK in a PTEN-deleted background blocks the enhanced sprouting effects usually seen with PTEN deletion, despite elevated pS6 signal levels in the brain. This result strongly suggests that while PTEN deletion is able to enhance sprouting, this induction of sprouting relies on the activity of DLK/LZK signaling. As with the regeneration, it appears as though the PTEN and DLK/LZK pathways function in parallel, but both are required for induced sprouting. Given that double deletion of LZK and PTEN resulted in sprouting that is comparable to PTEN deletion alone, LZK is dispensable for induced

sprouting. However, further study using DLK/PTEN knockout mice will be necessary to ascertain whether DLK is the more critical of the two kinases for the induced sprouting, or DLK/LZK as a whole is required but either kinase can be compensated by the other and thus is dispensable. Given the results from co-deletion of the two kinases in compensatory sprouting (Chapter 4), which resulted in sprouting that trended lower than wildtype mice, we hypothesize that, at least for sprouting, DLK and LZK have redundancy and are able to compensate for each other when only one kinase is deleted. Our present results comparing DLK/LZK/PTEN^{cKO} and LZK/PTEN^{cKO} to PTEN^{cKO} further support this hypothesis, since the triply deleted mice showed wildtype levels of sprouting while the LZK/PTEN knockouts were indistinguishable from PTEN single knockouts. Of course, as with the regeneration studies, the relative importance of DLK versus LZK in induced sprouting can only be further ascertained by a study conducted in DLK/PTEN mice.

The major takeaway from these series of experiments is that DLK and LZK are active in uninjured axons for both compensatory and induced models of sprouting. The relationship between these kinases and the PTEN pathway appears to be parallel, although it is presently unclear whether there may be cross-talk between signaling molecules in these two pathways. From the pS6 data, it is evident that deletion of DLK and LZK do not significantly interfere with the expected pS6 elevation subsequent to PTEN deletion via disinhibition of the AKT/mTOR pathway. But the reverse scenario regarding the effect of PTEN deletion on MAP3K signaling cannot be fully assessed from the present experiment, due to the caveats of staining for p-c-Jun in PTEN-deleted cells.

A clearer phenotype regarding changes in DLK and LZK signaling in uninjured sprouting axons may be more prominent in mice that do not also have a PTEN-deletion (e.g. DLK/LZK^{cKO} mice), so as to limit the observed biological effect on p-c-Jun expression that may be a result of the underlying PTEN-deletion. Additionally, analyzing p-c-Jun in DLK/LZK double knockouts can also serve to provide some baseline when comparing the expression between PTEN^{cKO} and DLK/LZK/PTEN^{cKO}. To further investigate the relationship between PTEN and MAP3K signaling,

it would be interesting to see how PTEN deletion affects p-c-Jun expression in DLK/LZK deleted injured neurons, given p-c-Jun's inherent relationship to injury signaling. As p-c-Jun is maximally elevated within a very short time frame after injury, it would be helpful to also choose an injury timeline that captures greater p-c-Jun expression (Repici et al., 2007; Yuan et al., 2010).

Given the literature that had shown that DLK is critical for initiating both pro-apoptotic and pro-regeneration transcriptional programs (Watkins et al., 2013), we had initially hypothesized that DLK and LZK would be critical for AKT/mTOR and downstream pS6 elevation. However, PTEN deletion in the presence of DLK/LZK co-deletion still resulted in the pS6 elevation that has been associated with a high level of regenerative competence (Lu et al., 2014). The findings from the present study demonstrate that DLK/LZK signaling as a whole is essential for the robust CST axon sprouting induced by PTEN deletion. While the current results indicate that LZK can be spared it remains to be seen if DLK can be spared as well, thus illuminating the functional redundancy between the two kinases. A key goal for the future would be to delineate the mechanism by which DLK and LZK signaling are involved in axonal repair in both sprouting and regeneration models.

