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Molecular Mechanisms of Axon Growth and Regeneration

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Molecular Mechanisms of Axon Growth and Regeneration

by

Dana Watt

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

December 2015

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ABSTRACT OF THE DISSERTATION

Molecular Mechanisms of Axon Growth and Regeneration

by Dana Watt

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Associate Professor Valeria Cavalli, Chair

Neurons are cells with unique and extremely polarized morphologies. The axon allows communication between the cell soma and the distantly located synaptic terminal and can extend up to one meter in humans. This exceptional cellular structure therefore has specialized biological processes dedicated to its growth, maintenance and regeneration. The structure that carries out axon elongation during development and regeneration is the growth cone, which features a cytoskeletal structure that is both highly dynamic yet consistent in overall organization. The growth cone coordinates a leading edge of dynamic actin with the microtubules of the growing axon, enabling directed outgrowth while maintaining the structural integrity of the axon. In the axon, microtubules are oriented uniformly with their plus-ends facing away from the cell body, providing directionality for cargo transport streams. Axonal transport is critical during growth and after the axon has reached its target to ensure proper distribution of organelles and other necessary components of the axonal biology machinery. Axon transport is especially important because the axon is capable of independently carrying out tasks ranging from protein synthesis to synaptic transmission. Therefore a thorough understanding of microtubule biology and axon transport is critical for understanding how axons grow during development and regeneration.

I first investigated the role of a neuron-specific molecular motor adaptor, JIP3, in axon elongation. JIP3 had been previously demonstrated to bind both the anterograde motor kinesin-1
and the retrograde motor complex dynein/dynactin, generating cargo-specific directional transport in the axon. Furthermore, our lab had also demonstrated that JIP3 was capable of binding directly and activating dimeric kinesin heavy chain (KHC) for motility. While the function of dimeric KHC is still debated, tetrameric kinesin-1 is known as the primary anterograde motor in axons. I therefore investigated the role of JIP3 in regulating tetrameric kinesin-1 motility, and the functional significance of this interaction in axons. I used a total internal reflection fluorescence (TIRF)-based single molecule imaging assay to demonstrate that JIP3 binds to the kinesin-1 tetramer in at least a 2:1 ratio. I also used this TIRF assay to show that JIP3 binding to the cargo-adapting kinesin light chain (KLC) activated tetrameric kinesin-1 for microtubule binding, while JIP3 binding to KHC activated tetrameric kinesin-1 motility along microtubules. Furthermore, while the JIP3/KLC interaction is dispensable for axon growth and regeneration in neurons in vitro, the JIP3/KHC interaction is necessary for both these activities. This work demonstrated that a single molecule can activate tetrameric kinesin-1 for microtubule binding and motility. Furthermore, this work demonstrated that regulation of molecular motor activity supports axon growth and regeneration.

I next investigated the small GTPase Ran and its role in axon elongation. Ran is best known for its canonical function as a nuclear transport protein, but it can also stabilize microtubules during mitotic spindle formation and is located in the axon. While Ran has a proposed role in regulating a retrograde injury signaling complex in adult axons, it is unknown whether Ran also regulates cytoskeletal dynamics in developing axons. I used Ran knockdown to demonstrate that Ran promotes axon elongation on growth-promoting substrates, and restricts axon elongation on growth-inhibiting substrates. This effect is at least partially mediated by microtubule dynamics, as microtubule imaging using a cell-permeable pan-tubulin marker
showed decreased microtubule dynamics in Ran knockdown cells on growth-promoting substrates. Furthermore, I demonstrated that a Ran activating protein, RanBP10, is enriched in growth cones and promotes axon elongation, and that both of these phenomena are Ran-dependent. This work raises the exciting possibility that Ran controls microtubule dynamics in the growth cone, distinct from its canonical role in nucleocytoplasmic trafficking.
CHAPTER 1

Introduction: molecular mechanisms of axon growth and regeneration
Introduction

Information in the brain is encoded and transmitted as an electrical signal by neurons, brain cells specialized for long-range communication. For information to travel quickly across long distances, neurons must be capable of rapidly transmitting information at up to 100 meters per second. The neuron accomplishes this feat by extending a cellular process over distances as great as one meter in the human body. This cellular process, called the axon, is a contiguous extension of the cytoplasm. All mature neurons only have one axon, and electrical signaling via the action potential occurs in only one direction: anterogradely, away from the cell soma and towards the axon terminal. While the extreme polarity that the axon conveys on neural morphology is necessary for neuronal function, development and maintenance of this compartment is a challenge. During development, neurons must extend axons across great distances to precise targets. After axon growth, the neuron needs to maintain the health of the axon as it carries out the complex task of long-range signal transduction. Finally, if an axon becomes injured, the neuron carries out a complex set of actions to attempt to regrow the axon and reestablish neural architecture. These processes of axon growth, maintenance and regeneration are accomplished via coordination of

(1) organellar structures which allow the axon to carry out complex cellular functions independently of the cell body,

(2) a temporary structure dedicated to axon growth, the growth cone,

(3) transient transcription programs which are activated or suppressed in response to extracellular signaling cues, and

(4) molecular motors which utilize the polarized microtubule structure of the axon to enable long-term communication between the axon and soma.
The axon coordinates and executes complex cellular functions to support developmental and regenerative growth

Axonal growth, maintenance and regeneration require complex cellular processes to function successfully. Axons need to respond to signals from their postsynaptic partners, environment and somas by transcribing or degrading proteins. And they must generate enough energy to carry out these tasks locally. Accordingly, axons contain much of the machinery localized to the cytoplasm of other cells, but specialized for use in the axon.

The large and relative somatic isolation of the axon makes the conclusion that the axoplasm can carry out protein translation almost obvious; however for a long time the absence of rough endoplasmic reticulum in the axon suggested that the axoplasm was incapable of independent translation (1). Instead, researchers surmised that surrounding glia provided axons with newly synthesized proteins, as they were better able to respond to local conditions than the far-removed cell body (2). However, a growing body of evidence that ribosomes were located in axons, mRNAs located to axons via specialized axon-directed untranslated regions, and that isolated synapses could synthesize proteins strongly suggested that mature axons, and especially the synapse, carry out protein translation (3–8). Further work on developing axons and growth cones supported the conclusion that protein synthesis was an integral axonal function at all stages. Now it is understood that even the growth cone contains the machinery necessary to translate mRNAs into proteins (9). Importantly, not all axonal proteins are locally synthesized; many are transported via axonal transport mechanisms described below. Why might an axon locally translate proteins? One obvious advantage of local translation is its rapidity compared to axon transport, which in some neurons could take days. The Jaffrey group, which has published extensively on the topic of intra-axonal protein synthesis and mRNA translation, has argued that
Axonal translation and protein degradation are linked (10). Proteins that need to undergo rapid turnover in the growing axon, especially those involved in local signaling responses such as growth cone collapse, would therefore be preferentially transcribed in axons. This hypothesis is supported experimentally: during axon growth, local protein synthesis is necessary to specifically enrich cytoskeletal components (11) and their regulators (12), signaling molecules (11, 13), and translational machinery (14). During development, axon-synthesized signaling factors can also contribute to retrograde signaling and subtype specification (15). Protein translation in the axon might also be important for growth cone response to environmental cues, although this conclusion is controversial (16–18). Asymmetrical translation of β-actin mRNA in response to NGF, for example, has been proposed to mediate growth cone turning, discussed in more detail below (19). Axonal protein synthesis is also necessary for axon regeneration (20, 21). In addition to generating proteins necessary to form a new growth cone, newly-transcribed proteins act both as retrograde injury signals (22–24) and to regulate retrograde injury signaling (25). Axonal translational machinery therefore enables not only autonomous protein synthesis in the distal axon, but also novel growth and injury signaling mechanisms.

In addition to carrying out protein translation, axons are also capable of degrading proteins independently of the soma. Autophagy is an especially critical function in neurons, which are postmitotic and therefore cannot rely on dilution and cell death to minimize the effects of misfolded or nonfunctional proteins. Perhaps not surprisingly, mutations in the autophagocytic pathway are often associated with neurodegenerative disease, underscoring the importance of these proteins for axonal homeostasis (26). Developmentally, axonal autophagy seems to be associated with a reduction in axon outgrowth (27). Autophagy is necessary for retrograde signaling and growth cone collapse and turning in response to inhibitory factors (28–30).
However, autophagy is also associated positively with axon growth, as autophagosomal machinery is upregulated in response to outgrowth-stimulating nerve growth factor (NGF) (31). The autophagic machinery supports axon outgrowth by enabling membrane and membrane receptor recycling within the growth cone (32–35). Additionally, maintenance of BiP-mediated autophagy is necessary for axon outgrowth, defasciculation and pathfinding, underscoring the importance of canonical protein quality control in axon outgrowth (36). The divergent effects of axonal autophagy are partially explained by its interplay with other cellular functions. In close cooperation with protein synthesis machinery, axonal autophagy machinery allows precision tuning of growth cone dynamism in response to a range of attractive or repulsive cues (37). In this model, the authors propose that local endocytosis enables rapid desensitization of the growth cone to extracellular cues via internalization of cell surface receptors. Protein-synthesis dependent resensitization of the growth cone then enables the axon to respond to increasing concentrations of chemotactic cues. Different stages of the autophagic pathway therefore play distinct roles in axon growth. Maintaining the machinery that enables localized translational control through protein synthesis and degradation is an energy-intensive task, in addition to the uniquely energy-intensive task of carrying out neurotransmission. The axon therefore also needs to generate energy independently of the soma to survive.

Mitochondria have been recognized as an integral part of the axoplasmic niche for over half a century (38). That axonal mitochondria have been implicated in neurodegenerative diseases highlights their importance in axon biology: Alzheimer’s disease, Parkinson’s disease, hereditary spastic paraplegia, multiple sclerosis, Huntington’s disease, and chemotherapy-induced neuropathic pain, to name a few, have all been linked to dysfunctional axonal mitochondria (39–41). A critical factor of axonal mitochondrial biology is the proper positioning
of mitochondria to sites of high ATP consumption, including the synapse and the nodes of Ranvier (6, 42). Mitochondrial localization along growing axons is particularly associated with growth cones and branch sites, where intensive ATP-dependent actin polymerization, a primary neuronal ATP sink, occurs (43). While growth cones are generally mitochondria-rich, extracellular signals can also stimulate accumulation of mitochondria within the growth cone, as is the case for NGF (44, 45). Mitochondrial enrichment by NGF is necessary for NGF-mediated axon outgrowth, as local enrichment of mitochondria enables the formation of actin patches that are precursors to filopodia (46). Similarly, mitochondrial capture along the length of developing axons dictates placement of axonal branches, as an infusion of energy is necessary to remodel the cytoskeleton and create a new growth cone (6, 42). Terminal axon branching is particularly sensitive to mitochondrial transport, and mitochondrial capture at nascent presynapses is a necessary prerequisite for this phenomenon in cortex *in vivo* (47). Mitochondrial localization can also be affected by its fission/fusion dynamics, and a reduction in mitochondrial fusion decreases axon elongation on permissive substrates by modulating growth cone lamellipodial dynamics (48). Therefore mitochondria provide energy not only for homeostatic axonal functions, but also for the actin polymerization-intensive task of axon outgrowth. While research on mitochondrial function in injured axons focuses primarily on degeneration, it is likely that mitochondrial function during developmental axon elongation and branching are conserved during regeneration, as cytoskeletal remodeling is a common feature of both.

The axon contains all the molecular machinery necessary to function almost independently from the cell body. Organellar structures present in the axon enable protein regulation beginning at the mRNA level and local regulation of metabolites for energy-dependent processes. The complex cellular functions that the axon is capable of enable its
remarkable structure. They particularly support the subcellular structure responsible for carrying out axon growth, the growth cone.

**The growth cone is the structure that carries out axon growth and regeneration**

The growth cone is a temporary structure that forms at the growing axon tip. In developing neurons, nascent growth cones develop at the tips of multiple neurites, and through the process of axon specification a single neurite is selected as the axon, undergoing growth-cone mediated elongation (49). In contrast, regenerating axons form a new growth cone near the severed tip of the injured, and already specified, axon (50). While the process of growth cone formation is distinct during development and regeneration, the biology of the formed growth cone is shared between the two. The growth cone dynamically moves through its environment, extending the tip of the axon while responding to extracellular growth cues. Growth cones thus rapidly change structure, extending and retracting filopodia or lamellipodia in a matter of seconds. This dynamism requires coordination of cytoskeletal dynamics and enables the axon to achieve growth rates of up to 5 mm/day.

The growth cone has stereotyped cytoskeletal structure that maximizes its dynamics while allowing maturation of the axon shaft (Figure 1) (51). Microtubule dynamics are greatly influenced by myriad posttranslational modifications, although the purpose of each of these
modifications is not clear (52). However, microtubule acetylation and tyrosination are well-characterized in growth cone function. In the microtubule-rich axon shaft, microtubules are heavily acetylated. Acetylation stabilizes microtubules, protecting the structural integrity of the shaft so that it can perform the critical function of bidirectional microtubule-based axon transport. In the dynamic body of the growth cone, cytoskeletal structures must rapidly polymerize or depolymerize as the growth cone changes shape. Here, microtubules are primarily tyrosinated, as tyrosination promotes disassembly, and therefore dynamism, of tubulin. The highly dynamic outer fringe of the growth cone is enriched for actin, as actin-rich filopodia and lamellipodia rapidly extend and retract. Coordination of these cytoskeletal elements is carried out by a coterie of cytoskeletal organizing proteins.

Tubulin acetylation is carried out on the Lys40 residue by tubulin acetyltransferases (TATs). The primary neuronal TAT is α-tubulin acetyltransferase 1 (αTAT1) (53). In C. elegans, loss of αTAT1 (MEC-17) results in an altered distribution of critical axonal components, including mitochondria (41). This results in spontaneous adult axonal degeneration, implying that tubulin acetylation is critical for axon stability. Interestingly, while targeted knockout of αTAT1 eliminates detectable tubulin acetylation in the mouse brain, it does not affect development or overall viability, with the exception of male fertility (54). This suggests that while tubulin acetylation may be critical for microtubule stability in lower eukaryotes, higher eukaryotes may have developed redundant mechanisms for promoting microtubule stability in the axon (i.e., via microtubule-associated proteins, the MAPs). A second TAT, Elp3, regulates axon shape and synaptic number in Drosophila (55). Elp3 is a subunit of the Elongator complex, which is primarily located in the nucleus where it stabilizes elongating RNA and displays histone acetyltransferase activity (56, 57). Interestingly, Elp3 also displays tubulin acetyltransferase
activity and is located axonally in *C. elegans* (58, 59). Cytoplasmic Elp3 is critical for acetylation of axonal tubulin in *C. elegans*, and this tubulin acetylation regulates velocity of vesicular traffic in the axon. In *Drosophila*, Elp3 knockout results in an axon and synaptic overgrowth and an overabundance of axonal branching, although whether these are the effects of cytoplasmic or nuclear Elp3 is not clear. The microtubule stability achieved via tubulin acetylation might be compensated for via other mechanisms, but seems to regulate some components of axonal shape.

While the purpose of axonal tubulin acetylation is unclear, defects in tubulin deacetylation have been linked to multiple neural degenerative disorders including Parkinson’s disease and amyotrophic lateral sclerosis (60, 61). Tubulin deacetylation is carried out by the class IIa histone deacetylases HDAC5 and HDAC6 and by Sirtuin2 (Sirt2) (62, 63). HDAC5 was originally characterized as nuclear-located regulators of histone stability, however HDAC5 is also located in the cytoplasm and axon, and HDAC6 is located exclusively in the cytoplasm. HDAC6 is present in all neurons but is especially enriched in Purkinje cells, highly arborized cells in the cerebellum (62). Axonal tubulin deacetylation by HDAC6 may therefore promote axonal branching by enabling tubulin remodeling and formation of new growth cones along the axon. While HDAC6 has been implicated as a tubulin deacetylase in developing, mature and regenerating axons (64), HDAC5 has well-defined roles as an axonal tubulin deacetylase after injury. After injury, HDAC5 is exported from the nucleus, increasing histone acetylation and activating a set of regeneration-associated genes (65). HDAC5 is then transported anterogradely into the axon, where it deacetylates tubulin specifically at the severed axon tip (63). This combination of transcriptional changes and local tubulin modifications promotes axon regeneration after injury. Sirt2 functions primarily in mature neurons to deacetylate tubulin,
although Sirt2 also plays roles in oligodendroglial differentiation and morphology (60, 62, 66). While the specific function of axonal Sirt2-based microtubule deacetylation remains unclear, accumulation of axonal Sirt2 has been implicated in aging and dementia (66). While much work has been done to illuminate the function of tubulin deacetylases in mature and regenerating neurons, whether tubulin deacetylation plays a role in axon development remains unclear.

