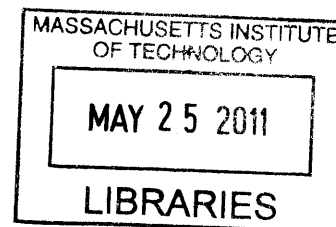**The Role of Ena/VASP and Associated Proteins in Regulation of Neuronal Morphology and Filopodia Architecture**

by

Leslie Marie Mebane

B.S. Cell and Molecular Biology
Texas A&M University, 2003

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT
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The Role of Ena/VASP and Associated Proteins in Regulation of Neuronal Morphology and Filopodia Architecture

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Leslie Marie Mebane

Submitted to the Department of Biology on May 17, 2011 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

Thesis Abstract

During central nervous system development cortical neurons extend a primary axon and multiple collateral branches to connect to numerous synaptic targets. While many guidance cues and their receptors have well-characterized roles in cortical axon guidance, the pathways that link these signals to cytoskeletal remodeling remain poorly understood. The Ena/VASP family of proteins function as key signaling molecules that influences actin reorganization in response to environmental cues, and has been implicated in many aspects of development.

My work has focused on defining the mechanisms by which the brain-specific ubiquitin ligase, Trim9, regulates cytoskeletal dynamics in response to the axon guidance cue Netrin-1 and its receptor DCC. I have shown Trim9 binds the cytoplasmic tail of DCC and also binds Ena/VASP proteins and Myosin-X, which are cytoskeletal effectors downstream of Netrin-1. I discovered that inhibition of Trim9 ubiquitin ligase activity specifically blocks Netrin-1 induced cortical branching. I uncovered an interaction between Trim9 and the microtubule-associated protein, Map1b, a regulator of microtubule stability and axon branching. My data demonstrates that Trim9 coordinates Netrin-1 induced axon branching via its interaction with the cytoplasmic tail of DCC and cytoskeletal-associated proteins.

I have also investigated the role of several actin-associated proteins in regulation of the actin ultra-structure. I used platinum replica electron microscopy to study the architecture of actin in neurons null for the Ena/VASP family, which failed to form axons. We determined the defect in axon formation is due to an inability to form bundled actin filaments and filopodia. In addition, splice isoforms Mena, a member of the Ena/VASP family, are tightly regulated during cancer metastasis and we determined these splicing changes influence the assembly of actin protrusions. My findings have helped to elucidate how environmental signals affect actin cytoskeletal dynamics and how changes in the cytoskeleton influence development.

Thesis Supervisor: Frank Gertler
Title: Professor of Biology

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Growing up in my small, rural Texas town I often watched Cheers on TV. Never did I think I would move to Boston and find my very own Cheers, at MIT, much less. The Muddy Charles Pub is such a fantastic, dirty place and I have experienced some of the best times of my life behind and in front of the bar. I must thank Mike Greiner for being such a great pub manager and helping us make the Muddy even better for years to come.

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Mom and Dad, this thesis is dedicated to you. I feel so fortunate to have such amazing parents, who encouraged me my whole life, and made anything seem possible with hard work. You are amazing role models and my inspiration. David, you are the best brother a mean older sister could ask for. I only hope to have the kind of happiness you, Brianna and Elli share someday.

Finally I would like to acknowledge my boyfriend, Dave. I am continually amazed by the amount of love and support you give me. I am truly lucky to have you. After reading my entire thesis, I am sure it is painfully clear I am not one for words, and I hope you understand how grateful I am for all that you do for me.

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Thesis Overview

Precise cell movement is a fundamental physiological process necessary for embryonic development. Individual motile cells must adhere to substrates, move past neighboring cells, migrate through tissues, and respond to extracellular cues. These cues result in polymerization or depolymerization of the actin and microtubule cytoskeleton leading to changes in cell shape and movement. My thesis work has focused on signaling pathways that modulate cytoskeletal dynamics required for cell motility, neuronal morphology, axon guidance and neuronal branch patterning.

In the following introduction I will give a basic overview of the central nervous system development and discuss several major classes of guidance systems. I will focus on signaling pathways and molecules that affect the cytoskeleton, giving special attention to mechanisms that will be of importance in experiments discussed later.