Acknowledgement

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References

Geoffroy, C. G., Lorenzana, A. O., Kwan, J. P., Lin, K., Ghassemi, O., Ma, A., Xu, N., Creger, D., Liu, K., He, Z., & Zheng, B. (2015). Effects of PTEN and Nogo codeletion on corticospinal axon sprouting and regeneration in mice. *Journal of Neuroscience*, *35*(16), 6413–6428. https://doi.org/10.1523/JNEUROSCI.4013-14.2015

Jin, Y., & Zheng, B. (2019). *Annual Review of Cell and Developmental Biology Multitasking: Dual Leucine Zipper-Bearing Kinases in Neuronal Development and Stress Management*. https://doi.org/10.1146/annurev-cellbio-100617

Li, L., Liu, F., & Ross, A. H. (2003). PTEN regulation of neural development and CNS stem cells. In *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.10312

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature Neuroscience*, *13*(9), 1075–1081. https://doi.org/10.1038/nn.2603

Lu, Y., Belin, S., & He, Z. (2014). Signaling regulations of neuronal regenerative ability. In *Current Opinion in Neurobiology* (Vol. 27, pp. 135–142). Elsevier Ltd. https://doi.org/10.1016/j.conb.2014.03.007

Repici, M., Centeno, C., Tomasi, S., Forloni, G., Bonny, C., Vercelli, A., & Borsello, T. (2007). Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. *Neuroscience*, *150*(1), 40–49. https://doi.org/10.1016/j.neuroscience.2007.08.021

Watkins, T. A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J. S., Tessier-Lavigne, M., & Lewcock, J. W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(10), 4039–4044. https://doi.org/10.1073/pnas.1211074110

Yuan, Q., Hu, B., Wu, Y., Chu, T. H., Su, H., Zhang, W., So, K. F., Lin, Z., & Wu, W. (2010). Induction of c-Jun phosphorylation in spinal motoneurons in neonatal and adult rats following axonal injury. *Brain Research*, *1320*, 7–15. https://doi.org/10.1016/j.brainres.2010.01.038

Yuan, Q., Su, H., Guo, J., Wu, W., & Lin, Z.-X. (2014). Induction of phosphorylated c-Jun in neonatal spinal motoneurons after axonal injury is coincident with both motoneuron death and regeneration. *Journal of Anatomy*, *224*(5), 575–582. https://doi.org/10.1111/joa.12165

Chapter 6:

Conclusion

The goal of identifying different molecular pathways and mechanisms involved with axonal repair is inherently tied with the clinical need for improving and broadening therapeutic options for individuals burdened with axonal CNS injury. While we in our lab focus heavily on the spinal cord as our model system, understanding different forms of axonal repair, and the signals that contribute to repair informs the regeneration field more broadly. The work presented in this dissertation is an extension of research conducted in worms (Hammarlund et al., 2009; Yan et al., 2009), as well as different models of nerve injury models in rodents, in order to identify how key genes are involved with CNS injury and repair. The identification of DLK-1 as being crucial for axon repair in worms has lent itself to the study of mammalian DLK in peripheral nerve injury in the rodent sciatic crush model, as well in CNS injury using the optic nerve crush model (Shin et al., 2012; Watkins et al., 2013; Welsbie et al., 2017).

The goal of this thesis, then, was to explore the effects of DLK and LZK conditional knockout on multiple models of axonal repair in the corticospinal tract. By leveraging two different surgical models of injury, we were able to investigate the effect of deleting these kinases in both injured and uninjured axons. The key takeaways from this series of experiments is firstly that co-deletion of DLK and LZK limited the effects of PTEN deletion on both regeneration and induced sprouting, and secondly, the effect of DLK/LZK co-deletion had no impact on the elevation of pS6 in response to PTEN deletion. Taken together, these findings demonstrate that both the disinhibition of AKT/mTOR pathway via PTEN deletion and functional activity of DLK/LZK signaling are required for CST axonal repair. To date, our study in regeneration is the first demonstration of failed axon regeneration in mice that exhibit elevated levels of pS6 in response to PTEN deletion, a finding which is in alignment with a previous study in the optic nerve that showed that deletion of PTEN did not enhance regeneration in DLK deleted mice (Watkins et al., 2013). This finding shows that pS6 elevation alone is not sufficient to drive regeneration in the CST.

Interestingly, LZK/PTEN co-deletion led to a phenotype that was identical to PTEN deletion alone for both PTEN-deletion induced regeneration and sprouting, implying that LZK is dispensable for these processes. It remains to be seen if DLK is also dispensable, in which case, DLK and LZK would be functionally redundant in supporting the regeneration seen in PTEN deleted mice; conversely, if DLK alone is required for PTEN deletion-induced CST axon regeneration, we would expect to see little to no regeneration in DLK/PTEN double mutants. It is also possible that co-deletion of DLK and PTEN will produce an intermediate phenotype, suggesting that LZK can partially compensate for DLK deletion.