Tubulin tyrosination is carried out at the α-tubulin C-terminal by tubulin tyrosine ligase (TTL) (67). Tyrosination of tubulin is generally associated with higher tubulin dynamic instability and is therefore thought to be critical for maintaining the dynamism of the shape of the growth cone (68). Loss of TTL and tubulin tyrosination results severe axon pathfinding defects in vivo (67). In vitro, loss of TTL causes highly dystrophic growth cones and aberrations in small GTPase signaling (69). A growing body of evidence suggests that tubulin tyrosination functions as a signal for localization of microtubule plus-end binding proteins (+TIPs), which are critical for regulating microtubule dynamics and initiating retrograde injury signaling. KIF3C, a microtubule depolymerizing member of the kinesin superfamily, is rapidly upregulated after injury and interacts specifically with EB3, a +TIP. This causes KIF3C to localize to growth cones of regenerating axons, where it depolymerizes microtubules and contributes to their dynamic instability. This localization of KIF3C is dependent on tubulin tyrosination. Work from our lab confirmed that axon injury-induced tubulin tyrosination is necessary for +TIP recruitment and retrograde injury signaling (70, 71). Tubulin tyrosination is therefore important for regulating growth cone shape and axonal transport. Tubulin detyrosination is carried out by an as-yet uncharacterized tubulin carboxypeptidase (TCP) (72). Since TCP has not yet been identified, any importance of tubulin detyrosination remains elusive.
How do tubulin dynamics direct axon outgrowth? As actin-rich filopodia explore the neuronal environment, so-called “pioneer” microtubules invade the base of the filopodium, providing the ground scaffolding for directional outgrowth (73–75). Dynamic, rapidly-polymerizing tyrosinated tubulin alone is capable of invading the rapidly growing filopodium. This tyrosinated tubulin is also enriched for +TIPs, which are often downstream proteins in canonical extracellular signaling cascades. The +TIP adenomatous polyposis coli (APC), for example, is a downstream effector protein in the glycogen synthase kinase 3β (GSK3β)-signaling cascade (76, 77). +TIPs can regulate microtubule stability via a number of mechanisms, including stabilizing the tubulin/actin interface, bundling individual tubulin filaments, and acting as a nucleation site for newly-synthesized cytoskeletal elements (73). Indeed, APC not only bundles tubulin filaments, it also binds cytoskeletal mRNAs, and therefore acts as a nucleating center for newly-transcribed cytoskeletal components and regulators (78, 79). As more tubulin filaments invade the filopodium, the +TIPs accumulate, stabilizing microtubules in the direction of axon outgrowth. While the elusive identity of TCP makes it impossible to say how microtubules are detyrosinated, eventually the earlier pioneer microtubules mature into their detyrosinated and eventually acetylated isoforms. Posttranslational modifications therefore treadmill along axonal microtubules as the growth cone advances.

In addition to posttranslational modifications of microtubules, the growth cone also consists of a variety of proteins that regulate the actin/microtubule interface. The actin/microtubule junction is especially critical for filopodial dynamics, as pioneer microtubules will invade actin-rich filopodia to lay the groundwork for axon outgrowth (73). The spectraplakin family of proteins contain characteristic actin- and microtubule-binding domains and are the primary proteins regulating actin/microtubule junctions (80). The spectraplakin
ACF7 (also known as MACF1, and with domain similarities to the *Drosophila* protein shortstop) binds both microtubules and actin, providing a link between the actin-rich fringe and microtubules (81). The microtubule/actin interface can also be modified indirectly. For example, the +TIP Navigator interacts with the Rac guanine exchange factor TRIO in neurons. Since Navigator is a +TIP, this allows selective enrichment of TRIO at growing microtubule ends in the growth cone. As a Rac GEF, TRIO then locally enriches for Rac-GTP, which enables actin remodeling and directed neurite outgrowth (82, 83). In addition to linking actin and tubulin protrusion at the leading edge of the growth cone, actin/microtubule interactions are also critical for tubulin bundling in the axon shaft. In the neck of the growth cone, the actin cytoskeleton is organized as a series of lateral bands (84). This is in stark contrast to its filamentous (filopodial) or meshwork (lamellipodial) organization at the leading edge of the growth cone, which is typically in the direction of axon growth. This lateral actin organization enables MyosinII-mediated contraction of the growth cone neck, as MyosinII movement along actin bands results in a medial flux of actin. This actin contraction at the growth cone neck facilitates microtubule bundling and therefore maturation of the microtubule cytoskeleton at the growth cone neck. Therefore actin/microtubule dynamics promote directed outgrowth of the cytoskeleton along the axis of axon growth and promote maturation of the cytoskeletal network at the stabilizing axon shaft.

At the leading edge, the growth cone is at its most dynamic, rapidly cycling between filopodial and lamellipodial morphologies carried out by its actin-rich fringe. The “treadmilling” of actin polymerization to generate actin-rich structures and forward force has been well-characterized in multiple cell types (85, 86). In short, actin filaments rapidly polymerize in a meshwork, wherein anterograde-facing barbed ends of filaments act as sites of profilin-mediated
actin polymerization, and retrograde-facing pointed ends of filaments act as sites of cofillin-mediated actin depolymerization. Both profilin and cofillin are necessary for neuronal development and are linked to neuronal disease. New actin filaments are nucleated off existing actin filaments by the Arp2/3 complex, which is necessary for growth cone function. Filamin promotes the gelating, or lateral crosslinking, of the actin meshwork in the body of the growth cone and the lamellipodium, and was recently shown to be critical for axon regeneration (87). Fascin bundles actin filaments, thereby promoting filopodial extrusion, and is also critical for axon growth (88). Actin therefore comprises the majority of the leading edge of the growth cone, and is the primary cytoskeletal force-generator at the cell membrane. Membrane-imbedded extracellular signaling receptors are therefore often upstream regulators of actin dynamics.

The growth cone completes its task by carefully regulating cytoskeletal dynamics to promote outgrowth while enabling maturation of the axon shaft. Highly dynamic filopodia and lamellipodia advance the cell membrane and are achieved primarily through canonical actin treadmilling. As actin advances the body of the growth cone forward, dynamic tyrosinated tubulin invades actin-rich structures with the help of the actin/microtubule bridging +TIPs. +TIPs facilitate bundling of the advancing tubulin filaments, which is facilitated in the neck of the growth cone by MyosinII-mediated contraction of lateral actin bands. As microtubules bundle, detyrosination and acetylation mark maturing tubulin as MAPs stabilize the tubulin bundles and complete the process of axon maturation. This process is similar in development and regeneration, although regenerating axons need to carry out the additional step of generating a new growth cone from the mature axon shaft at the site of injury. This requires tubulin deacetylation, tubulin tyrosination and the recruitment of +TIPs to severed microtubule tips, and the reestablishment of cytoskeletal treadmilling (63, 70). Misregulation of the cytoskeletal
network during development or regeneration can lead to aberrant axon growth rates or branching; additionally, misregulation of cytoskeletal remodeling after injury leads to the formation of a retraction bulb and the failure of axon regeneration (89). Understanding growth cone and axonal cytoskeletal regulation therefore contributes to our understanding of neural development and repair.

**Axon growth and regeneration are parallel processes dependent on intracellular and extracellular signals**

Axonal organellar and growth cone functions exist to carry out the needs of the neuron. What does a neuron “need”? The most basic answer to this question is “to survive.” In order to survive, the neuron activates whole-cell transcriptional programs in response to extracellular cues. These transcriptional programs enable directed cell responses, such as axon elongation or regeneration. Extracellular cues provide context and feedback for these responses, allowing a neuron to maximize its function and survival.

*Dedicated neuronal transcriptional programs promote axon growth and regeneration*

Cell identity and certain cell activities are dictated by the availability of transcripts that can be translated into proteins necessary for that identity or activity. The coterie of transcripts available for translation in any cell is decided by transcriptional programs established by transcription and epigenetic factors. Likewise, neurons are subject to several distinct transcriptional programs depending on their developmental and environmental needs. Transcriptomic analysis of neurons suggest distinct translational programs for axon development (3, 90, 91) and injury (92–95), among other activities. Specificity of transcriptional programs is
established via differential regulation of transcription factors (96–98), expression of specific transcript isoforms (99, 100), and spatial regulation of transcripts (4, 101).

During development neurons have distinct needs. They must support axon outgrowth at a rate of up to 5 mm/day. After a period of rapid axon outgrowth and the establishment of the physical organization of circuitry, neurons must establish synapses and throughout its lifetime regulate synaptic plasticity. Furthermore, neurons must carry out these complex tasks while the axoplasm is physically remote from the soma. For this reason, axons are capable of carrying out protein translation independently from the cell body (3, 102). The neuron establishes an axon-specific transcriptome by specifically trafficking mRNAs and translational machinery into the axon via mRNA zip codes (3–5, 23). During development, the axonal transcriptome is enriched for processes necessary for rapid outgrowth, especially mitochondrial components and translational machinery (3). Within the axon, the translational machinery is specifically localized to promote branch formation (6), growth cone function (9, 103) or synaptogenesis (21). Furthermore, the developing axon may appropriate the translational machinery for a novel purpose, as proposed by the Fainzilber group and explained in more detail below (104). In short, timed anterograde trafficking of mRNA and the resultant timing of retrogradely transported protein products of that mRNA would allow for somatic sensing of axonal length. Indeed, the developing neuron not only has a unique transcriptome, but also may use the extreme lengths of its axon to repurpose the transcriptional machinery to novel ends.

In addition to a specific developmental transcriptome, the neuron also possesses a specialized regenerative transcriptome. This was discovered in part via the unique properties of dorsal root ganglion (DRG) neurons, psuedobipolar neurons which extend one axon branch into the periphery and another into the spinal cord. As was expected based on their extracellular
environments, discussed in more detail below, peripheral branches of DRG neurons are well-known to exhibit some regenerative capabilities, while central branches do not regenerate after injury. However, a prior “conditioning” injury to the peripheral branch can enhance regeneration of the central branch (105, 106). This conditioning injury is dependent on transcription, as inhibition of mRNA synthesis abolishes the response (107). Differential regulation of so-called regeneration-associated genes (RAGs) is therefore a cell-intrinsic prerequisite for axon regeneration. While the injury-responsive transcriptome in DRGs shares some similarities with the developmental neural transcriptome, including differential regulation of metabolic machinery, it also displays unique characteristics, including upregulation of immunological transcripts and differential regulation of the transcriptional machinery and especially transcription factors (108–111). Furthermore, central nervous system (CNS) neurons, which are typically incapable of significant axon regeneration, fail to activate the regeneration-associated transcriptome, partially explaining their failure to regenerate (112, 113). Ectopic activation of RAGs in CNS neurons can promote axon regeneration after injury, underscoring their functional importance (112, 114, 115). The neuronal injury-induced transcriptome is therefore specialized to the needs of a regenerating, rather than developing, axon, and is a prerequisite for successful regeneration.

The extracellular neuronal environment directs axon growth and regeneration

While neurons maintain intracellular transcriptional programs, they ultimately need to respond to extracellular cues guiding axon growth during development or regeneration. In the growth cone, cell-surface receptors respond to both growth-promoting and growth repulsive cues, which trigger long-term transcriptional effects via retrograde signaling or more immediate effects on local cytoskeletal dynamics. Both attractive and repulsive chemotactic cues are
necessary for directed axon growth during development and regeneration, as axons must grow towards specific targets. However, the extracellular environment established during development is often not optimized to support axon elongation.

One interesting means of efficiency is the axon’s integration of its translational regulation machinery into its chemotactic response mechanisms. Growth-attractive and growth-repulsive cues can stimulate translation of cytoskeletal proteins or their upstream regulators, with rapid effects on growth cone shape. For example, the growth-attractive cues Netrin-1 and BDNF promote translation of β-actin (116, 117) and asymmetric β-actin translation results in growth cone turning towards these cues (19). Additionally, the microtubule-stabilizing protein β-catenin is translated in response to the attractive cue neurotrophin-3, resulting in axon elongation (118). Concomitantly, protein turnover via endocytosis is necessary for growth cone collapse in response to repulsive cues (119). Spatially regulated protein translation can also mediate growth cone collapse in response to repulsive cues, as in the case of the actin depolymerizer cofilin in response to Slit2 (30), and the actin regulator RhoA (120) and the microtubule modulator MAP1B in response to Semaphorin 3A (121, 122). Therefore the ability of the growth cone to locally synthesize proteins is not only necessary due to the extreme remoteness of the growing axon tip, but also to maximize cytoskeletal motility and responsiveness there.

In addition to changes of cytoskeletal dynamics in the immediate vicinity of the growth cone, extracellular signaling can also have larger scale effects on neuronal biology, including regulation of somatic responses. Often times, extracellular cues will activate proteins which act both as immediate regulators of cytoskeletal dynamics at the growth cone, and as long-term signaling cascades at the soma. GSK3, for example, is activated by extracellular Wnt via the frizzled receptor in the axon (123–125). Activated GSK3 then locally regulates the activity of
microtubule stabilizing proteins such as MAP1B and APC in the growth cone (126, 127).

Additionally, activated GSK3 is retrogradely transported to the soma, where it participates in multiple axon growth- and regeneration-associated signaling pathways, including c-Jun, CREB, β-catenin, p53, and SMAD signaling (122, 127, 128). These signaling pathways ultimately regulate transcriptional programs, which dictate what transcripts are available in the axon. Extracellular signaling can therefore have far-reaching effects on neuronal cell biology.

The extracellular signal caused by axon injury deserves special mention. This traumatic response results in arguably the most significant cellular change a neuron will experience in its mature life: the cell will activate the multitude cytoskeletal and transcriptional programs discussed above, resulting in either axon regeneration or atrophy; cell survival or death. Additionally, axon injury is the only time in a neuron’s lifetime when extracellular signaling is activated by destruction of the diffusion barrier between the cytoplasm and the extracellular matrix. Axotomy therefore causes the mixture of the intracellular and extracellular milieu at the site of injury. This most immediately manifests in a loss of membrane polarization caused by an influx of calcium (65, 129). This results in a back-propagating electrical signal which is rapidly relayed to the cell body, activating early-stage injury signaling (65). Meanwhile, the axon locally activates multiple signaling cascades at the site of injury which engage in reactivation of the protein synthesis machinery and motor-dependent retrograde signaling (25, 130). Axons are exceptionally poor at resealing membrane injuries, resulting in sustained ion influx and membrane depolarization (131–133). The faster an axon can reseal its membrane, and reestablish homeostatic intracellular and extracellular milieus, the more likely it is to regenerate. Otherwise, sustained injury signaling results in the formation of a retraction bulb, mitophagy, and in many
neurons, death. In the event of growth cone reformation, the axon must regenerate through its extracellular environment, which may be permissive or inhibitory to axon growth.

The CNS, which consists of the brain and spinal cord, is highly inhibitory to axon growth, while the peripheral nervous system (PNS, which classifies all non-CNS neurons) is generally permissive for axon growth. This is partially attributable to the non-neuronal cells in these distinct milieus. After injury the CNS inhibits axon regeneration by activating microglia and astrocytes to form a glial scar (134–136). Rapidly proliferating glial cells physically inhibit axon regeneration, and myelin debris and reactive astrocytes secrete growth-inhibitory signaling molecules, including chondroitin sulfate proteoglycans (see chapter 3). In the PNS, however, axon injury results in macrophage activation and efficient myelin debris clearance, and de- or transdifferentiated Schwann cells secrete axon growth factors to promote directed functional regeneration (reviewed in 135, 136). Extracellular signaling in the CNS can also block retrograde injury responses and RAG activation. Importantly, the conditioning injury paradigm in DRG neurons discussed above is nonreversible: an injury to the central branch will not prime the peripheral branch for regeneration, suggesting a deficiency in retrograde injury signaling specifically in the CNS (105). Therefore axon regeneration and growth are not only dependent upon intrinsic neuronal growth capabilities, but also extracellular signals.

A recurring theme in axonal biology is the critical role of axon transport. Axon transport positions axonal organelles where they are needed and delivers cargoes critical for axon growth or synaptic transmission to the axon tip. Axon transport enables retrograde signaling to direct transcriptional programs in response to extracellular growth or injury cues.
Microtubule-based molecular motors are responsible for long-range axonal transport

*Kinesin-1 and cytoplasmic dynein are the canonical MT-based molecular motors in axons*

A defining feature of axons compared to unspecified neurons or dendrites is their highly polarized microtubule structure. Microtubules have intrinsic directionality based on the structure of the tubulin dimer (139). Microtubules are spiral oligomers made of individual dimers of α- and β-tubulin. The tubulin dimers polymerize end-to-end, with the α and β subunits organizing towards distinct ends of the tubulin oligomer. This directionality of microtubules is described by “plus” and “minus” ends: α-tubulin lines up oriented towards the minus end, while β-tubulin orients towards the plus end. In axons, the plus-ends of microtubules are uniformly oriented away from the cell body, towards the distal axon. This microtubule orientation allows for specific trafficking streams based on the directionality of molecular motor, since plus-end directed motors will move anterogradely, while minus-end directed motors will move retrogradely towards the cell body.