In the body of the thesis, I will address outstanding questions in the field through data that elucidates the function of several cytoskeletal-associated proteins. In particular, in Chapter 2, I will introduce a novel E3 ubiquitin ligase, Trim9, its required function in shaping neuronal morphology in response to guidance cues. In Chapter 3 and Appendix A, I will describe the role of the Ena/VASP protein family plays in cytoskeletal remodeling.

1.1 Development of the Cortex

The nervous system is an intricate organ that coordinates formation of specific connections between 10^{10}-10^{11} neurons (Tessier-Lavigne and Goodman, 1996). A neuron's distinct morphology is the foundation for a functioning brain. In the following section I will outline the specific stages of cortex development, and in particular, I will focus on the morphological changes a post-mitotic cortical neuron undergoes to form proper neural circuits.

The starting point in my research is the study of the Ena/VASP family of proteins that are known to influence actin remodeling and are implicated in axon guidance signaling pathways. We found that a novel Ena/VASP binding partner, a brain specific E3 ligase Trim9, also plays an important role in axon branching. Ena/VASP proteins are function in many aspects of development, but together, Trim9 and Ena/VASP, regulate cytoskeletal dynamics in response to the axon guidance cue netrin-1 and its receptor DCC during axonal branching.

1.2 Axon Guidance

As neurons migrate to their proper positions within the cortex, they extend multiple processes called neurites. In most cortical neurons, one of the neurites, which trails behind the migrating cell body, continues to elongate, becoming the axon. As they extend, axons respond to cues from the environment that guide them to their specific synaptic targets. The remainder of the neurites will form the dendrites, which remain in relatively close proximity to the cell body.

Growing axons encounter a complex milieu of extracellular signals as they navigate to their targets (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001). At the tip of each axon is a growth cone, a highly dynamic, actin-rich structure that contains all the machinery necessary to receive, integrate, and respond to the plethora of diffusible and substrate bound guidance cue signals. Growth cones constantly sample the microenvironment, dynamically remodeling the growth cone cytoskeleton to drive the appropriate response, leaving an elongating axon shaft behind (Dent et al., 2010). Guidance cues are bound by receptors on the growth cone that activate signaling cascades, ultimately leading to reorganization of both the cytoskeleton and cell adhesions which together underlie motility of the structure (Mitchison and Kirschner, 1988). The ability of a growth cone to respond quickly and accurately to multiple sources of spatial information allows axons to innervate their targets with remarkable accuracy (Lowery and Van Vactor, 2009).

1.2.1 Growth Cone Structure

The growth cone is a fan-like structure that can assume many shapes and sizes as it samples the environment during guidance (Dent et al., 2010). Growth cone movement is driven by the polymerization and depolymerization of the actin and microtubule cytoskeleton, discussed in detail below. As seen in Figure1-1, the peripheral (P) domain of the growth cone consists of actin microspikes, called filopodia, and mesh-like lamellipodia, which are protrusive, flat sheets of actin veils. Dynamic microtubules explore the perimeter of the growth cone and track into filopodia tips. The central (C) region of the growth cone consists mainly of stable, bundled microtubules that provide structure to the motile domain. Lastly, the transition (T) domain is at the interface