The relative effect of individual kinase deletion is still an incomplete story, but the present data indicate that LZK in particular is dispensable for these findings. However, a follow-up study in DLK/PTEN co-deleted mice is necessary to determine whether DLK is fully redundant with LZK. While DLK and LZK single mutants do not significantly alter compensatory spontaneous sprouting, co-deletion of both kinases shows a trend for a decrease in sprouting to below baseline levels, indicating that these kinases may share some redundancy in this mechanism of repair. Further exploration of gene expression in single versus double knockouts are necessary to truly confirm the redundancy of these two kinases.

PTEN deletion is not sufficient for regeneration

The major finding of Chapter 3 is that deletion of DLK, LZK and PTEN results in WT levels of regeneration, i.e. no detectable regeneration. Historically, PTEN deletion was identified to cause robust regeneration in CST axons, as well as in many other cell types. The effect of PTEN deletion on enhancing regenerative competence has been established as a gold-standard for axon regeneration studies, with many experiments investigating whether other genetic manipulations in addition to PTEN deletion enhances regeneration (Lu et al., 2014). We were surprised to see such a clear-cut loss of regenerative phenotype in these triply deleted mice.

Thus, we wondered whether the effect of DLK and LZK co-deletion attenuated the effects of PTEN deletion on AKT/mTOR signaling.

Previous literature has strongly suggested that mammalian DLK is crucial for retrograde injury signaling from the axon to the cell soma. This retrograde signal, in turn, would initiate response-to-injury programs that are cell type and injury type specific. For example, in the sciatic nerve, where regeneration is a naturally occurring phenomenon, injury induces a DLK-dependent transcriptome, which largely includes genes involved with axon regeneration (Shin et al., 2019). In the optic nerve, deletion of DLK prior to crush injury stunts both pro-apoptotic and pro-regenerative gene expression changes (Watkins et al., 2013). Given this framework, we had hypothesized that the deletion of DLK and LZK might interfere with the effect of PTEN deletion on AKT/mTOR activation, which is one of the key signaling mechanisms involved with axon regeneration (Liu et al., 2010; Park et al., 2008). To answer this question, we investigated pS6 expression in DLK/LZK/PTEN^{cKO} and PTEN^{cKO} mice compared to wildtype, using pS6 as a proxy for AKT/mTOR activation. To our surprise, all knockout genotypes had an average of 4-5x increase in pS6 measured in the PTEN-deleted hemisphere, regardless of whether DLK or LZK was also co-deleted. To our knowledge, this is the first demonstration of pS6 elevation not resulting in CST regeneration. This led us to revise our hypothesis: DLK/LZK signaling is not upstream but in a parallel pathway to AKT/mTOR, and in order for CST axonal repair to occur, both pathways must be activated and functional. Activating mTOR alone is insufficient for axon regeneration.

Effects DLK and LZK deletion on compensatory and induced sprouting

The fourth and fifth chapters of this dissertation, while presented separately, are closely related in terms of the question they seek to answer: do DLK and LZK have a role in mediating axonal repair in uninjured axons? Initially, the main rationale to investigate this arose from the general concept that molecular factors involved in regeneration are also involved in sprouting.

However, DLK in particular is known to become activated in response to injury, and our model for sprouting investigates the cellular response of uninjured neurons.

Fundamentally, both compensatory and induced sprouting investigates the response of spared neurons in CNS repair. Conceptually, the key difference between spontaneous compensatory sprouting and induced sprouting is the relative regenerative competence of the spared neurons. While sprouting can occur under wildtype conditions, the baseline level of sprouting is low due to the inherently low regenerative competence of adult CNS neurons. By comparison, using a mechanism of induction, such as the genetic deletion of PTEN, can robustly enhance sprouting of spared uninjured neurons in response to injury. With induced sprouting, it is possible to clarify otherwise subtle effects that can be missed by looking at just spontaneous sprouting. Given that the roles of DLK and LZK in sprouting of uninjured neurons is fairly unexplored territory, with the bulk of literature focusing exclusively on axon regeneration or cell death in response to direct cellular injury, we decided to explore deletion of these kinases both in a wildtype and PTEN-deleted background.