Rapid anterograde transport delivers vesicular cargoes to the axon tip, which during growth contain the machinery for axon growth and after development are precursors for neurotransmitter-containing vesicles. In contrast, slow axonal transport of certain cargoes, such as organellar and cytoskeletal components, occurs up to fifty times slower than rapid axonal transport (140, 141). Rapid retrograde transport allows for near real-time signaling of axonal conditions: axon-to-soma signaling cascades are well-characterized in response to repulsive signals, attractive signals, and axon injury (130, 142–146). Some cargoes, including mitochondria, are bidirectional. These bidirectional cargoes might bind anterograde and retrograde motors simultaneously and select a prevailing direction based on the number of motors or on regulating specific motor activity, described (in the case of autophagosomes) in
more detail below. These distinct cargo streams suggest complex axonal “highways”: the crowded microenvironment of the axoplasm must be coordinated enough to allow transport of vesicular cargoes as fast as 400 mm/s.

Interestingly, the anterograde and retrograde molecular signaling streams themselves may provide valuable information to neurons. Recently the Fainzilber group suggested a model of axon length-sensing whereby timed release of anterograde cargoes and their subsequent retrograde products generated an oscillating signal (103). Based on the frequency of this signal, the neuron might sense how far it has grown and if it needs to continue on its growth trajectory or begin the process of axonal maturation and synapse formation. The authors proposed an mRNA-based signaling mechanism, where an axonally-directed mRNA is transported to the axon terminal, where it is translated and its protein product is sent back to the soma via retrograde transport. Since the frequency of this oscillating signal will decrease as axon length increases, the cell can sense the length of the axon. This model is possible only if the activity of molecular motors can be tightly regulated. Indeed, the axon has multiple layers of motor regulation which enables both rapid transport and precise positioning of cargoes.

*Molecular motor activity in the axon is tightly regulated via multiple mechanisms*

A primary mechanism of motor regulation is autoinhibition: neither kinesin-1 nor cytoplasmic dynein, common anterograde and retrograde axonal motors, are capable of anything other than diffusive motility in the absence of binding partners. This mechanism is conserved between different kinesin motors and cytoplasmic dynein because of its elegant simplicity: motor trafficking uses ATP, and therefore intrinsic inhibition in the absence of binding partners prevents fruitless energy expenditures (147). For kinesin, autoinhibition is conferred by both the light and heavy chains. The tail of the heavy chain folds over and interacts directly with the
motile head, inhibiting its interaction with microtubules (148). Additionally, KLC pushes apart the two motile heads of the KHC dimer, preventing the release of ADP and the completion of the ATP hydrolysis cycle (149). Therefore to initiate movement of kinesin-1 along microtubules, regulatory proteins must bind both the KHC and KLC subunits (150, 151). Dynein is autoinhibited via a slightly different mechanism than kinesin. When not bound by cargo, the two motile dynein heads “stack” alongside one another; binding of the neck linker region pushes the motile heads apart, enabling their directional movement along microtubules (152). Several neuron-specific cargo adaptors exist which regulate both the autoinhibition and motility of microtubule-based motors.

In addition to direct binding by cargo adapters and regulatory proteins, molecular motors are also regulated by the number of motors attached to a single cargo. This is especially true for large organellar structures, such as endosomes. The Holzbaur group has recently made a case study of autophagosomal trafficking in axons (153–157). Autophagy is necessary for mitochondrial turnover in axons: as mitochondria age or become damaged, they must be efficiently cleared from the axon. In the distal axon, autophagosomes bind both kinesin-1 and dynein and move in short bidirectional bursts, but do not sustain directed motility over long periods. As the autophagosome matures, it undergoes a switch and begins directed retrograde movement towards the cell body. As the autophagosome is transported back to the cell body, it fuses with lysosomes and efficiently degrades mitochondria and other cargoes. This retrograde transport of autophagosomes is necessary for their maturation and efficient degradation of mitochondria. Interestingly, retrogradely transported autophagosomes are also bound to kinesin-1. How is the directionality of the autophagosome, which binds both kinesin-1 and dynein, regulated? Huntingtin acts in concert with HAP1 as a cargo adaptor, loading autophagosomes
onto dynein. Additionally, JIP1 acts as a regulator of both dynein and kinesin-1 motors on the autophagosome, but importantly, cannot bind both dynein and kinesin simultaneously. JIP1 binding to autophagosomes is mediated by the autophagosomal cargo adaptor LC3. While JIP1 ordinarily activates KHC for motility, interaction with LC3 abolishes this activation. JIP1 can therefore act as a switch on autophagosome directionality, inhibiting KHC motility specifically when LC3 is recruited to the autophagosome. Therefore sustained retrograde transport of autophagosomes occurs only when efficient docking of autophagosomes to dynein by Huntingtin/HAP1 and LC3-mediated JIP1 inhibition of KHC motility occur simultaneously. The specific example of the autophagosome demonstrates the complexity of directional transport in the axon, which often has multiple layers of regulatory control. Indeed, recent work has suggested that vesicular cargoes might regulate the activity of their own motors by producing ATP via glycolysis, adding the additional layer of metabolite regulation onto the already complex transport machinery (158). An additional in-depth look at motor regulation by the JIP3 scaffolding protein is explored in Chapter 2. The complexity surrounding regulation of microtubule-based motors in axons underscores their importance in axon growth.

Conclusions

The extreme polarity of the axon presents the neuron with unique biological challenges. Developmentally, the neuron undergoes a dramatic morphological change, establishing short- and long-range connections which might extend across distances thousands of times the length of its cell body. After a period of rapid growth, neurons need to keep these cytoplasmic compartments alive for the lifetime of the organism, which might last more than a century. This extreme morphology also leaves the axon uniquely susceptible to damage, and catastrophic axon
injury is often met with attempts at regeneration. These feats are all the more impressive when one considers that the neuron is postmitotic, and therefore accomplishes broad changes in transcriptional programs, cellular biology and architectural remodeling within the lifetime of a single cell. The work that follows are in-depth delves into two critical aspects of axonal biology: molecular motor function and growth cone microtubule dynamics.
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CHAPTER 2

JIP3 activates kinesin-1 motility to promote axon elongation

This chapter contains a manuscript previously published:

Abstract

Kinesin-1 is a molecular motor responsible for cargo transport along microtubules and plays critical roles in polarized cells such as neurons. Kinesin-1 can function as a dimer of two heavy chains (KHC), which harbor the motor domain, or as a tetramer in combination with two accessory light chains (KLC). To ensure proper cargo distribution, kinesin-1 activity is precisely regulated. Both KLC and KHC subunits bind cargoes or regulatory proteins to engage the motor for movement along microtubules. We previously showed that the scaffolding protein JIP3 interacts directly with KHC in addition to its interaction with KLC and positively regulates dimeric KHC motility. Here we report that JIP3 activates tetrameric kinesin-1 motility. Using an in vitro motility assay, we show that JIP3 binding to KLC engages kinesin-1 with microtubules, and that JIP3 binding to KHC promotes kinesin-1 motility along microtubules. We tested the in vivo relevance of these findings using axon elongation as a model for kinesin-1-dependent cellular function. We demonstrate that JIP3 binding to KHC, but not KLC, is essential for axon elongation in hippocampal neurons as well as axon regeneration in sensory neurons. These findings reveal that JIP3 regulation of kinesin-1 motility is critical for axon elongation and regeneration.
Introduction

The kinesin-1 molecular motor is essential for cellular function, transporting cargoes along microtubules to distinct locales with precision timing. Kinesin-1 conventionally refers to a heterotetramer consisting of two kinesin heavy chains (KHC), which harbor the motor domain, and two non-motile light chains (KLC) (1–3). However, kinesin-1 can also function as a dimer of two motile KHC (4–13). Although KHC contains the force–generating component and provides motility for the kinesin-1 molecule, KHC also inhibits its own motility in the absence of cargo or regulatory proteins (14–17). The KHC tail can bind to the motor head domain and microtubules, creating a paused state for kinesin-1 (14). The KLC subunits act both as a cargo adapter for KHC and inhibit microtubule binding and motility of KHC in the absence of cargo (18, 19). Since the kinesin-1 tetramer is dually autoinhibited in the absence of binding partners, regulatory proteins must bind both the KHC and KLC to relieve inhibition and promote binding and movement along microtubules (20).

Neurons depend on kinesin-1 to transport cargoes along the extreme length of axons for efficient long-distance intracellular communication. Kinesin-1 plays critical roles during axon specification (21–23), growth (24), maintenance (25–27), organelle positioning (28), and regeneration (29–31). Defects in kinesin-1 cargo delivery or motility lead to neurological disorders (32–40), underscoring the importance of regulating kinesin-1 motility for neuronal function. However, the cellular mechanisms that control kinesin-1 motile properties remain poorly understood.

We previously showed that the c-Jun terminal kinase (JNK)-interacting protein 3, JIP3 (also known as JSAP1 or Sunday Driver) interacts with both KHC and KLC and promotes motility of dimeric KHC in vitro (5), suggesting that JIP3 regulates kinesin-1 motility in axons.
In agreement with this notion, previous studies have shown that JIP3 mutation leads to axonal transport defects in *C. elegans* (41–43) and *D. melanogaster* (44). Both JIP3 null mice and neuronal specific conditional JIP3 knockout mice are perinatal lethal and display severe axon guidance defects of the telencephalic commissure (45–47). At the cellular level, absence of JIP3 in cerebellar granule neurons reduces axon length but increases axon branching (48). In cortical neurons, JIP3 absence stimulates neurite elongation and branching (49). Although the localization of JIP3 to axon tips by kinesin-1 correlates with axon elongation in hippocampal neurons (50), whether modulation of kinesin-1 motile properties by JIP3 contributes to axon elongation remains unknown. We therefore sought to directly determine whether JIP3 regulates tetrameric kinesin-1 *in vitro*, and how JIP3 interaction with kinesin-1 subunits regulates axon elongation.
Results

JIP3 differentially regulates kinesin-1 for microtubule binding and motility

By virtue of its non-overlapping KLC and KHC binding domains, JIP3 has the potential to activate tetrameric kinesin-1 for microtubule binding and motility (5, 20). To test this possibility, we used a total internal reflection fluorescence microscopy (TIRF) based *in vitro* motility assay, as previously described (5, 20, 51) (Figure 1A). COS-7 cells were used to express wild-type JIP3 or JIP3 lacking the KHC or KLC binding site (5), as well as KLC-mCit and Flag–KHC (Figure 1B,C). Cell lysates were mixed before the motility assay and an excess of FLAG–JIP3 (WT or deletion mutants) was used (Figure 1D). Since KLC has no intrinsic motile activity, mCit-positive puncta moving along rhodamine-labeled microtubules represent KLC-KHC complexes (i.e., tetrameric kinesin-1) (20).

When mixed with control lysates lacking JIP3, KLC-mCit rarely displayed motile events (Supplemental video S1), in agreement with previous findings (20). This was expected since KLC inhibits the microtubule binding and motility of KHC (19, 20, 52). Under control conditions, the binding frequency of KLC-mCit is 0.11 ± 0.01 events/µm and the motile efficiency is 0.22 ± 0.04 events/10µm of microtubule (Figure 1F-G). The presence of WT JIP3 lysate doubled both the binding frequency (0.22 ± 0.02 events/µm) and motile efficiency (0.50 ± 0.07 events/10µm) of KLC-mCit (Figure 1F-G and Supplemental video S2), indicating that WT JIP3 activates kinesin-1 for microtubule binding and motility. JIP3ΔKLC lysates were unable to significantly increase the binding frequency of KLC-mCit (0.15 ± 0.02 events/µm) compared to control lysates, but they increased the motile efficiency of KLC-mCit close to the levels obtained using WT JIP3 lysates (0.42 ± 0.08 events/10µm) (Figure 1F-G and Supplemental video S3). In contrast, JIP3ΔKHC lysates resulted in a high binding frequency that is similar to WT JIP3
lysates (0.21 ± 0.02 events/µm), but only modestly increased the motile efficiency compared to control lysates (0.34 ± 0.05 events/10µm) (Figure 1F-G and Supplemental video S4). Together, these data suggest that JIP3 interaction with KLC promotes binding of kinesin-1 to microtubules, and that JIP3 interaction with KHC binding promotes motility of kinesin-1 along microtubules.

To explore further how JIP3 regulates kinesin-1 motility, we measured the velocity and run length of motile events (Figure 1H-I, and supplemental figure S5). Rare motile events observed in the presence of control lysates moved at an average velocity of 0.17 ± 0.01 µm/sec and an average run length of 0.44 ± 0.02 µm. In the presence of WT JIP3, we observed a significant increase in the velocity (0.21 ± 0.01 µm/sec) and run length (0.64 ± 0.03 µm) of motile events compared to control lysates. The velocity of KLC-mCit puncta were comparable to those reported using a similar in vitro assay (20), but were lower than those we measured previously for dimeric KHC motility (5). To determine if the lower velocities were due to the presence of KLC or due to slight differences in experimental conditions, we measured dimeric KHC velocity by imaging GFP-JIP3 puncta in the presence of Flag-KHC, as described (5). We observed that dimeric KHC moved at 0.25± 0.02 µm/sec in the presence of WT JIP3 (Supplemental figure S5A-C). The overall low velocity we measured thus likely reflects slight differences in experimental conditions. Nonetheless, we observed statistically significant differences in kinesin-1 velocity and run length in the presence of the different JIP3 constructs. JIP3ΔKLC lysate increased the velocity of KLC-mCit puncta similar to WT JIP3 lysate, while JIP3ΔKHC lysate did not (Figure 1H). These data indicate that JIP3 binding to KHC, but not KLC, contributes to regulation of kinesin-1 velocity. Run lengths of KLC-mCit puncta were highest in the presence of WT JIP3 lysate and were modestly lower in the presence of both
JIP3ΔKHC and JIP3ΔKLC lysates (Figure 1I), indicating that the binding of JIP3 to both KHC and KLC regulates the off-rate of kinesin-1.

Given the important role of JIP3 binding to KHC for kinesin-1 motility, we next sought to determine the stoichiometry of JIP3 and KHC complexes in vitro. To this end, we immobilized dimeric KHC and its associated WT GFP-JIP3 molecules on rhodamine-labeled microtubules by using AMPPNP and conducted single-molecule photobleaching assays as described previously (53). In these assays, the most frequent number of photobleaching steps was 4, indicating 4 JIP3 molecules per KHC dimer (Figure 1J). We also measured up to 8 photobleaching steps, which may result from two unresolved KHC dimers bound to a microtubule and photobleaching of all or some of the associated GFP-JIP3 molecules. We rarely measured photobleaching steps less than 4. Based on this analysis, we conclude that at least four JIP3 molecules bind per KHC dimer, and therefore the stoichiometry for JIP3:KHC is likely to be at least 2:1.

**JIP3 is required for axon elongation**

To test whether modulation of kinesin-1 motile properties by JIP3 contributes to axon elongation, we first evaluated axon morphology of cultured hippocampal neurons isolated from JIP3 knockout (KO) (45) or littermate control E18 embryos (Figure 2A,B). After 5 days in culture, neurons were fixed and stained for tau, an axonal marker, and MAP2, which is enriched in dendrites (Figure 2C). Given the possible function of JIP3 at the axon initial segment (43), and the role of the JIP1 in axon specification (54), we first determined whether absence of JIP3 affects axon specification by measuring the number of tau-positive axons. No effect on axon number was detected between the groups, indicating that JIP3 is dispensable for axon specification (Figure 2D). We next measured the length and branching of both axons and
dendrites. Absence of JIP3 significantly reduced axon length (379 ± 18 µm WT versus 322 ± 15 µm KO) (Figure 2E), in agreement with what was reported in cerebellar granule neurons (48). JIP3 heterozygous neurons also showed a reduction in axon length compared to WT (332 ± 13 µm). We observed no effect on axon branching (ANOVA \( p=0.23 \), Figure 2F), in contrast to what has been reported in cerebellar granule and cortical neurons (48, 49). Importantly, neither length nor branching of MAP2-positive dendrites were affected by the absence of JIP3 (ANOVA \( p=0.81 \) and 0.86, respectively, Figure 2G-H), indicating that JIP3 contributes specifically to axon elongation in mouse hippocampal neurons.

**JIP3 binding to KHC is required to promote axon elongation**

To determine whether binding of JIP3 to KHC and KLC differentially affects axon elongation, we performed rescue experiments with our JIP3 deletion mutants. EGFP-tagged WT JIP3, JIP3ΔKLC, JIP3ΔKHC and JIP3ΔΔ (Figure 1B) were expressed in hippocampal neurons isolated from JIP3 KO E18 embryos. At DIV5, neurons were fixed and stained for tau and MAP2 to assess axon morphology (Figure 3A and Supplemental figure S6). We note that electroporated JIP3 KO neurons consistently grew more robustly than non-electroporated neurons (compare figure 2C and 3A). Only neurons with EGFP signal evident in the axon were selected for analysis. Both JIP3ΔKLC and JIP3ΔKHC localized to the axon and were enriched at the axon tip, similar to WT JIP3, while JIP3ΔΔ primarily localized to the cell body and initial portion of the axon (Figure 3A), as previously reported (5, 50). Compared to the EGFP vector control, neurons expressing WT JIP3 and JIP3ΔKLC extended longer axons, indicating that both constructs rescued the axon elongation defects (441 ± 40 µm, 577 ± 53 µm and 636 ± 62 µm respectively, Figure 3A-B). In contrast, expression of JIP3ΔKHC or JIP3ΔΔ failed to rescue axon length defects (498 ± 44 µm and 505 ± 54 µm, respectively). We observed no significant
effect on axon branching (ANOVA $p=0.35$, Figure 3C) or on the length and branching of MAP2-positive dendrites (ANOVA $p=0.25$ and 0.33 respectively, Figure 3D, E). These results suggest that JIP3 binding to KHC, but not KLC, is sufficient to promote axon elongation.