between the central and peripheral zone where integration between theses two distinct regions takes place (Dent and Gertler, 2003).
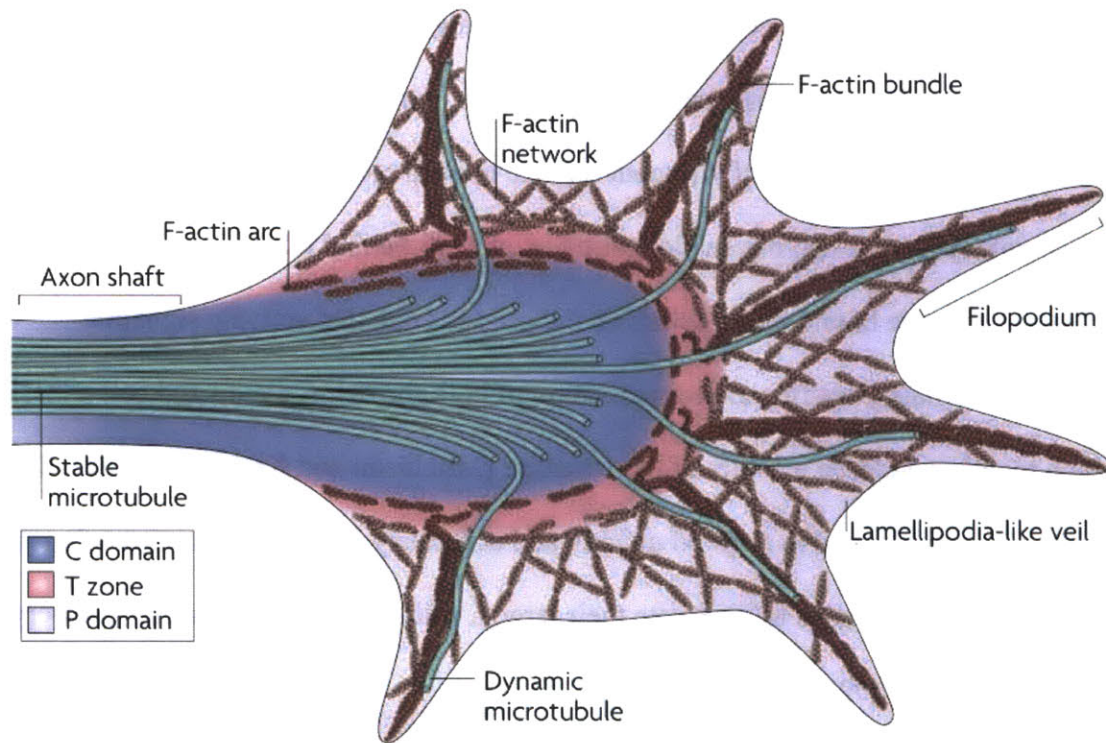
Figure 1-1: Structure of the Growth Cone
From (Lowery and Van Vactor, 2009)

1.2.1.1 Actin Dynamics in Growth Cone Protrusion

The two main actin structures at the leading edge of the growth cone are filopodia and lamellipodia (Figure 1-6). Filopodia are bundled actin microspikes and lamellipodia are dense actin meshwork (Mitchison and Kirschner, 1988). In both of these structures, actin filaments are oriented with their growing barbed ends directed toward the periphery.

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This places force generating structures at sites where rapid response to guidance cues will result in immediate morphological changes (Dent et al., 2010).

Actin polymerization is the core mechanism that controls growth cone guidance. Actin filaments are polar, helical filaments with a barbed and pointed end (Figure 1-2). Actin monomer can be added on either end but monomer addition is ten fold higher at the barbed end, and monomer dissociation occurs at the pointed end (Chhabra and Higgs, 2007). Most actin-based structures have substantial flux resulting in a treadmilling effect within actin filaments (Pollard and Borisy, 2003).
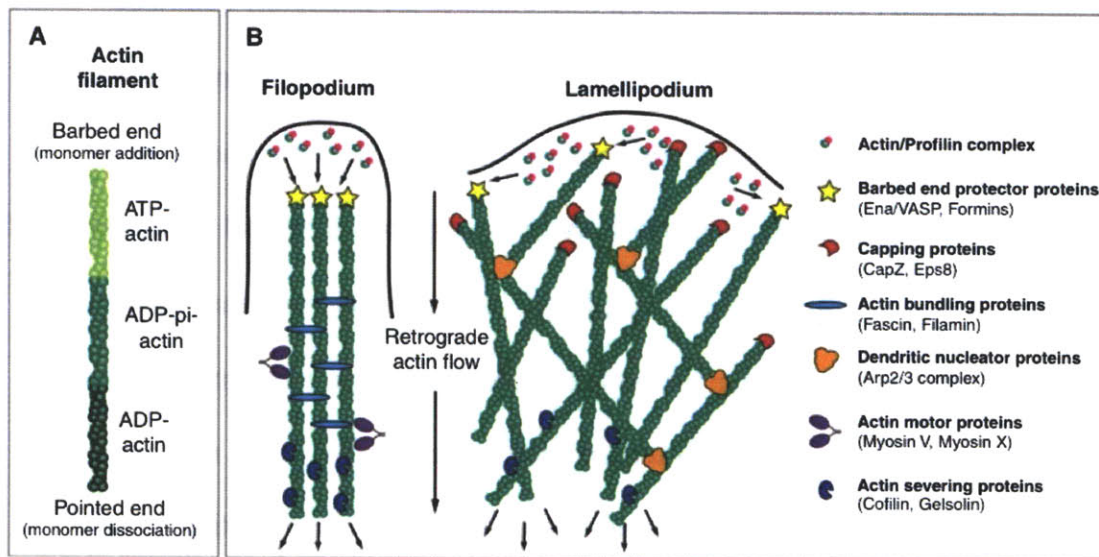
Figure 1-2: F-actin and associated proteins.
From (Dent et al., 2010).