By interpreting the results from both compensatory and induced sprouting together, we can appreciate a fuller picture of the roles of these kinases in uninjured neurons: in the wildtype background, we observed no change in sprouting phenotype when deleting either one of the two kinases, but co-deletion produced a statistically trending effect of lowered sprouting compared to wildtype mice. With the PTEN deletion, we observed that triply deleted mice showed wildtype levels of sprouting, significantly lower than what was observed in PTEN deleted mice. Meanwhile, co-deletion of LZK/PTEN resulted in sprouting that was largely comparable to PTEN deletion alone. While we still need to explore the DLK/PTEN knockout, but the evidence so far supports that DLK and LZK are involved in the sprouting phenotype observed in uninjured neurons. Though LZK is dispensable for induced sprouting, the planned follow-up experiments in DLK/PTEN knockouts can clearly answer whether DLK is the more critical kinase, or if the two kinases can be compensated by one another, requiring both to be deleted to interfere with sprouting.

Again, these findings largely parallel the regeneration results, wherein LZK/PTEN co-deletion regenerated in a manner comparable to PTEN deletion, but triply deleted mice failed to regenerate entirely, both rostrally and caudally to the site of injury. Taken together, the DLK/LZK signaling pathway is critical for neuronal response to injury in both injured and uninjured neurons.

Do PTEN and DLK/LZK pathways interact with each other?

Across multiple chapters of the dissertation, we attempted to understand the relationship (if there is one) between DLK/LZK signaling and the PTEN pathway. We attempted to investigate this by looking at downstream markers of the MAP3K and AKT/mTOR pathways, respectively. In analyzing downstream markers, we sought to investigate whether deletion of DLK/LZK impacted the PTEN pathway and vice versa. In both the regeneration and induced sprouting model, we assayed the expression of pS6 to interrogate the effect of DLK/LZK deletion on AKT/mTOR activity following PTEN-deletion. As described earlier, we were surprised to see consistently profound elevations in pS6 expression within the AAV-Cre injected hemispheres across PTEN, DLK/LZK/PTEN and LZK/PTEN deleted mice for both regeneration and induced sprouting studies. This finding shows that the deletion of DLK/LZK does not obstruct AKT/mTOR signaling.

Understanding the reverse situation has proven to be a considerably harder task. Unlike pS6, which is elevated upon PTEN deletion, no molecule downstream of the MAP3K cascade shows an inverse relationship with DLK and LZK expression, due to the fact that these upstream regulators of the MAP3K cascade are not inhibitory. Nevertheless, we attempted to explore this question by using p-c-Jun expression as a proxy for MAP3K activity. p-c-Jun is known to elevate in response to cellular injury, reaching maximal expression shortly after cellular injury (Repici et al., 2007; Yuan et al., 2010). While its expression can last for a period of time after injury, it generally decreases over time. We decided to probe the brains of mice that underwent pyramidotomy to investigate the relationship between PTEN deletion and p-c-Jun.

Though we were unsure if even this time frame of four weeks would be sufficiently quick to capture differences in p-c-Jun signal, especially since the neurons of interest in these studies were uninjured, we were surprised to see not only clear p-c-Jun signal in the axotomized hemisphere, but also in the uninjured but genetically manipulated contralateral hemisphere, albeit at considerably lower levels. Particularly in our GFP-injected controls, we saw strong dense nuclear p-c-Jun staining in both the injured and uninjured tracts, although the total number of cells in the uninjured hemisphere was lower than that of the injured tract. An interesting and unexpected observation from this preliminary analysis was that $PTEN^{cKO}$ and $DLK/LZK/PTEN^{cKO}$ mice appeared quite similar. Both exhibited far fewer cells with dense nuclear p-c-Jun signals in the AAV-injected hemisphere compared to what was observed in the GFP-injected hemispheres of wildtype controls. Instead, we observed a more dim, diffuse staining pattern in neurons throughout the uninjured but genetically altered hemisphere. This low-expressor pattern of staining was restricted to only the genetically manipulated hemisphere, with dense nuclear p-c-Jun signal observed in the injured tract. At present, we cannot rule out that this pattern may be the effect of PTEN deletion present in both genotypes. If this phenotype holds, it may suggest that PTEN does have some influence on p-c-Jun expression in uninjured neurons. To date, literature spanning the realms of both axon regeneration and cancer signaling pathways have discussed PTEN and MAP3K as two distinct pathways. It would be extremely surprising if there is a relationship between the two, but this initial foray implies that there might be an effect of PTEN deletion on p-c-Jun expression.