**Deletion of the KHC binding domain in JIP3 has a dominant-negative effect**

Because JIP3 can homo-oligomerize (5, 55) we next determined if JIP3 lacking the KHC binding domain had dominant-negative effects on axon morphology in a WT background. For these experiments, we used rat hippocampal neurons and assessed axon and dendrite morphology at DIV5 with tau/MAP2 staining as above (Supplemental figure S7). Only neurons with EGFP signal present in the axon were analyzed. As observed in mice hippocampal neurons, both JIP3ΔKLC and JIP3ΔKHC localized to the axon and were enriched at the axon tip, while JIP3ΔΔ primarily localized in a punctate pattern in the cell body and initial portion of the axon (Figure 4A). Whereas expression of WT JIP3 and JIP3ΔKLC had no effect on axon length when compared with vector controls (655 ± 39 µm, 626 ± 38 µm and 562 ± 36 µm, respectively, Figure 4A-B), expression of JIP3ΔKHC or JIP3ΔΔ essentially halved the total axon length compared to vector controls (347 ± 25 µm and 326 ± 24 µm respectively). These results suggest that JIP3ΔKHC and JIP3ΔΔ act as dominant-negative mutants. The dominant-negative effect of JIP3ΔΔ on axon growth has been reported previously (50) and attributed to its inability to localize to axon tips. However, we observed that JIP3ΔKHC, which localized to the axon tip, also reduced axon elongation (Figure 4A,B). The dominant-negative effect therefore seems attributable to lack of JIP3 interaction with KHC and not solely due to the mislocalization of JIP3. In this rat hippocampal assay, JIP3ΔKHC and JIP3ΔΔ reduced axon branching compared to vector controls (6.8 ± 0.4, 6.5 ± 0.5 and 9.8 ± 0.4 branches respectively, Figure 4C), suggesting that JIP3 can regulate axon branching in certain cases, as reported previously (48,
Importantly, dendrite length was not different between any of the groups (ANOVA $p=0.42$), and dendrite branching, while reduced by the expression of JIP3ΔKHC and JIP3ΔΔ compared to controls ($4.9 \pm 0.2$, $4.6 \pm 0.2$ and $5.7 \pm 0.3$ branches respectively), was affected to a much smaller degree than axon branching (Figure 4D-E). These data provide further evidence for the importance of JIP3 binding to KHC, but not KLC, for axon elongation and, in some cell types, branching.

**JIP3 interaction with KHC is required for axon regeneration**

To test if JIP3-dependent regulation of kinesin-1 contributes to axon growth following injury, we used axon regeneration assays. Axon growth after injury is distinct from axon growth during development, but both processes depend on kinesin-1 driven transport (29–31). We used embryonic dorsal root ganglion (DRG) neurons to test for axon regeneration. This neuronal type extends only axons (56) and can be cultured in a “spot”, which leads to large numbers of radially growing axons that are amenable to measuring growth and regeneration (57). To establish a baseline for the effects of JIP3 mutants in this neuronal subtype, we used a lentiviral system to infect DRGs with EGFP-tagged wild type and mutant JIP3 on DIV1 and then assayed their growth on DIV3. Wild type JIP3 and JIP3 deletion mutants were expressed at a similar level (Figure 5A). However, none of the JIP3 mutants had an effect on axon outgrowth during normal development of DRG neurons (Figure 5B, C), in contrast to our hippocampal neuron results (Figures2-4). This finding indicates that JIP3 function likely differs in different neuronal subtypes.

Nevertheless, we tested the effect of JIP3/kinesin-1 interactions on axon regeneration, using an assay we developed previously (57). Embryonic DRG neurons were plated in a spot culture and infected with lentiviral EGFP-tagged WT or mutant JIP3 at DIV3. At DIV7, axons
were injured mechanically and 12h later cultures were fixed and stained with the regenerating axon marker SCG10 (58). Axon regeneration was assessed by measuring the regeneration index, normalizing SCG10 intensity distal to the site of injury to SCG10 intensity proximal to the site of injury. A regeneration index of 0 would indicate a complete lack of regeneration. Vector treated embryonic DRGs produced a regeneration index of 36 ± 4%, indicating that these neurons produce robust axon regeneration in response to injury (Figure 5D, E). WT JIP3 and JIP3ΔKLC produced modestly but not significantly lower regeneration indices of 28 ± 3% and 28 ± 3%, respectively, compared to vector controls. Expression of either JIP3ΔKHC or JIP3ΔΔ significantly reduced the regeneration index compared to vector transfected controls (21 ± 2% and 22 ± 3%, respectively). Importantly, the reduction in the regeneration index was not due to mislocalization of the regeneration marker SCG10 (Supplemental figure S8).

Since JIP3 also plays a role in retrograde injury signaling and activation of the pro-regenerative program (58, 59), we tested whether JIP3ΔKHC and JIP3ΔΔ reduce axon regeneration by decreasing the phosphorylation of c-Jun, a regeneration-associated protein (60). DRG neurons were cultured and infected as described above and immunostained for phosphorylated c-Jun (p-c-Jun) (Figure 5F). Compared to uninjured controls, injury elicited a robust p-c-Jun staining in all cases. These results indicate that the reduced axon regeneration observed in the presence of JIP3ΔKHC or JIP3ΔΔ does not result from defects in retrograde signaling after injury, and likely involves other activities that rely on the JIP3-KHC interaction.
**Discussion**

Here, we show that JIP3 activates tetrameric kinesin-1 in two different ways depending on whether it binds to KLC or KHC. Specifically, JIP3’s interaction with KLC promotes binding of kinesin-1 to microtubules, whereas JIP3’s interaction with KHC promotes motility of kinesin-1 along microtubules. While this dual action of JIP3 might be important for maximally activating kinesin-1 motility, our *in vivo* experiments show that JIP3 binding to KHC, but not KLC, is sufficient to rescue axon elongation defects caused by the loss of JIP3 in cultured neurons. In addition, expression of a JIP3 mutant lacking the KHC binding domain reduces axon elongation and axon regeneration. Our data reveal that regulation of kinesin-1 motility by JIP3 is critical for axon growth. Together with the recent observation that modulation of dynein’s velocity affects axon elongation (61), our study underscores the importance of regulation of intracellular transport for neuronal development.

Binding of JIP3 to KLC enhanced kinesin-1 binding to microtubules *in vitro*, indicating that JIP3 likely relieves KLC-based inhibition of KHC-microtubule binding. This is consistent with previous findings that KLC inhibits binding of KHC to microtubules (19) and that KLC must be bound by regulatory proteins in order for kinesin-1 to efficiently dock on microtubules (20). However, the absence of JIP3-KLC binding did not have any effect on axon elongation or regeneration. One possible reason for this observation is that cargoes associated with KLC might compensate for loss of JIP3 interaction with KLC. Binding to the tetratricopeptide (TPR) domain of KLC is sufficient to activate kinesin-1 transport (62) and several neuronal proteins interact with KLC via its TPR domain, including amyloid precursor protein (63), calsyntenin (64), alcadein (25) and caytaxin (65). Alternatively, other JIPs may compensate for loss of JIP3-KLC interactions in cells. Indeed, JNK-interacting protein 1 (JIP1), a protein functionally and
structurally related to JIP3, also regulates kinesin-1 microtubule binding and motility (20), regulates cortical axon development (54) and can partially rescue the axon projection defects present in JIP3 KO mice (47). Therefore, neurons seem to have multiple means of ensuring kinesin-1 docking to microtubules.

JIP3 interaction with KHC specifically promoted kinesin-1 motility in our TIRF assay. This is in agreement with reports that the tail domain of KHC autoinhibits its motility along microtubules and must be bound by regulatory proteins for efficient motility (14–17, 20). Exactly how JIP3 regulates kinesin-1 motility remains to be studied, but our stoichiometry experiments, which show that at least 4 JIP3 molecules bind per KHC dimer, suggest that JIP3 oligomerization may be important. Our finding that neurons cultured from heterozygous JIP3 KO mice show significant axon elongation defects also suggests that the amount of JIP3 protein is important for its function. Our experiments in neurons further indicate that JIP3 binding to KHC is a key event for axon growth and elongation.

Previously, we reported that JIP3 lacking both KHC and KLC binding domains fails to localize to axon tips (5) and subsequently this inability was posited to be responsible for the failure of JIP3ΔΔ neurons to promote axon elongation (50). However, we show that JIP3ΔKHC, which is present in axon tips, is defective for axon growth and regeneration. These results suggest that regulation of kinesin-1 motility by JIP3 rather than its subcellular localization is important for axonal outgrowth. Our data do not rule out the possibility that the axonal growth defects of JIP3ΔKHC may be due to compromised ability to serve as a JNK scaffolding protein, although we think this is unlikely because the KHC and JNK binding domains of JIP3 do not overlap (5, 66).
We found that JIP3 promotes axon elongation in mouse hippocampal neurons but not in mouse DRG neurons, indicating that JIP3 functions differentially in different cell types. In addition, the effect of JIP3 can also vary between species since JIP3 promotes axon elongation but not axon branching in mouse hippocampal neurons, whereas it induces both axon elongation and branching in rat hippocampal neurons. Our findings are consistent with previous work, which showed that JIP3 promotes axon length in rat hippocampal neurons (50), but reduces axon length and branching in mouse cortical neurons (49), and restricts axon branching in rat cerebellar granule neurons (48). The diversity of neuronal responses to JIP3 is probably a reflection of JIP3’s multiple functions as a JNK scaffolding protein (55, 67–70), a transport regulator (41, 42, 44), and an axon gatekeeper (43). We propose that different neuronal subtypes in different species may be selectively sensitive to one or more functional modules of JIP3.

Why does modulation of kinesin-1 motility on microtubules affect axon growth, especially axon length? A simple explanation is that delivery of trophic cargoes to the axon tip is disrupted when kinesin-1 motility is disrupted. However, our results are interesting in light of a recent model of axon length-sensing proposed by Rishal and colleagues (24). This model posits a frequency-based signaling module, whereby a cell senses the length of an axon based on the frequency of an oscillating signal sent anterogradely to the axon tip and returned via retrograde transport. Given that JIP3 associates with both kinesin-1 and dynein during axon growth and after injury (59, 71–73), JIP3 may be a promising lead to elucidate possible oscillating signaling modules.
Materials and Methods

Antibodies and reagents

Rhodamine- and biotin-labeled microtubules were generated as described using porcine rhodamine- and biotin-tubulin and bovine unlabelled tubulin, all obtained from Cytoskeleton (Denver, CO). The antibodies used were as follows: anti-MAP2 (Millipore, Temecula, CA), anti-Tau clone 5E2 (Millipore), anti-SCG10 (Novus, Littleton, CO), anti-JIP3 (Bowman et al., 2000, Cavalli 2005), anti-GFP (Life Technologies, Grand Island, NY), anti-FLAG (Sigma-Aldrich, St. Louis, MO), anti-α-tubulin (Abcam, Cambridge, MA), p-cJun (Cell Signaling, Danvers, MA) and anti-βIII-tubulin (Sigma-Aldrich). JIP3 knockout mice were previously described (Kelkar 2003). CD10 timed pregnant mice were obtained from Charles River Laboratories (O’Fallon, MO) for isolation of embryonic DRGs. Sprague-Dawley timed pregnant rats were obtained from Charles River Laboratories for isolation of embryonic hippocampal neurons.

Plasmid construction

KLC-mCit was a generous gift from Dr. Kristen Verhey. FLAG-KHC and EGFP-JIP3 mutants were described previously (5). Briefly, the cDNA coding sequences were cloned in-frame downstream of their respective tags in the multicloning sites of pcDNA3 and EGFP-C1 vectors, respectively. Lentiviral JIP3 constructs were generated by excising the JIP3-EGFP fusion construct from their C1 vectors and inserting them into the FUGW vector using Age1/EcoRI cloning sites.

Lentiviral production

Lentiviral particles were concentrated from HEK293T culture medium as described previously (57). Briefly, HEK293T cells were transfected using Lipofectamine 2000 with the FUGW-JIP3 construct of interest, pVSV-G and dR8.91 in a 1:1:3 ratio according to the manufacturer’s
specifications (Invitrogen, Grand Island, NY). After 2 days, culture medium was collected and filtered through a 0.45 μm pore. Filtered culture medium was incubated overnight with Lenti-X lentiviral concentrator according to the manufacturer’s instructions (Clontech, Mountain View, CA) and centrifuged at 1,000xg for 45 minutes to pellet lentiviral particles. Pellets were resuspended in opti-mem before adding to cultures.

**In vitro motility assay**

In vitro motility assay was performed as described (5, 20) with minor modifications. COS-7 cells were obtained from ATCC (Manassas, VA) and cultured as recommended. Cells were transfected using Lipofectamine 2000 (KLC-mCit, all FLAG-JIP3 and EGFP-JIP3 constructs) or Lipofectamine 3000 (FLAG-KHC), both from Invitrogen according to the manufacturer’s instructions. Twenty-four hours after transfection, COS-7 cells were lysed in buffer (500 mM PIPES, 1 mM EGTA, 1 mM MgSO4, 0.1% Triton X-100, Roche protease inhibitor tablet, pH6.9) and supernatant was collected by centrifugation at 16,000xg for 10 min at 4°C. Flow chambers of approximately 20µl volume were assembled by adhering silanized coverslips to glass slides using double-sided tape. 250 nM rhodamine- and biotin-labeled microtubules were adhered to the coverslips using 0.01µg/µl streptavidin (Sigma-Aldrich). The coverslip surface was then blocked with 5% pluronic (Sigma-Aldrich). Motility mix was generated by adding 5µl FLAG-JIP3 (or untransfected control lysate), 1µl FLAG-KHC, 1µl KLC-mCit and 1µl 40 mM Na-GTP to 8µl motility assay buffer (10 mM PIPES, 50 mM KOAc, 4 mM MgSO4, 1mM EGTA) supplemented with 1µl 1 M DTT, 1µl oxygen scavenging system (250µg/µl glucose oxidase, 35µg/µl catalase, and 1µl 4.5mg/mL glucose), and 1µl 0.5 mM taxol. KLC-mCit molecules were subsequently visualized at 25°C using TIRF microscopy on an inverted microscope (Olympus IX81). 488 and 532 nm diode-pumped solid-state lasers (Melles
Griot) were used at 5 mW power to visualize mCit and rhodamine, respectively. Images were captured at 1s intervals on a back-thinned electron multiplier-CCD camera (ImagEM, Hamamatsu). KLC-mCit motility was analyzed by kymographs generated and measured in Slidebook (Intelligent Imaging Innovations). Vertical lines in the kymographs were scored as nonmotile events, whereas diagonal lines that lasted longer than two frames were scored as motile events. Only lines with a clear on and off event during the 100s movie were analyzed to avoid inclusion of aggregates bound to microtubules. We also did not score landing events for puncta that were unusually bright, since these most likely represent aggregates.

For the KHC dimer experiments in supplement S5, the experimental setup was as above except 1 μl each WT JIP3-GFP and FLAG-KHC were used. For the stoichiometry experiments, we modified our dimeric KHC assay by adding AMPPNP instead of Mg-ATP. Since AMPPNP is non-hydrolyzable, it locks KHC and associated WT JIP3-GFP molecules to the microtubules. We then photobleached WT JIP3-GFP puncta by applying continuous laser power; this causes the JIP3-GFP molecules to photobleach randomly, and a unitary photobleaching step can be determined by measuring the smallest drop in fluorescence intensity. We then calculated the number of GFP molecules in individual puncta as (initial fluorescence intensity of a puncta – background fluorescence intensity)/fluorescence intensity of a single GFP molecule.

**Cell culture, transfection, staining and image analysis**

**Hippocampal electroporation and culture**

Hippocampuses were isolated from pups and dissociated with papain (Worthington, Lakewood, NJ) and DNAse for 30 minutes and triturated in neurobasal culture medium supplemented with B-27 (1%), penn/strep (1%) and L-glutamate (0.25%). Neurons were isolated by centrifugation and resuspended in electroporation solution. Electroporation was carried out using 5 μg DNA in
100 µl isotonic solution (2% Buffer I (2 g ATP-disodium salt, 1.2 g MgCl2•6H2O into 10 mL H2O) into Buffer II (6 g KH2PO4, 0.6 g NaHCO3, 0.2 g glucose into 300 mL H2O, pH 7.4)) using an Amava Nucleofector II (Lonza, Basel, Switzerland) factory preset for mouse hippocampal neurons. Neurons were rapidly centrifuged after electroporation and resuspended in culture medium for plating. Neurons were cultured on poly-D lysine (PDL) coated glass coverslips that had been etched for 3 hours in nitric acid. Neurons were allowed to grow for 5 days before fixing and staining.