The growth cone leading edge protrusion results from a combination of substrate adhesion, F-actin treadmilling and F-actin retrograde flow (Medeiros et al., 2006; Suter and Forscher, 2000). Actin retrograde flow is driven in two ways: by new F-actin polymerization in the P domain of the growth cone and contractility of the motor protein

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myosin II which crosslinks actin filaments in the T zone (Lin et al., 1996). Myosin II

filament compression across the T zone causes break down of the actin bundles aided by

actin-depolymerizing factors. Breakdown of F-actin in the T zone increases the

availability of actin monomers to the growth cone's leading edge (Sarmiere and

Bamburg, 2004).

1.2.1.2 Microtubule Dynamics in the Growth Cone Guidance

Microtubules (MTs) have generally received less attention in growth cone

guidance than actin, yet the growth cone cannot move forward without proper

microtubule function (Tanaka et al., 1995). Microtubules are inherently polarized

structures due to their construction (Figure 1-3), α and β tubulin form dimers that are

assembled into linear arrays which gives rise to a tubular structure (Luduena, 1998).

Neuronal microtubules are heterogeneous polymers composed of several combinations of

α β isotypes. Generally, βIII tubulin is found only in post-mitotic neurons (Caccamo et

al., 1989). Microtubules are known for being unstable structures, constantly undergoing

growth, collapse, and rescue, which is termed dynamic instability. In the nervous system,

microtubule-binding proteins (MAPs) regulate the stability and organization of this

cytoskeletal component to maintain cell shape and respond to changes in extracellular

cues.

A key mechanism of microtubules regulation is the posttranslational modification

of tubulin. Microtubules that become tyrosinated are characterized as dynamic,

polymerizing into the distal regions of the growth cone P domain, while acetylated

microtubules are localized to the growth cone C domain and usually do not co-localize

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with F-actin (Brown et al., 1993). These differential modifications can span different regions of a single microtubule and correlate directly with the age of the microtubule, yet do not confer stability to the structure (Khawaja et al., 1988).
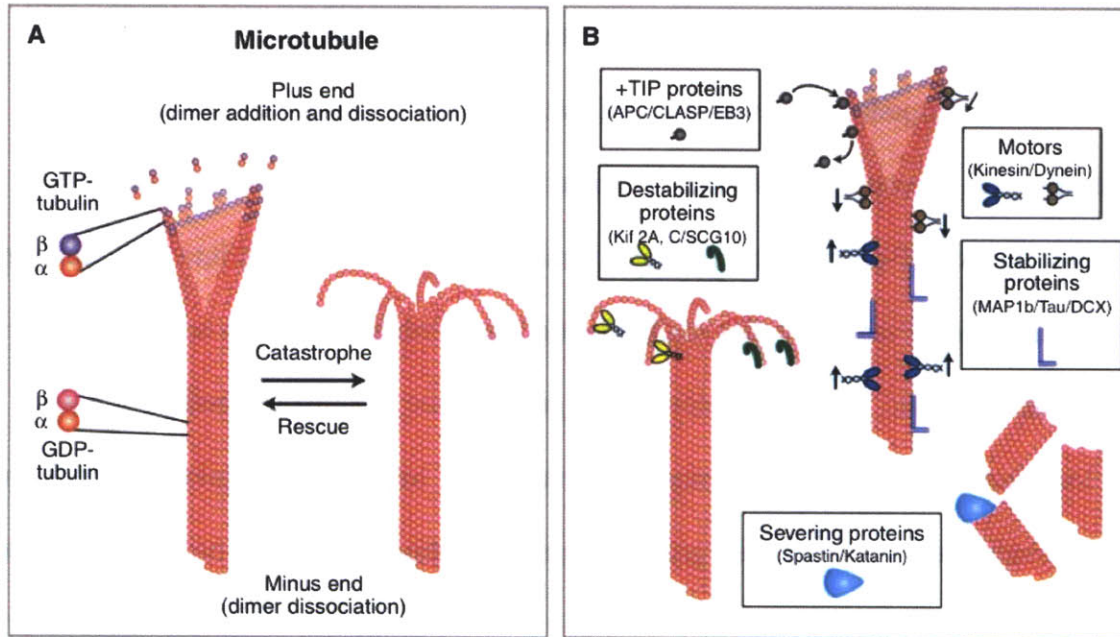
Figure 1-3: Microtubule Structure and Associated Proteins
From (Dent et al., 2010).