Overall, through the various questions investigated in this thesis, we have begun to open the door on demystifying the role of DLK and LZK in axonal repair in spinal cord injury. These findings are merely a first step into understanding the relationship between DLK/LZK signaling and axonal repair. There is much left to be understood; for example, how does co-deletion of DLK and LZK prevent PTEN deletion from inducing regeneration and sprouting? Why and how do uninjured axons upregulate p-c-Jun in response to a nearby CNS injury? Is there a way we can

leverage the DLK/LZK pathway to synergize with PTEN deletion to further enhance axonal repair? Filling the holes in this story will undoubtedly lead to even more questions, but the hope remains that with these findings, we will be able to translate our knowledge of molecular pathways involved in repair to a clinical setting, and provide patients with CNS injury some relief from their otherwise long-term impairments.

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References

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E. M., & Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. *Science*, *323*(5915), 802–806. https://doi.org/10.1126/science.1165527

Liu, K., Lu, Y., Lee, J. K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K. K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., & He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nature Neuroscience*, *13*(9), 1075–1081. https://doi.org/10.1038/nn.2603

Lu, Y., Belin, S., & He, Z. (2014). Signaling regulations of neuronal regenerative ability. In *Current Opinion in Neurobiology* (Vol. 27, pp. 135–142). Elsevier Ltd. https://doi.org/10.1016/j.conb.2014.03.007

Park, K. K., Liu, K., Hu, Y., Smith, P. D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., & He, Z. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science*, *322*(5903), 963–966. https://doi.org/10.1126/science.1161566

Repici, M., Centeno, C., Tomasi, S., Forloni, G., Bonny, C., Vercelli, A., & Borsello, T. (2007). Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. *Neuroscience*, *150*(1), 40–49. https://doi.org/10.1016/j.neuroscience.2007.08.021

Shin, J. E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., & DiAntonio, A. (2012). Dual Leucine Zipper Kinase Is Required for Retrograde Injury Signaling and Axonal Regeneration. *Neuron*, *74*(6), 1015–1022. https://doi.org/10.1016/j.neuron.2012.04.028

Shin, J. E., Ha, H., Kim, Y. K., Cho, Y., & DiAntonio, A. (2019). DLK regulates a distinctive transcriptional regeneration program after peripheral nerve injury. *Neurobiology of Disease*, *127*, 178–192. https://doi.org/10.1016/j.nbd.2019.02.001

Watkins, T. A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J. S., Tessier-Lavigne, M., & Lewcock, J. W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(10), 4039–4044. https://doi.org/10.1073/pnas.1211074110

Welsbie, D. S., Mitchell, K. L., Jaskula-Ranga, V., Sluch, V. M., Yang, Z., Kim, J., Buehler, E., Patel, A., Martin, S. E., Zhang, P. W., Ge, Y., Duan, Y., Fuller, J., Kim, B. J., Hamed, E., Chamling, X., Lei, L., Fraser, I. D. C., Ronai, Z. A., … Zack, D. J. (2017). Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. *Neuron*, *94*(6), 1142-1154.e6. https://doi.org/10.1016/j.neuron.2017.06.008

Yan, D., Wu, Z., Chisholm, A. D., & Jin, Y. (2009). The DLK-1 Kinase Promotes mRNA Stability and Local Translation in C. elegans Synapses and Axon Regeneration. *Cell*, *138*(5), 1005–1018. https://doi.org/10.1016/j.cell.2009.06.023

Yuan, Q., Hu, B., Wu, Y., Chu, T. H., Su, H., Zhang, W., So, K. F., Lin, Z., & Wu, W. (2010). Induction of c-Jun phosphorylation in spinal motoneurons in neonatal and adult rats following axonal injury. *Brain Research*, *1320*, 7–15. https://doi.org/10.1016/j.brainres.2010.01.038