**DRG infection and culture**

DRG neurons were isolated from e13 mouse pups and dissociated with 0.05% Trypsin-EDTA for 20 minutes. The trypsin was replaced with neurobasal culture media supplemented with B27 (2%), pen/strep (1%), Glutamax (1%), FDU (0.5%) and NGF (0.1%), and neurons were triturated 25 times with a p1000 pipette tip. Media was removed and neurons were triturated an additional two cycles. Neurons were resuspended in enough medium to plate 5 µl/10,000 cell spot and allowed to sit on the PDL- and laminin-coated culture surface for 5 minutes before a full volume of culture medium was added to the culture surface. For growth assays, neurons were infected at DIV1 and fixed and stained at DIV3 for alpha tubulin. For injury assays, at 3 DIV neurons were infected with lentiviral JIP3 constructs. At DIV7, neurons were lysed for western blots or injured with a metal probe. 12h after injury, neurons were fixed and stained for the axonal regeneration marker SCG10 or βIII-tubulin.

**Fixing and staining**

Culture media was removed and replaced with 4% phosphate-buffered paraformaldehyde for 15 minutes to fix neurons. Neurons were washed with PBS thrice before blocking in 10% goat serum/0.1% Triton X-100 in PBS. Primary antibody was diluted in blocking solution and added
to cultures after 20 minutes. Cultures were washed thrice with PBS before the addition of secondary antibodies (1:1000). All antibody incubation times were 30 minutes. Cultures were washed thrice with PBS after secondary antibody incubation before mounting using Prolong mounting medium (Life Technologies).

*Image acquisition and analysis*

Images were acquired with fluorescence microscopy (Nikon, Eclipse TE 200-E) and analyzed using ImageJ. For hippocampal axon morphology, neurites were traced and length was measured using the NeuronJ plugin. NeuronJ-produced tracings were then used as input for the Sholl Analysis plugin. For DRG axon regeneration, regeneration index was measured as previously described (57). Briefly, SCG10 images were thresholded to create a binary image that eliminated background fluorescence. Then, fluorescence intensity distal to the site of injury was measured as a percentage of fluorescence intensity proximal to the site of injury for a given area (that is, fluorescence intensity was measured over an equal sized area for proximal/distal comparisons).

**Acknowledgements**

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Figures

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**Figure 1: JIP3 regulates distinct aspects of kinesin-1 motility via differential binding to KLC and KHC subunits.** (A) Schematic representation of the TIRF-based kinesin-1 motility assay. Rhodamine- and biotin-labeled microtubules (red line) are adhered to coverslips (black line) with streptavidin. A motility mix with FLAG-JIP3, KLC-mCit and FLAG-KHC protein lysates is added to the chamber. Since KLC has no intrinsic motility along microtubules, mCit puncta move along microtubules only when kinesin-1 is functioning as a heterotetramer. (B) Schematic representation of JIP3 mutants used in this study. JIP3ΔKLC removes aa429-459 corresponding to the KLC binding domain. JIP3ΔKHC removes aa5-81 corresponding to the KHC binding domain. JIP3ΔΔ removes both the KLC and KHC binding domains. KBD, KHC Binding Domain; JBD, JNK binding domain, LZ, leucine zipper (KLC binding domain). (C) Representative western blot of KLC-mCit and FLAG-KHC constructs. FLAG-KHC and KLC-mCit were both present in their expected size ranges (n=3). (D) Representative western blot of FLAG-JIP3 lysates, including a control lysate lacking JIP3, and FLAG-KHC for comparison. 1µl of each of the JIP3 mutants and control lysate and 3µl of the FLAG-KHC were loaded in the gel. All JIP3 constructs were expressed at much greater levels than FLAG-KHC. (E) Representative kymographs from TIRF experiments. Each Y-axis represents 100s of imaging time. Scale bar, 5 µm. (F) Binding frequency was measured as the number of motile and nonmotile binding events per micron of microtubule. WT JIP3 and JIP3ΔKHC significantly increased the microtubule binding frequency of kinesin-1 compared to untransfected controls, but JIP3ΔKLC did not. (F-G) n=74 (control), 93 (WT JIP3), 85 (JIP3ΔKLC) and 92 (JIP3ΔKHC) microtubules analyzed in four replicates. ANOVA $p=1.65\times10^{-5}$, $p<0.001$ compared to control, Tukey HSD. (G) Motile efficiency was measured as the number of motile events per 10 microns of microtubule. All JIP3 mutants increased motile efficiency compared to control, but JIP3ΔKHC produced a
significantly lower motile efficiency than WT JIP3. ANOVA $p=0.01$, $*p<0.05$, $**p<0.001$ compared to control, Tukey HSD. (H) Velocity of motile events. WT JIP3 and JIP3ΔKLC significantly increase run speed compared to control, but JIP3ΔKHC did not. (H-K) n=41 (control), 108 (WT JIP3), 80 (JIP3ΔKLC) or 86 (JIP3ΔKHC) motile events in four replicates. ANOVA $p=9.65\times10^{-5}$, $**p<0.01$, $***p<0.001$ compared to control, Tukey HSD. (I) Run length of motile events. WT JIP3, JIP3ΔKLC and JIP3ΔKHC all significantly increase run length compared to controls, but JIPΔKHC does not increase run length to the extent that WT JIP3 does. Kruskal-Wallis $p=4.2\times10^{-5}$, $*p<0.05$, $**p<0.01$, $#p<<0.001$ compared to Velocity, Mann-Whitney U test. (J) Histogram of the number of GFP molecules in JIP3-GFP bound to FLAG-KHC. Counts below 4 were rarely observed, suggesting that JIP3 associates with KHC with at least 2:1 stoichiometry. (K) Representative trace of the fluorescence intensity over time of a JIP3-GFP puncta. Fluorescence intensity decreases in distinct steps (red lines), representing photobleaching of single GFP molecules. The total number of JIP3-GFP molecules can then be calculated by dividing the overall drop in fluorescence intensity (blue line) by the intensity of a single GFP molecule. This example shows a puncta that is calculated to contain 4 GFP molecules.
A. Western blot analysis of JIP3 and tubulin expression levels in different genotypes.

B. Bar graph showing the ratio of JIP3 to tubulin expression.

C. Representative images of neurons stained with tau, MAP2, and merge of both stains for different genotypes (+/+ and +/-).

D. Bar graph showing the percentage of neurons with two axons and one axon for different genotypes (+/+ and +/-).

E. Bar graph showing axon length (in μm) for different genotypes (+/+ and +/-).

F. Bar graph showing axon branch counts for different genotypes (+/+ and +/-).

G. Bar graph showing dendrite length (in μm) for different genotypes (+/+ and +/-).

H. Bar graph showing dendrite branch counts for different genotypes (+/+ and +/-).
**Figure 2: JIP3 is required for axon elongation.** (A) Representative western blots from JIP3 WT (+/+), het (+/-) and KO (-/-) littermate embryonic whole brain lysates. (B) Quantification of (A). JIP3 heterozygosity results in an approximately 50% reduction of JIP3 expression compared to WT. n=5 pups each group, litter-matched. (C) Tau (axons) and MAP2 (dendrites) staining of littermate WT, het and KO embryonic hippocampal neurons at DIV5. Most neurons produce one tau-positive axon and multiple MAP2-enriched dendrites. Arrowheads indicate cell bodies, arrows indicate tip of the longest axon. Scale bar 50µm. (D) Quantification of axon number from (C). No difference in axon number was detected between any of the groups. Kruskal-Wallis \( p=0.22 \). (D-H) n=100 neurons per group over 2 replicates. (E) Quantification of total axon length from (C). Het and KO axons are significantly shorter than WT. ANOVA \( p=0.018 \), \(*p<0.05\), \(**p<0.01\) compared to WT, Tukey HSD. (F) Quantification of axon branching from (C) by Sholl analysis. No differences in axon branching were detected in any of the groups. ANOVA \( p=0.23 \). (G) Total dendrite length quantification of (C). No differences in dendrite length were detected in any group. ANOVA \( p=0.81 \). (H) Dendrite branching quantification of (C) by Sholl analysis. No difference in dendrite branching was detected in any group. ANOVA \( p=0.87 \).
Figure 3: JIP3 binding to KHC is required to promote axon elongation. (A) Rescue of axon length defects in JIP3 KO mouse hippocampal neurons by JIP3 WT and mutants shown in Figure 1B. Top panel, tau (axons) and MAP2 (dendrites) staining of mouse JIP3 KO hippocampal neurons. Bottom panel, EGFP signal produced by the JIP3-EGFP fusion constructs. As in Figure 2C, most neurons extend a single axon. Arrowheads indicate cell bodies, arrows indicate tip of the longest axon. Scale bar 50µm. (B) Quantification of total axon length from (A). WT JIP3 and JIP3ΔKLC rescued axon length defects of the JIP3 KO, but JIP3ΔKHC and JIP3ΔΔ did not. (B-D) n=59 (Vector), 66 (WT JIP3), 71 (JIP3ΔKLC), 67 (JIP3ΔKHC) or 53 (JIP3ΔΔ) neurons from 3 replicates. ANOVA p=0.065, *p<0.05, **p<0.01 compared to Vector, Tukey HSD. (C) Quantification of axon branching from (A) by Sholl analysis. No changes in axon branching was detected amongst any of the groups. ANOVA p=0.35. (D) Total dendrite length quantification from (A). No change in dendrite length was detected between any of the groups. ANOVA p=0.25. (E) Dendrite branching from (A) analyzed by Sholl analysis. No change in dendrite branching was measured between any groups. ANOVA p=0.34.
Figure 4: Mutations in JIP3-KHC binding are dominant-negative. Analysis of JIP3 deletion mutants shown in Figure 1B in WT rat hippocampal neurons. (A) WT rat hippocampal neurons electroporated with the indicated constructs were fixed and stained for tau/MAP3 (axons/dendrites) at DIV5 (top panel). Arrowheads indicate cell bodies, arrows indicate axon tips. Scale bar 50µm. (B) Total axon length quantification of (A). Expression of JIP3ΔKHC or JIP3ΔΔ, but not WT JIP3 or JIP3ΔKLC, significantly reduced axon length compared to Vector controls. (B-E) n=70 (Vector), 68 (WT JIP3), 72 (JIP3ΔKLC), 73 (JIP3ΔKHC) or 66 (JIP3ΔΔ) neurons from 5 replicates. ANOVA p<<0.001, #p<<0.001 compared to vector, Tukey HSD. (C) Axon branching quantification by Sholl analysis of (A). Expression of JIP3ΔKHC and JIP3ΔΔ reduced axon branching, but expression of WT JIP3 or JIP3ΔKLC did not, ANOVA p<<0.001, #p<<0.001 compared to vector, Tukey HSD. (D) Total dendrite length quantification of (A). No difference in dendrite length was measured between any groups, ANOVA p=0.42. (E) Dendrite branching of (A) by Sholl analysis. Expression of JIP3ΔKHC and JIP3ΔΔ significantly reduced the number of dendrite branches, but to a much smaller extent than axon branches (see 4C), ANOVA p=2.0x10^{-4}, **p<0.01, Tukey HSD.
Figure 5: JIP3 binding to KHC is required for axon regeneration. (A) Representative western blot of spot cultures infected with lentiviral vector, EGFP-tagged WT JIP3, JIP3ΔKLC, JIP3ΔKHC or JIP3ΔΔ. GFP was used to probe for transgenic JIP3 expression. Note that the gel used did not allow for resolution of molecular weight changes between mutants and WT. (B) Axon growth of DRG neurons infected at DIV1 with mutant JIP3 lentivirus and assessed for axon growth at DIV3. Dotted lines indicate boundary of the cell body spot and the point from which axon length was assessed. Expression of JIP3 mutants did not affect growth of DRG neurons. The grid-like overlay is an artifact of stitching images together to visualize the entire spot field. (C) Quantification of (B). No significant differences in maximum axon projection from the spot culture boundary were detected between different conditions. n=17 (Vector), 19 (WT JIP3), 24 (JIP3ΔKLC), 21 (JIP3ΔKHC) or 17 (JIP3ΔΔ) fields of view analyzed from 3 replicates (4 spots per group per replicate), ANOVA p=0.30. (D) Representative images of infected spot cultures 12h after axon injury, stained for the regenerating axon marker SCG10. Vector-, WT JIP3- and JIP3ΔKLC-infected neurons undergo robust regeneration after injury, which is markedly reduced with expression of JIP3ΔKHC or JIP3ΔΔ. Dashed lines indicate the site of injury. Scale bar 100µm. (E) Quantification of (D). WT JIP3 and JIP3ΔKLC slightly, but not significantly, reduce axon regeneration compared to Vector controls (p=0.077 and 0.088, respectively). In contrast, JIP3ΔKHC and JIP3ΔΔ attenuate axon regeneration after injury. n=15 (Vector), 18 (WT JIP3), 23 (JIP3ΔKLC), 30 (JIP3ΔKHC), or 31 (JIP3ΔΔ) fields of view analyzed from 4 replicates (4 spots per group per replicate), ANOVA p=0.002. **p<0.01, ***p<0.001, Tukey HSD. (F) Top two rows: staining of activated phosphorylated cJun (p-cJun) after axotomy in JIP3 mutant-expressing neurons. All groups display strong nuclear staining of
p-cJun as indicated with the DAPI counterstain. Bottom row: Uninjured controls display no p-c-Jun staining.
Supplemental material

Supplemental video S1: Representative video of kinesin-1 motile events on microtubules in the presence of untransfected control COS7 lysates. Untransfected control lysates rarely produce microtubule binding or motile events, and microtubule binding events are short-lived when they do occur. (Supplemental videos S1-4) First frame shows rhodamine-labeled microtubules, all subsequent frames show mCit-positive KLC puncta. 100 frames of a 1 FPS video are shown here at a 7 FPS rate (7x speed). Arrows indicate start points of motile events, arrowheads indicate nonmotile events. Scale bar 5 µm.

Supplemental video S2: Representative video of kinesin-1 motile events on microtubules in the presence of WT JIP3 COS7 lysates. Binding events are long-lived and often result in motility. Motile events are frequently robust, fast and long-lived. Arrows indicate start points of motile events, arrowheads indicate nonmotile events. Scale bar 5 µm.

Supplemental video S3: Representative video of kinesin-1 motile events on microtubules in the presence of JIP3ΔKLC COS7 lysates. Binding events are infrequent but often result in motile events. Motile events are not as long-lived as those produced in the presence of WT lysates, but are relatively long-lived and fast compared to untransfected controls. Arrows indicate start points of motile events, arrowheads indicate nonmotile events. Scale bar 5 µm.

Supplemental video S4: Representative video of kinesin-1 motile events on microtubules in the presence of JIP3ΔKHC COS7 lysates. Binding events are frequent but rarely result in motility. Motile events are short and slow relative to those produced in the presence of WT JIP3 lysates. This video shows a looped bundle of two microtubules, hence the motility of the highlighted event is bidirectional. As nonmotile events are abundant under these conditions, only some are
highlighted in this video. Arrows indicate start points of motile events, arrowheads indicate nonmotile events. Scale bar 5 µm.
Supplemental figure S5: (A) Schematic representation of a KHC dimer experiment. (B) Run speeds from (A). WT fully activates the KHC dimer to a run speed of 0.25 µm/sec, while JIP3ΔKHC performs no better than control conditions without added KHC. JIP3ΔKLC partially activates the dimer. ANOVA $p=3.98 \times 10^{-8}$, #p<<0.001; (B-C) n=51 (WT), 88 (ΔKLC), 161 (ΔKHC), 46 (-KHC). (C) No difference in run length was detected between any groups. ANOVA $p=0.18$. (D) Histogram of velocity data from Figure 1H. (E) Histogram of run length data from figure 1I.
Supplemental figure S6: (A) Split channels of images shown in Figure 3A. As in Figure 1, Tau is restricted to the axon and MAP2 is enriched in the dendrites. (B) Axon number data from neurons analyzed in Figure 3. No difference in axon number was detected between groups. \( p=0.15 \), Kruskal-Wallis.
Supplemental figure S7: (A) Split channels of images shown in figure 4A. (B) Axon number data from neurons analyzed in Figure 4. No difference in axon number was detected between groups. $p=0.08$, Kruskal-Wallis.
**Supplemental figure S8:** SCG10 as a marker for axon regeneration in JIP3 mutant-expressing neurons. SCG10 is shown here with a β-III tubulin counterstain. SCG10 is present in the regenerating axons in all conditions, as shown with the tubulin counterstain. However, SCG10 is not present in the degenerating axons past the site of injury, highlighted with arrowheads. Tubulin is present in these degenerating axons and is therefore not an appropriate axon regeneration marker. Axotomy site indicated with dotted line. Scale bar, 50 µm.
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CHAPTER 3

Neuronal Ran promotes axon elongation by promoting growth cone filopodial dynamics
Abstract

Axon growth is a critical feature of both neural development and repair. Microtubule dynamics are critical for axon growth; however the molecular mechanisms governing microtubule dynamics in developing axons are complex and not fully understood. The small GTPase Ran, originally characterized as a nucleocytoplasmic trafficking protein, also has roles in ciliary transport and mitotic spindle formation. Several Ran-dependent cytoskeletal remodelers active during mitotic spindle formation are also known to control axon cytoskeletal dynamics. Since Ran is present in developing and adult axons, we explored the possibility that Ran regulates axon growth by controlling microtubule dynamics. Here we present evidence that neuronal Ran levels are precisely tuned to promote axon growth. Furthermore, we show that Ran promotes axon growth by controlling microtubule dynamics in filopodia. Interestingly, we found that Ran knockdown in sensory neurons plated on and inhibitory substrate that mimics the environment of the injured central nervous system promotes axon elongation, an effect also mediated by filopodial dynamics. We also investigated proteins that might modulate Ran function in the axon, focusing particularly on the only known cytoplasmic Ran guanine exchange factor, RanBP10. We demonstrate that RanBP10 is enriched in growing axon tips in a Ran-dependent manner and interacts with Ran and tubulin in neurons. RanBP10 knockdown significantly reduces axon elongation, an effect that is rescued by expression of Ran, suggesting that RanBP10 promotes axon elongation in a Ran-dependent manner. These results indicate that Ran is a key player in the modulation of axon elongation and might differentially affect axon growth in the peripheral and central nervous systems.
Introduction

Axon growth is a critical feature of neuronal development and repair. In both development and repair, the structure responsible for axon growth is the growth cone, a temporary structure that forms at growing axons tips. The growth cone is highly dynamic and rapidly extends and retracts to explore its environment and propel axon growth. A critical component of growth cone function is the modulation of cytoskeletal dynamics. The actin-rich lamellipodium provides mechanical force to propel the plasma membrane forward through an environment, allowing rapid responses to environmental cues (1, 2). The actin- and microtubule-rich filopodia provide a structure on which microtubules can nucleate and mature, directing axon growth, while maintaining a degree of instability, allowing the growth cone to rapidly change shape in response to its environment (1, 3, 4). Precision modulation of cytoskeletal dynamics is therefore critical for axon growth during development and repair.

The small GTPase Ran was originally characterized as a nucleocytoplasmic trafficking protein. At the nuclear pore, nuclear import and export are driven by local gradients of GTP- or GDP-bound Ran (5, 6). These local nucleotide-bound Ran gradients are achieved through selective localization of Ran accessory proteins (7). The guanine exchange factors (GEFs) generate Ran-GTP, and the primary Ran GEF, RCC1, binds chromatin and is therefore found exclusively in the nucleus (8, 9). Localization of Ran regulatory proteins is therefore a critical feature of Ran biology, as it enables local enrichment of Ran-GTP. Recently, the Verhey group described a Ran-based transport operation at the ciliary port with striking similarities to the nuclear pore, suggesting that Ran functions in distinct niches based on subcellular enrichment of Ran-GTP (10, 11). In addition to its role in nucleocytoplasmic and ciliary transport, Ran also functions as a microtubule modulating protein during mitotic spindle formation (12). During
mitosis, the nuclear envelope disintegrates, however local enrichment of Ran-GTP around chromatin is maintained via the chromatin binding feature of RCC1 (9, 13–19). This local enrichment of Ran-GTP releases microtubule effector proteins from importin-based molecular sequester and allows stabilization of microtubules specifically around chromatin (20–23). In addition to distinct local niches, then, the Ran cycle can also have distinct temporal niches, functioning at the nucleus as a trafficking protein during interphase and a microtubule modulator during mitotic spindle formation.

Here, we explore the possibility that the axon growth cone is a novel Ran niche, and that Ran modulates microtubule dynamics in growth cones. Several pieces of evidence make this hypothesis especially compelling. First, microtubule-stabilizing proteins regulated by the Ran cycle during mitotic spindle formation also have well-characterized roles in axon elongation. Adenomatous polyposis coli (APC), for example, promotes microtubule stability during mitotic spindle formation and modulates microtubule dynamics in growth cones (24–29). Second, Ran protein is present in adult axons, and Ran mRNA is present in developing axons and growth cones (30–32). This means that Ran has an opportunity to regulate microtubule dynamics, although this possibility has not been explored to date. We present evidence here that neuronal Ran levels are tuned to promote axon elongation, that neuronal Ran modulates filopodial and microtubule dynamics in growth cones, and that a cytoplasmic Ran GEF is specifically enriched in growth cones. This evidence is consistent with Ran functioning as a microtubule modulating protein in growth cones.
Results

Neuronal Ran promotes axon elongation

To investigate the role of Ran in axon elongation in developing neurons, we modified its levels in laminin-plated mouse embryonic DRG neurons. These neurons are highly growth-competent and robustly extend axons (33). We used a spot culture assay as described previously, plating DRG cell bodies in a “spot” on the growth substrate and allowing axons to extend radially, which allows us to analyze growth of many axons simultaneously (34). We infected neurons at DIV1 with control or a murine-specific Ran shRNA, which reduced Ran expression approximately 70% (Figure 1A-B). At DIV3, control cells extended axons 1375 ± 130 µm. Compared to control cells, Ran knockdown neurons greatly reduced axon elongation, growing only 705 ± 57 µm (Figure 1C-D). This effect was completely rescued by expression of human WT Ran (1278 ± 196 µm), suggesting that the reduction in axon elongation witnessed in Ran knockdown cells was specific to Ran function. Next, we determined if Ran overexpression would also modulate axon growth, and infected neurons with lentiviral constructs containing human WT Ran or vector (Figure 1E). Vector-infected neurons extended their axons 1468 ± 87 µm, comparable to control shRNA-infected neurons. In contrast, WT Ran-infected neurons extended their axons 1303 ± 70 µm, a small but significant reduction in axon elongation compared to vector controls (Figure 1F-G). Taken together, these data suggest that neuronal Ran levels must be properly coordinated to promote axon elongation.

Ran promotes growth cone filopodial and microtubule dynamics

To determine why Ran knockdown neurons reduced axon elongation, we employed live imaging of axon growth cones. We infected neurons at 6h post-plating with control or Ran shRNA together with farnesylated EGFP (f-EGFP). Farnesylation robustly localizes proteins to
the plasma membrane, and allowed us to image the extending growth cones of axons (e.g. 32) (Figure 2A). On DIV2, we imaged growth cones of neurons and measured the overall rate of growth cone area change per second to determine how dynamic growth cones were. Control growth cones were highly dynamic, with a change in growth cone area of \(0.99 \pm 0.03 \, \mu m^2/s\). This is comparable to growth cone area change rates reported previously (34). In contrast, Ran knockdown reduced the rate of growth cone area change to \(0.74 \pm 0.03 \, \mu m^2/s\) (Figure 2B). We then determined if Ran knockdown had any effect on filopodial dynamics, since these are the only motile growth cone structures that contain microtubules and since Ran is known to modulate microtubule dynamics in other contexts. To this end, we manually tracked filopodia and measured their speed. Filopodia of control growth cones were highly motile, moving \(3.1 \pm 0.7 \, \mu m/sec\). In contrast, Ran knockdown neurons had much less motile filopodia, averaging a speed of \(2.0 \pm 0.6 \, \mu m/sec\) (Figure 2C). This suggests that the reduction in axon growth observed in Ran knockdown neurons can be attributable to a reduction in growth cone and, more specifically, filopodial dynamics.

While growth cones contain filopodia and lamellipodia, only filopodia contain microtubules (36). Since we detected changes in filopodial speed in Ran knockdown neurons, we also measured overall microtubule dynamics using the SiR-tubulin probe, a cell-permeable marker for microtubules. We measured the overall rate of change of SiR-tubulin positive area as a function of time to determine if overall microtubule dynamism was changed by Ran knockdown (Figure 2D). Overall, the SiR-tubulin positive area was less motile than the f-EGFP positive area, changing at a rate of \(0.58 \pm 0.02 \, \mu m^2/s\) in control neurons (Figure 2E). This is consistent with growth cone biology, as the cell membrane would change with both lamellipodial and filopodial dynamics, and since the microtubule cytoskeleton must stabilize to promote cargo
delivery to growing axon tips. Ran knockdown significantly reduced overall microtubule motility, eliciting a rate of SiR-tubulin area change of $0.41 \pm 0.02 \mu m^2/s$. These data are consistent with Ran functioning in growth cones to, at least in part, modulate microtubule dynamics.

**Ran knockdown promotes axon growth on inhibitory substrates by increasing filopodial dynamics**

Laminin supports axon growth during development, where it functionschemotactically to promote axon extension. However, environments inhibitory to axon growth are a primary concern for axon regeneration. After spinal cord injury, an inflammatory glial scar forms which blocks axon regeneration, partially via enrichment of chondroitin sulfate proteoglycans, CSPGs (37–40). We therefore decided to test whether neuronal Ran also supported axon growth on CSPG-coated substrates. To this end, we plated embryonic DRG neurons on substrates coated with CSPG. For this experiment we employed dissociated cultures since spot cultures did not adhere to the CSPG-coated substrates, CSPG potently inhibited axon outgrowth, reducing axon length approximately 90% ($77 \pm 10 \mu m$) compared to laminin-plated neurons (Figure 3A-B). Unexpectedly, neurons infected with Ran shRNA had longer axons than control shRNA-infected neurons ($195 \pm 24 \mu m$). This effect was mitigated by expression of WT Ran ($50 \pm 15 \mu m$), indicating that Ran specifically inhibits axon elongation on CSPG-coated substrates.

To determine how Ran knockdown promotes axon growth on inhibitory substrates, we performed live imaging of axon tips growing on CSPG using microfluidic chambers to pattern CSPG coating. Live imaging dishes were plated with PDL before adhering microfluidic chambers. Then, laminin and CSPG were coated on different sides of the microfluidic device via differential osmotic pressure. Cell bodies were plated on the laminin-coated side of the device to
promote neuronal survival and axon extension onto the CSPG-coated side. At DIV1, neurons were co-infected with f-EGFP and control or anti-Ran shRNA. At DIV4, all axons extending into the CSPG compartment were imaged, unless they were undergoing degeneration, marked by a fragmentation of the axon f-EGFP signal. Growth cones on these axons were largely immobile, and never displayed lamellipodia (Figure 3C). The rate of growth cone area change in control neurons was $0.27 \pm 0.01 \, \mu m/sec$, a reduction compared to laminin controls of approximately 75% (Figure 3E). Filopodial tracking indicated that CSPG coating reduced filopodial speed to $0.03 \pm 0.002 \, \mu m/sec$, compared to more than $3 \, \mu m/sec$ on laminin-coated substrates (Figures 2C, 3F). This dramatic reduction in filopodial displacement on CSPG versus laminin correlates well with the reduction in axon elongation, and is likely partially responsible for that phenotype.

Importantly, knockdown of Ran partially mitigated the growth cone and filopodial dynamics phenotype induced by CSPG substrates. In contrast to control neurons, on CSPG Ran knockdown neurons exhibited a $0.38 \pm 0.03 \, \mu m$ rate of growth cone area change, a significantly greater rate of area change than control-infected neurons. Filopodial speed of Ran knockdown growth cones on CSPG were more than double that of control-infected neurons, averaging $0.08 \pm 0.005 \, \mu m/sec$. In addition to increasing filopodial dynamics, we also observed a number of filopodial faults in Ran knockdown cells plated on CSPG, which were present at lower frequencies in control shRNA neurons (Figure 3D). For our purposes, a filopodial “fault” was a notable morphological change in a CSPG-plated filopodium, which were rarely observed in filopodia of laminin-plated neurons. Specifically, we observed buckling, splitting and degeneration of filopodia, as well as basal motility in some filopodia (that is, the tip of the filopodium was stationary but the base of the filopodium moved along the body of the axon, a reversal of the usual motile pattern). All of these faults were more prevalent in Ran knockdown
(93% of filopodia display a fault) than control neurons (84%), suggesting an abnormality in filopodial dynamics was responsible for the increase in filopodial dynamics and, concomitantly, axon elongation. This, coupled with the fact that only MT-rich filopodia and never actin-rich lamellipodia were observed in CSPG-plated growth cones, suggests that the increase in axon elongation observed in Ran knockdown neurons on CSPG is at least partially attributable to changes in microtubule dynamics.

**RanBP10, a cytoplasmic Ran GEF, is enriched in growing axon tips in a Ran-dependent manner**

Ran cycles between its inactive GDP-bound state and its active GTP-bound state via its accessory proteins, the GAPs and the GEFs. While multiple Ran GAPs exist, only two Ran GEFs have ever been reported. Of those, RCC1 is restricted to the nucleus, but RanBP10 is known to function in the cytoplasm (14, 41). Furthermore, RanBP10 mRNA is present in embryonic axons and growth cones (31, 32). We therefore decided to investigate if RanBP10 is present in neurons, and whether it interacts with Ran to regulate axon growth. First, we used a RanBP10 antibody to investigate its distribution in neurons. RanBP10 was present in neurons, and especially at axon tips and in the soma (Figure 4A and data not shown). In contrast, RCC1 was only present in the nucleus (data not shown). We compared RanBP10 expression to the f-EGFP tag, which localizes to cell membranes. Theoretically, f-EGFP should be evenly distributed throughout the cell membrane, and not specifically enriched at axon tips. Compared to this neutral membrane marker, RanBP10 was enriched approximately 80% specifically at axon tips (Figure 4B). This suggests that active Ran-GTP is produced at growing axon tips, where it might regulate Ran function.
In megakaryocytes, Ran and RanBP10 interact with tubulin to regulate platelet formation and shape (41–43). Therefore, we also investigated whether Ran and RanBP10 might interact with cytoskeletal components to regulate axon shape in neurons. To this end, we employed a reciprocal coimmunoprecipitation using anti-Ran and anti-RanBP10 antibodies in cultured embryonic DRG neurons (Figure 4C). In DIV8 neurons, Ran and RanBP10 reciprocally coimmunoprecipitated, suggesting that Ran and RanBP10 indeed interact in neurons. Furthermore, Ran and RanBP1 antibodies also coimmunoprecipitated tubulin, suggesting that Ran, RanBP10 and tubulin exist in a complex in neurons. These data are consistent with RanBP10 functioning as a link between Ran and microtubule dynamics, perhaps by acting as a GEF to produce active Ran in axon tips. To further explore the relationship between Ran and RanBP10 in neurons, we measured Ran and RanBP10 distribution in Ran knockdown neurons. Ran is slightly enriched at the growing tips of axons, however this enrichment is significantly reduced in Ran knockdown neurons (Figure 4D-E). Interestingly, Ran knockdown increased growth cone enrichment of RanBP10 (Figure 4F-G). These data are consistent with Ran regulating the retrograde transport of RanBP10, similar to its role in importin-β mediated retrograde injury transport (30).

**RanBP10 promotes axon growth in a Ran-dependent manner**

We next decided to see if axonal RanBP10 had any functional significance. To this end, we expressed a RanBP10 shRNA in embryonic DRG neurons. In these neurons, expression of the RanBP10 shRNA reduced RanBP10 expression approximately 50%. Concomitantly, axon elongation was reduced from 1828 ± 81 µm in control-infected neurons to 1557 ± 138 µm in shRBP10-infected neurons, a small but significant effect. Interestingly, this reduction in axon elongation could be rescued completely with expression of WT Ran (1860 ± 140 µm, compared
to 1640 ± 131 µm in Vector-infected knockdown neurons). This suggests that RanBP10 regulates axon elongation in a Ran-dependent manner. We also investigated whether RanBP10 modulates axon elongation of CSPG-coated substrates, and to that end repeated our dissociated culture experiments described above. On CSPG-coated substrates, RBP10 knockdown did not promote axon elongation compared to control neurons (203 ± 57 µm in control neurons versus 139 ± 14 µm in RanBP10 knockdown neurons), consistent with RBP10 functioning upstream of Ran in axons. Taken together, these data are consistent with RanBP10 acting as a Ran GEF.
Preliminary Data

In addition to investigating the effect of Ran knockdown on neurons, I have also completed preliminary studies using nucleotide-locked Ran mutants. RanT24N loses its ability to release a spent GDP, locking it in its GDP-bound state. RanL43E loses its ability to hydrolyze GTP to GDP, locking it in its GTP-bound state. Since Ran function is dependent on local enrichment of Ran-GTP or –GDP, overexpressing nucleotide-locked mutants can bias the system towards one binding state or the other, changing Ran biology in a more subtle way than whole-cell knockdown. For these data, it is important to keep in mind that only Ran-GTP has known binding partners, and is therefore referred to as “active” Ran.

Nucleotide-locked Ran mutants differentially effect axon branching and elongation

I used dissociated DRG cultures to investigate nucleotide binding state-dependent Ran effects, since subtleties in axon branching patterns are easier to see in these cells (preliminary Figure 1). Dissociated embryonic DRGs grow in a characteristic pattern, extending two axons approximately 1-1.5 mm before generating terminal branching arbors with short axon branches. In vector infected neurons, the longest axon grew approximately 1475 ± 89 µm and 74% of neurons underwent terminal branching. Nucleotide-locked Ran mutants all localized to the axon as well as the soma, as evidenced by their EGFP tags presence there (data not shown) Expression of Ran-GDP, which cannot bind effector proteins, reduced axon elongation (1195 ± 81 µm) and branching (63% terminally branch), suggesting that active Ran-GTP supports both of these activities. Indeed, expression of Ran-GTP had no effect on terminal axon branching (69%), but curiously reduced the length of the longest axon (1163 ± 79 µm). Upon further examination, Ran-GTP expression increased the mode of axonal branch points (5 versus 3 for vector control) and decreased the length to the first branch point (582 ± 73 µm versus 828 ± 83 µm for vector
control). This suggests that an overabundance of Ran-GTP causes ectopic axon branching, which reduces elongation of the longest axon when the neuron expends resources to elongate secondary branches. WT infected neurons had no effect on axon elongation (1335 ± 88 µm), but significantly reduced the incidence of terminal axon branching (55%). I posit that overexpression of WT Ran partially mimics overexpression of Ran-GDP: if the axonal Ran GEF nears maximal activity in control neurons, then an abundance of WT Ran would bias the system towards Ran-GDP. The specific and differential effects of nucleotide-locked Ran mutants on axon shape support the fact that Ran acts locally within the axon to control growth: since axon growth was largely intact but exhibited subtle differences between the nucleotide-locked mutants, we doubt that large scale misregulation of nucleocytoplasmic transport is occurring in these neurons. Taken together, these data suggest that Ran-GTP is the primary actor in axon growth cones.

Ran-GTP, but not Ran-GDP, rescues Ran knockdown

To validate if Ran-GTP is critical for axon growth, I employed the spot culture assay described above and determined which nucleotide-locked Ran mutants rescued the axon elongation defects of Ran knockdown (preliminary Figure 2). As demonstrated previously, Ran knockdown reduced axon growth significantly (705 ± 57 µm) compared to control neurons (1376 ± 131 µm), and WT Ran expression rescued this effect (1279 ± 196 µm). Expression of Ran-GTP (1743 ± 238 µm) enhanced axon elongation beyond control levels, and Ran-GDP (974 ± 166 µm) failed to completely rescue knockdown of Ran, consistent with the conclusion that active Ran-GTP is necessary for axon elongation. Furthermore, these data also support the conclusion that Ran functions in an axonal niche distinct from its nucleocytoplasmic transport niche. Ectopic Ran-GTP would be produced in the cytoplasm, but Ran is transported into the nucleus only in its GDP-bound state. Therefore any large-scale nucleocytoplasmic trafficking
defects caused by whole-cell Ran knockdown would be unlikely to be rescued by Ran-GTP, since ectopic Ran-GTP would be unable to dynamically relocalize as necessary for the nucleocytoplasmic trafficking cycle. This strongly suggests that Ran-GTP specifically promotes axon elongation when it is localized to the axon.
Discussion

Here, we present evidence that Ran promotes axon elongation. First, we demonstrate that neuronal Ran levels are tuned to promote axon elongation on permissive substrates, as knockdown or overexpression of Ran on laminin reduces axon elongation. We trace these effects to changes in filopodial dynamics, as a Ran shRNA-induced reduction in filopodial speed and motility correspond to an overall reduction in growth cone dynamism and axon elongation. This effect is at least partially attributable to changes in microtubule dynamics, as dynamics of the microtubule-specific SiR-tubulin probe were also reduced by Ran knockdown. Interestingly, Ran knockdown has the opposite effect on the inhibitory substrate CSPG, where it promotes axon elongation. Again, filopodial dynamics are altered on CSPG, where Ran seems to increase axon elongation by increasing filopodial motility and speed. Finally, we demonstrate that the only known cytoplasmic Ran GEF, RanBP10, is enriched in growth cones, where it promotes axon elongation in a Ran-dependent manner. These data are an exciting preliminary look into the possibility that the axon might be a distinct Ran niche. Functional Ran niches have two defining characteristics: local enrichment of Ran-GTP or –GDP, accomplished by the localization of Ran GAPs and GEFs; and the release of proteins from karyopherin-based molecular sequester.

Our data do not specifically conclude whether Ran-GTP or –GDP is locally enriched in subcompartments of the axon. However, the enrichment of RanBP10 in growth cones suggest that Ran-GTP might be generated there and locally enriched compared to Ran-GDP. Interestingly, the existence of a Ran-RanBP10-tubulin complex raises the possibility that Ran-GTP might be specifically enriched near RanBP10-bound microtubules, and selectively stabilize them via effector proteins. Indeed, the Ran-regulated protein APC promotes stability of microtubules in growth cones, and also regulates the actin/microtubule interface (27–29, 44–47).
Although APC is understood as a Ran-regulated protein during mitotic spindle formation, it is unknown if its activity is Ran-dependent in the axon. It would be interesting to see APC or other proteins with dual mitotic spindle/growth cone functions are regulated by Ran in both contexts. We are currently performing experiments to determine if Ran nucleotide binding state is locally and dynamically regulated in growth cones.

Our data also do not determine whether Ran releases proteins from karyopherin-based sequester. However, some evidence exists which supports this conclusion. First, as stated above, proteins known to be regulated by Ran to promote microtubule stability in the mitotic spindle are present and active in the axon growth cone. Second, karyopherin mRNA, including importin-β mRNA, is present in embryonic growth cones and axons, suggesting that their proteins might be present there as well (31, 32). Furthermore, importin-α was shown to control axon pathfinding and synaptic bouton number in Drosophila larva (48). These data, combined with our live imaging results, are consistent with Ran functioning locally to control microtubule dynamics.

While filopodial “faults” were not observed in laminin-plated growth cones of control knockdown neurons, their occurrence was significantly increased compared to control neurons when Ran was knocked down in CSPG-plated neurons. The filopodial fault phenotype is significant because filopodia are the motile growth cone subcompartment that contain microtubules, in contrast to lamellipodia (36). Since we only ever observed filopodia on CSPG, and since these filopodia displayed the greatest number of morphological abnormalities, it is quite possible that modifications of neuronal Ran effect growth cone microtubule dynamics.

Indeed, our SiR-tubulin live imaging on laminin-coated substrates attributes at least part of the growth cone motility phenotype to microtubule dynamics. These data are consistent with a model.
wherein local generation of Ran-GTP by RanBP10 in the growth cone controls microtubule dynamics by releasing effector proteins from karyopherin-based molecular sequester.

A primary limitation of the experiments presented here is that they do not distinguish axonal and somatic Ran functions. After all, a critical function of Ran in any nucleated cell is to regulate nucleocytoplasmic trafficking; how can we then be sure that the effects we note in, for example, whole cell Ran knockdown are not attributable to changes in nucleocytoplasmic trafficking? We think it is unlikely that the effects noted here are exclusively due to changes in nucleocytoplasmic trafficking. First, while we do see dramatic changes in axon elongation in Ran knockdown neurons, the cells are remarkably healthy, not only surviving but reliably generating axons, a highly complex task. Recent studies have linked amyotrophic lateral sclerosis to inhibition of nucleocytoplasmic trafficking (49–51). These models suggest that elongated transcripts physically block nucleocytoplasmic trafficking, leading to neurodegeneration. Since our Ran knockdown neurons show no signs of degeneration as late as DIV8, and since Ran knockdown would presumably alter nucleocytoplasmic trafficking more acutely than the slow buildup of aberrant transcripts, we think these effects are at least partially attributable to axonal, not somatic, Ran. Furthermore, immunocytochemical analysis of our Ran knockdown neurons confirms that while axonal and growth cone Ran levels are reduced, somatic Ran levels are maintained in these neurons (Figure 4 and data not shown). Finally, we present data here that RanBP10, the only known cytoplasmic Ran GEF, is enriched specifically in growth cones. RanBP10s only known function outside of the neurons is to regulate platelet formation in megakaryocytes, a Ran-dependent function (41–43). We think it is unlikely that neurons specifically localize this microtubule-associated Ran GEF to growing axon tips if its function isn’t at least partially to produce Ran-GTP or control cytoskeletal dynamics there. Still,
we are currently exploring ways to manipulate Ran expression specifically in the axon or soma to more conclusively address this limitation.

One exciting possibility that our data raises is that Ran might perform distinct functions in the axon depending on the temporal needs of the axon. Our data are consistent with a model wherein Ran promotes microtubule stability locally in the growth cone. What might Ran be doing in the adult axon, where we know its mRNA is present and it is primarily GTP-bound, at least in healthy axons? The Fainzilber group developed an elegant model wherein Ran-GTP in the adult axon acts as a brake on an importin-based retrograde injury signaling complex (30). After injury, RanGAP mRNA is rapidly transcribed, and Ran-GTP is converted to Ran-GDP, releasing the retrograde injury signaling complex, which transports NLS-containing injury signals to the cell body, which responds by activating a pro-regenerative transcriptional program. In this model, newly converted Ran-GDP remains in the growth cone. It is possible that a delayed trafficking of RanBP10 in the growth cone might then reestablish the microtubule-regulating Ran role. A delayed enrichment of RanBP10 could be achieved by upregulation of RanBP10 mRNA or increased translation of RanBP10 mRNA after injury. Indeed, increased translation of specific transcripts after injury is common in the axon (30, 52, 53) Our data lay the groundwork for the exciting possibility that the axon is a distinct Ran niche, which modifies canonical Ran function to novel ends during development, in the adult axon and after axon injury.
Materials and Methods

Antibodies and reagents

The antibodies used were as follows: anti-Ran (mouse BD Biosciences, San Jose CA; Rabbit Abcam, Cambridge MA), anti-α-tubulin (Abcam), anti-RanBP10 (ProSci, Poway CA), anti-IgG (Abcam), anti-βIII-tubulin (Sigma-Aldrich, St. Louis, MO), anti-H3 (Abcam). CD10 timed pregnant mice were obtained from Charles River Laboratories (O’Fallon, MO) for isolation of embryonic DRGs.

Plasmid construction

Ran shRNA and RanBP10 shRNA were selected from a panel of five shRNAs per gene provided by the McDonnel Genome Institute at Washington University in St. Louis. Farnesylated EGFP was a generous gift from Naren Ramanan (Indian Institute of Science) and was cloned into a lentiviral vector using AgeI and EcoRI restriction sites. WT Ran was a generous gift from Jon Lane (University of Bristol) and was cloned into a lentiviral vector using BamHI and AgeI restriction sites.

Lentiviral production

Lentiviral particles were concentrated from HEK293T culture medium as described previously (34, 54). Briefly, HEK293T cells were transfected using Lipofectamine 2000 with the FUGW-JIP3 construct of interest, pVSV-G and dR8.91 in a 1:1:3 ratio according to the manufacturer’s specifications (Invitrogen, Grand Island, NY). After 2 days, culture medium was collected and filtered through a 0.45 µm pore. Filtered culture medium was incubated overnight with Lenti-X lentiviral concentrator according to the manufacturer’s instructions (Clontech, Mountain View, CA) and centrifuged at 1,000xg for 45 minutes to pellet lentiviral particles. Pellets were resuspended in opti-mem before adding to cultures.
Cell culture, transfection, staining and image analysis

DRG culture and infection

DRG neurons were isolated and cultured as previously described (54). Briefly, DRG neurons were isolated from e13 mouse pups and dissociated with 0.05% Trypsin-EDTA. Neurons were plated in neurobasal culture media supplemented with B27 (2%), penn/strep (1%), Glutamax (1%), FDU (0.5%) and NGF (0.1%). Neurons were plated on substrates coated in poly-D lysine and either laminin 3.3 µg/mL (ThermoFisher, Grand Island NY) or chicken whole nervous system CSPG at 1.5 µg/mL (Millipore). Spot culture assays were plated in media at a concentration of 5 µl/10,000 cells and allowed to sit for 5-15 minutes before the full volume of plating media was added, and neurons were infected at DIV1 and fixed and stained at DIV3 for α-tubulin. Dissociated cultures were resuspended in full plating medium volume before plating. For staining of endogenous proteins, neurons were fixed at DIV1 (untransfected) or infected at DIV1 and fixed at DIV2. For expression analysis by western blot, neurons were infected at DIV1 and fixed at DIV3.

Fixing and staining

Culture media was removed and replaced with 4% phosphate-buffered paraformaldehyde for 15 minutes to fix neurons. Neurons were washed with PBS thrice before blocking in 10% goat serum/0.1% Triton X-100 in PBS. Primary antibody was diluted in blocking solution and added to cultures after 20 minutes. Cultures were washed thrice with PBS before the addition of secondary antibodies (1:1000). All antibody incubation times were 30 minutes. Cultures were washed thrice with PBS after secondary antibody incubation before mounting using Prolong mounting medium (Life Technologies).

DRG live imaging
DRG neurons were dissociated and plated on live imaging dishes as described above. At 6h post-plating, neurons were infected with f-EGFP virus. At DIV1, neurons were infected with ctrl or Ran shRNA. For SiR-tubulin experiments, SiR tubulin reagent was suspended in plating media and added to neurons at 1 µm 2h prior to imaging on DIV2. Images were acquired using fluorescence microscopy (Nikon, Eclipse TE 200-E) and analyzed using ImageJ. Images were acquired every 3s over a 5-10min imaging timeframe for each movie. The Δ area per time was calculated as described previously (34). Briefly, images were thresholded and the magic wand tool was used to calculate growth cone area for each movie frame. Microtubule motility of the SiR-tubulin probe was analyzed similarly. Filopodial tracking was performed manually using the Manual Tracking plugin for ImageJ.

For CSPG live imaging, neurons were plated using microfluidic chambers (Xona Microfluidics, Temecula, CA), since the CSPG caused DRGs to fail to adhere to the live imaging dishes. Live imaging dishes were coated with PDL before adhering the microfluidic device according to the manufacturer’s specifications. Then, laminin and CSPG were coated on opposite sides of the chambers in different volumes to generate osmotic pressure and coating specificity. Neurons were plated on the laminin side and infected at DIV1. Live imaging was performed at DIV4. Despite plating 10,000 neurons in each device, fewer than 10 axons were detected on the CSPG side of the device, and so all available growth cones were analyzed in each of two replicates.

Image acquisition and analysis

Cellular images were acquired via fluorescence microscopy and analyzed using ImageJ. For dissociated cultures, axon length was analyzed using the NeuronJ plugin. For RanBP10 line scans (Figure 4A-B), the tip of the axon was traced using the segmented line tool and RanBP10 intensity was normalized to f-EGFP intensity. For Ran and RanBP10 growth cone intensity
(Figure 4D-G), the growth cone was defined as the point where the tubulin stain splayed. A box was drawn around the growth cone at this point, and Ran or RanBP10 intensity was normalized to βIII-tubulin intensity.

**Coimmunoprecipitation**

Coimmunoprecipitation was carried out using rabbit αRanBP10 and mouse αRan antibodies, with mouse αIgG serving as a negative control. We used Dynabeads protein G-coupled magnetic beads (Millipore, Temecula CA) according to the manufacturers’ protocol. We also employed a BS$_3$ crosslinking step for the RanBP10 coimmunoprecipitation to eliminate IgG bands on the western blot, since only rabbit RanBP10 antibodies are commercially available. BS$_3$ crosslinking reagent (Millipore) was used according to manufacturer’s specifications. For input, approximately 100 DIV8 DRG spot cultures were collected and split evenly between the three immunoprecipitations (approx. total lysate volume: 1mL).
**Figure 1: Neuronal Ran promotes axon elongation.** (A) Representative blot of DRG cultures infected with control or Ran shRNA. (B) Quantification of (A). Ran knockdown reduced neuronal Ran levels approximately 70%. Error bars ± S.E.M, n=3 replicates. (C) Representative images of DRG spot cultures infected with the indicated constructs. The bars across the image (and all spot culture images) are an artifact of stitching together multiple images. Scale bar 100 µm. (D) Quantification of (C). The ten longest axons were measured from the nearest edge of the spot. Ran shRNA reduced axon elongation approximately 50%, an effect which was rescued with expression of human WT Ran. Error bars ± S.E.M., ANOVA $p<0.001$, #p$<<0.001$ Tukey HSD, n=12 (ctrl shRNA), 6 (Ran shRNA + Vector) or 8 (Ran shRNA + WT Ran) spots analyzed over three replicates. (E) Representative western blot of DRG cultures infected with vector or human WT Ran. The top band in the Ran blot is the GFP-Ran fusion construct, the bottom band is endogenous Ran. n=2. (F) Representative images of DRG spot cultures infected with GFP vector or WT Ran. Scale bar 100 µm. (G) Quantification of (F). Ran overexpression reduced axon elongation slightly, but significantly. Error bars ± S.E.M., #p$<<0.001$ Student’s t-test, n=4 spots per group over two replicates.
**Figure 2: Ran knockdown reduces growth cone filopodial and microtubule dynamics.** (A) Representative time projections of growth cones imaged with f-EGFP over five minutes. In these images, all images from the movie were collapsed, so a larger area indicates more motility and not necessarily a larger growth cone. Scale bar 5 µm. (B) Overall growth cone motility was measured as the rate of area change per time calculated from (A). A greater rate of area change per time indicates a more motile growth cone. Error bars ± S.E.M., \#p<<0.001 Student’s t-test, n=11 (ctrl shRNA), 9 (Ran shRNA) growth cones analyzed over 3 replicates. (C) Filopodial speed measured by manually tracking filopodia in (A). The first five filopodia in each growth cone was analyzed or, if fewer than five filopodia were present in a video, all filopodia were analyzed. Ran knockdown significantly reduced filopodial speed. Error bars ± S.E.M., \#p<0.001 Student’s t-test, n=60 (ctrl) or 52 (Ran shRNA) filopodia analyzed over two replicates. (D) Representative still images of SiR-tubulin stain in f-EGFP transfected neurons. The SiR-tubulin signal is never detected outside the f-EGFP positive cell membrane. (E) Quantification of microtubule motility in (D). The change in area for the SiR-tubulin signal was significantly reduced in Ran knockdown cells, indicating a reduction in microtubule motility. Error bars ± S.E.M., \#p<<0.001 Student’s t-test, n=9 (ctrl shRNA), 10 (Ran shRNA) growth cones analyzed over two replicates.
Figure 3: Ran knockdown promotes filopodial motility and axon growth on CSPG-coated substrates. (A) Representative images of DIV3 DRG neurons plated on CSPG and infected with the indicated constructs. Scale bar 50 μm. (B) Quantification of axon length in (A). Neurons extended their axons much further when Ran was knocked down compared to control cells, and this effect was rescued with expression of WT Ran. Error bars ± S.E.M., ANOVA $p<0.001$, $p<0.001$, n.s., not significant, Tukey HSD, n=121 (ctrl shRNA), 116 (Ran shRNA + Vector), 10 (Ran shRNA + WT Ran) neurons analyzed over three replicates. (C) Representative time projections of growth cones imaged with f-EGFP over five minutes, as in Figure 2A. Scale bar 5 μm. (D) Representative filopodial faults from (C), and a quantification of the incidence of each fault. Ran knockdown neurons were more likely to display filopodial faults than their control counterparts. **$p<0.01$, Chi square test. (E) Overall growth cone motility measured from (C) as the rate of area change per time was reduced approximately tenfold compared to laminin-plated neurons (compare to Figure 2B), but Ran knockdown partially rescued this phenotype. Error bars ± S.E.M., #p<<0.001 Student’s t-test, n=14 (ctrl) or 8 (Ran shRNA) growth cones analyzed over two replicates. (F) Filopodial speed measured from (C) using manual tracking. Filopodial speed was reduced on CSPG approximately 100-fold compared to laminin-plated neurons (Figure 2C), but was significantly increased in Ran knockdown cells compared to controls. Error bars ± S.E.M., #p<<0.001 Student’s t-test, n=71 (ctrl) or 40 (Ran shRNA) filopodia analyzed over 2 replicates.
**Figure 4: Ran BP10 is enriched in growth cones in a Ran-dependent manner.** (A) Representative images and line traces of RanBP10 in axon growth cones. Top panel, f-EGFP signal, middle panel, RanBP10 intensity (shown as a heat map), traces, RanBP10 signal corrected for f-EGFP signal in arbitrary units. RanBP10 is consistently enriched in axon tips. Scale bar 5 µm. (B) Quantification as in (A), averaged across neurons. RanBP10 is enriched approximately 80% at the axon tip compared to the axon. Error bars ± S.E.M., n=40 neurons over two replicates. (C) Coimmunoprecipitation of cultured DRG lysates using RanBP10, Ran or IgG (negative control) antibody. While RanBP10 is not very abundant in neurons, both RanBP10 and Ran antibodies robustly immunoprecipitate endogenous RanBP10. Ran is also inefficiently immunoprecipitated by both RanBP10 and Ran antibodies, which is expected based on its abundance in the soma. Interestingly, a small quantity of tubulin is also specifically coimmunoprecipitated by both RanBP10 and Ran, suggesting that these proteins exist in a complex in neurons. Representative western blot from 2 replicates. (D) Ran staining in growth cones of control and Ran knockdown cells. Ran is slightly enriched in growth cones of control cells, but is mostly absent from growth cones of Ran knockdown cells. Scale bar 5 µm. (E) Quantification of (D). Ran knockdown significantly reduces Ran staining, correcting for growth cone size with βIII-tubulin counterstain. Error bars ± S.E.M., **p<0.01 Student’s t-test, n=20 growth cones analyzed per group over 1 replicate. (F) RanBP10 enrichment in growth cones is increased in Ran knockdown cells. Scale bar 5 µm. (G) Quantification of (F). Ran knockdown increases RanBP10 localization to the growth cone, implying a Ran-dependent transport mechanism for RanBP10. Error bars ± S.E.M., **p<0.01 Student’s t-test, n=60 growth cones analyzed per group over 3 replicates.
Figure 5: RanBP10 promotes axon elongation in a Ran-dependent manner. (A) Representative western blot of DRG lysates infected with control or RanBP10 shRNA. (B) Quantification of (A). RanBP10 shRNA reduced expression approximately 50%. Error bars ± S.E.M. (C) Representative images of spot cultures infected with the indicated constructs. RanBP10 knockdown reduces axon elongation, and interestingly this effect is rescued by expression of WT Ran. Scale bar 100 µm. (D) Quantification of (C). That RanBP10 can be rescued by expression of WT Ran suggests a Ran-dependent role for RanBP10 in promoting axon elongation. Error bars ± S.E.M., ANOVA $p<<0.001$, $\#p<<0.001$ Tukey HSD, $n=10$ (ctrl shRNA), 6 (RanBP10 shRNA), 7 (RanBP10 shRNA + Vector or + WT Ran) spots analyzed per group over two replicates. (E) Representative images of control and RanBP10 knockdown neurons plated on CSPG. Scale bar 50 µm. (F) Quantification of axon length from (G). RanBP10 knockdown did not affect axon elongation on CSPG, again suggesting a Ran-dependent role for RanBP10 in axon elongation. Error bars ± S.E.M., n.s., not significant Student’s $t$-test, $n=70$ (ctrl) or 35 (RanBP10 shRNA) neurons analyzed per group over two replicates.
Preliminary Figure 1: Neuronal Ran promotes axon branching and elongation in a nucleotide binding-state dependent manner. (A) Representative images of dissociated DRG neurons expressing the indicated construct. Scale bar 100 µm. (B) Quantification of the length of the longest axon in (A). Axon elongation is significantly reduced by expression of Ran-GTP or Ran-GDP. ANOVA $p=0.03$, *$p<0.05$, **$p<0.01$ Tukey HSD, $n=89$ (ctrl) 91 (WT Ran, Ran-GTP) or 96 (Ran-GDP) neurons analyzed over three replicates. (C) Quantification of axon terminal branching from (A). Expression of WT Ran or Ran-GDP reduces the percentage of neurons undergoing terminal branching. **$p<0.01$, $#p<0.001$, Chi square test, $n$ as in (B). (D) Quantification of branch number from (A). Expression of Ran-GTP significantly increased branch number compared to control. *$p<0.05$, Mann-Whitney U-test following Kalmogarov-Smirnov test for normality, $n$ as in (B). (E) Quantification of length to first branch point from (A). Expression of Ran-GTP caused premature ectopic branching. ANOVA $p=0.11$, *$p<0.05$ Tukey HSD, $n$ as in (B).
Preliminary Figure 2: Ran-GTP promotes axon elongation. (A) Representative images of nucleotide-locked Ran mutants in a Ran shRNA background. Scale bar 100 µm. (B) Quantification of (A). While expression of WT Ran rescued Ran knockdown, but expression of Ran-GDP did not. Interestingly, expression of Ran-GDP increased axon elongation above control levels, implying that a high relative Ran-GTP concentration promotes axon elongation. ANOVA $p<0.001$, $\#p<0.001$ compared to control Tukey HSD, $n=12$ (ctrl), 8 (Ran shRNA +WT Ran), 7 (Ran shRNA +Ran-GDP), or 6 (Ran shRNA +Vector, Ran shRNA +Ran-GTP) spots analyzed over two replicates per group.
References


CHAPTER 4

Discussion: molecular complexity and versatility are emerging themes in axon biology
Conclusion

The field of axon biology is incredibly complex, encompassing elements of epigenetics, cytoskeletal biology, molecular motor biology, bioenergetics, translational regulation, membrane biology, and many other fields. Whittling the field down to axon growth does not change the scale of complexity, as most elements that are necessary for axon homeostasis are also necessary for axon growth during development or regeneration. Therefore any examination of axon biology will be underrepresentative of the field, but careful study of components of this delicate system can lead to insights into how the axon functions generally. The work presented here supports two increasingly clear phenomena of axon biology. First, regulatory systems in the axon are multi-layered, which enables fine-tuning of diverse systems, including the axon transport system. Second, proteins in the axon are versatile, serving distinct functions based on their localization or the temporal needs of the neuron.

Regulating the molecular motor regulators

The regulation of axonal transport remains a primary concern for neurobiology. Neurons are postmitotic and therefore uniquely susceptible to aberrations in their cell biology. Furthermore, neurons are the largest cells in the body and therefore rely heavily on directed transport to survive and respond to their environments. Therefore understanding how axonal transport is regulated is key to understanding axonal biology.

The work presented here in Chapter 2 contributed importantly to the field of neuronal transport regulation, demonstrating that JIP3 was capable of independently activating tetrameric kinesin-1 for motility, and that differential binding to the KLC and KHC subunits has distinct effects on kinesin-1 function. Interestingly, JIP3 also binds dynein and regulates retrograde
signaling (1). Indeed, JIP3 seems to be a bidirectional motor regulator depending on its cargo and motor binding partners (2). How does JIP3 regulate the transport of multiple cargoes and multiple motors? Interesting insights into this question have recently come from the Holzbaur group as they study JIP1, a protein that is structurally and functionally related to JIP3. JIP1 also regulates kinesin-1 motility via direct binding to its KHC subunits (3). Interestingly, JIP1 also functions as a switch, enabling long-range retrograde transport of autophagosomes (4). JIP1 therefore both relieves autoinhibition of KHC to promote anterograde transport and enhances KHC autoinhibition to promote retrograde transport. JIP1 achieves these opposing functions by binding other cargo adaptors, specifically LC3, and via phosphorylation-dependent activity regulation. Preliminary data in our lab has identified multiple sites of in vivo phosphorylation on JIP3, at least one of which promotes axon elongation (preliminary data not shown). It will be interesting to see how activity of JIP3 and other cargo adaptor proteins is precisely tuned to support multiple modes of transport.

**Novel functions for nuclear proteins**

A fundamental question in the axon biology field is how the axon and cell body coordinate their activities to support axon growth, homeostasis and injury response. As focus in the field has shifted towards epigenetic regulation of neural growth state, a growing contingent of proteins is being recognized as having distinct function in the axon versus the cell body, and especially the nucleus. It seems the remote location of the axon has enabled repurposing of nuclear proteins for novel purposes in the axon. Localization-dependent protein versatility maximizes efficiency in the neuron, especially enabling efficient signaling between the cell body and axon.
Epigenetic modulators are interesting candidates for coupling nuclear activity to axonal biology, since they are partially responsible for the large-scale transcriptional changes that often accompany axon outgrowth during development or injury. The growing tip of an axon is usually far removed from the nucleus, and the growth cone is a temporary structure, the presence of which typically coincides with large-scale transcriptional changes, i.e. during neuronal development or after axonal injury. Additionally, temporary large-scale cytoskeletal reorganization is a hallmark of both nuclear and axonal biology during mitosis and outgrowth, respectively. Therefore it would be efficient for the neuron to couple epigenetic regulation to microtubule remodeling. The case of HDAC5 during axon regeneration is an example of this phenomenon (5–7). HDAC5 was originally studied as an epigenetic modulator of chromatin compaction via its histone deacetylase function before being appreciated as a microtubule deacetylase. After injury, a fast calcium wave induces HDAC5 export from the nucleus, causing injury-associated epigenetic changes to promote axon regeneration somatically. HDAC5 is then transported to the axon, where it functions as a tubulin deacetylase, promoting microtubule instability and axon regeneration axonally. The redistribution of HDAC5 happens in multiple phases, yoking the short-term injury response of cytoskeletal reorganization in the axon to more long-term transcriptomic injury responses. Further, the model of post-injury HDAC5 redistribution could theoretically be generalized to other nuclear proteins (Figure 1). This model efficiently couples nuclear transcription programs to axonal regeneration, as redistributed nuclear proteins would exert cellular effects more quickly than changes in transcriptional programs. A biphasic response to modify or generate growth cones results: an initial, fast response as nuclear proteins are redistributed from nucleus to axon tip, causing chromatin remodeling and beginning axonal tubulin reorganization; and a slower response wherein newly activated growth cone-
modulating genes are transcribed, translated and trafficked to the growing axon tip. This coupling mechanism could be used in either growth or development. Importantly, this establishes a paradigm whereby axon-specific functions of primarily nuclear proteins are a feature of neuronal signaling.

Since Ran has known functions as both a nucleocytoplasmic trafficking protein and as a microtubule organizing protein, it is a prime candidate for coupling somatic and axonal biology (Figure 2). The Fainzilber group has developed a model wherein the nucleotide binding state of Ran functions as a switch for retrograde transport of injury signaling. In adult axons, Ran-GTP is highly enriched and binds an importin/dynein complex, preventing its binding to nuclear localization signal (NLS)-containing cargoes. After injury, newly-transcribed RanGAP rapidly hydrolyzes Ran-GTP to Ran-GDP, allowing the importin/dynein complex to bind NLS cargoes, which are transported back to the cell body as positive injury signals (8). The data presented in Chapter 3 build on this model by defining the role of Ran in axon development as opposed to injury. Interestingly, these data are also consistent with a Ran nucleotide binding state-dependent switch on retrograde transport. There are two main differences between the Fainzilber model of Ran function in axon injury signaling and the model presented here for Ran function in axon growth: the direction of the Ran nucleotide binding switch, and the identity of the retrograde cargo. The Fainzilber model proposes enrichment Ran-GDP at injured axon tips, while we propose enrichment of Ran-GTP at growing axon tips. The enrichment of Ran-GDP is generated by newly transcribed Ran GAP in the Fainzilber model, while active Ran-GTP in our model is generated by the enrichment of the Ran GEF RBP10 at growing axon tips. The enrichment of Ran-GTP at growing axon tips during development could theoretically serve two purposes: growth cone-enriched Ran-GTP might modulate microtubule dynamics via effector proteins, or
conversion of Ran-GDP to –GTP might aid in maturation of the axon (after all, Ran-GTP is the predominant binding state in adult axons, per Fainzilber). Both of these functions are supported by the data presented here: Ran knockdown both reduces growth cone microtubule dynamics as measured by SiR-tubulin and causes ectopic enrichment of RBP10 in growth cones. This second piece of evidence suggests that RanBP10 is a Ran-dependent retrograde axonal cargo, as opposed to the importin cargo proposed in injury signaling. Why is RBP10 a retrograde cargo? It is interesting to ruminate on this question in the context of another model proposed by the Fainzilber group, the oscillating signal model for axon length sensing (9), since RBP10 could theoretically function as a self-regulating oscillating signal. That is, RBP10 can promote its own retrograde transport by converting Ran-GDP to –GTP, since Ran GEFs preferentially bind Ran-GDP. An oscillating RBP10 retrograde injury signal could be achieved in one of two ways. First, RBP10 mRNA might be transported to the axon in timed waves and subsequently translated there. Alternatively, RBP10 protein at the axon tip could generate its own oscillating signal via Ran-GTP: as RBP10 is retrogradely transported to the cell body, production of Ran-GTP in the axon tip would diminish, creating local enrichment of Ran-GDP, which would bind RBP10 ad inhibit its retrograde transport to the soma, which would cause increased accumulation of RBP10 at the axon tips, which would increase production of Ran-GTP at the axon tip, which would increase retrograde transport of RBP10 to the cell body, and so on. If Ran indeed functions as a retrograde signaling sequester protein during development as it does during injury, the exact function of this retrograde cargo is indeed worthy of further exploration.
Figure 1: Proposed mechanism of coupling epigenetic regulation to axonal signaling via dynamic relocalization of dual-purpose proteins. (A) Summary of recent work out of the Cavalli lab establishing the dynamic relocalization of HDAC5 in response to injury signaling. Suppression of HDAC5’s canonical function in the nucleus is achieved when the protein is exported from the nucleus and anterogradely transported to the distal axon tip. This has dual effects, activating a pro-regenerative transcriptional program in the soma and remodeling microtubules in the distal axons. (B) A generalized model coupling epigenetic regulation to axonal biology. Dynamic relocalization of chromatin modeling proteins creates a biphasic response: relocalization of nuclear proteins to the axon could enable short-term effects via noncanonical functions (for example, regulating microtubule dynamics) while simultaneously triggering long-term changes in transcriptional programs.
Figure 2: Proposed role for Ran regulating axonal retrograde signaling during injury and development. (A) Summary of recent work from the Fainzilber group establishing Ran as a retrograde injury signaling regulator. (1) After injury, newly transcribed RanGAP converts Ran-GTP to Ran-GDP. (2) A dynein-coupled complex is released via release of importin, which preferentially binds Ran-GTP. (3) Retrograde transport of the importin-dynein injury signal enables pro-regenerative transcriptional reprogramming. (B) Proposed role for Ran-GTPase in axon development. (1) RBP10, either transcribed in the growth cone de novo or transported to the growing axon tip, converts Ran-GDP to Ran-GTP. (2) Growth cone-enriched Ran-GTP promotes microtubule remodeling via effector proteins. (3) Oscillating retrograde transport of RBP10 informs somatic sensors of axon length.
References