Twenty years ago, live cell imaging of neurons showed that fluorescently labeled MTs were capable of exploring the periphery of the growth cone and that the orientation of the MTs often predicted the direction of outgrowth (Sabry et al., 1991; Tanaka et al., 1995). Recent work has shown that asymmetrical delivery of drugs within the growth cone, which stabilize MTs, is sufficient to induce turning. However, this turning movement is lost if actin dynamics are blocked by cytochalasin D, a drug that prevents F-actin polymerization. Therefore, even if extracellular cues directly influence MT dynamics, growth cone turning must utilize the actin cytoskeleton (Buck and Zheng, 2002).

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1.2.1.3 Actin and Microtubule Coordination

Generally, the organization of actin and microtubules filaments in the growth cone regulates its coordinated movement. Distinct classes of actin structures regulate populations of microtubules. Dynamic microtubules explore the growth cone periphery and track into filopodia and it was thought that the bundled F-actin of filopodia provide tracts to guide microtubules into the P domain (Zhou and Cohan, 2004). A recent study demonstrated that F-actin is not required for microtubule advancement into the P domain and, furthermore, F-actin bundles inhibit their advance when coupled to actin retrograde flow (Burnette et al., 2007). Therefore in the growth cone P domain, F-actin determines MT localization by acting as a barrier to premature MT invasion and exploratory MTs in the P domain must uncouple their growth from actin retrograde flow in order to track into filopodia.

The absence of F-actin bundles in the P domain does not lead to the advancement of C domain microtubules to the periphery suggesting MTs in the C domain are restricted by the contraction of actin arcs of the T zone (Burnette et al., 2007). Disruption of these actin arcs results in an abnormally large C domain and a defect in axon consolidation during outgrowth. Myosin II plays a role in transporting MT to the C domain where they are further stabilized and crosslinked by MAPs (Bielas et al., 2007; Burnette et al., 2008). Myosin II in the growth cone neck is also important for the suppression of F-actin protrusions to allowing for axon consolidation (Loudon et al., 2006).

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1.2.2 Axon Extension

Both the actin and microtubule cytoskeleton are necessary for axon guidance. Dissociated neurons in culture treated with cytochalasin B, which results in F-actin depolymerization, showed continued axon outgrowth but the growth cone path was looped and misdirected. Drugs that induce depolymerization of microtubules, such as nocodazole, did not affect protrusive actin dynamics in the growth cone but eventually lead to a retraction of the entire axon (Dent and Kalil, 2001; Tanaka et al., 1995; Yamada et al., 1971). This provides evidence that F-actin maintains growth cone shape and guidance while microtubules give the axon the structure necessary for elongation.

Growth cone extension to form new axon segments occurs by protrusion, followed by engorgement and consolidation, outlined in Figure 1-4. Protrusion is characterized as an elaboration of filopodia and lamellipodia at the leading edge of the growth cone. Engorgement then follows with the invasion of microtubules to the peripheral domain bringing along vesicles and organelles. Finally during consolidation the neck of the growth cone assumes a cylindrical shape around the stable microtubules due to a loss of actin polymerization in this region. These three continuous and overlapping stages are the mechanism of axon elongation (Dent and Gertler, 2003; Lowery and Van Vactor, 2009).
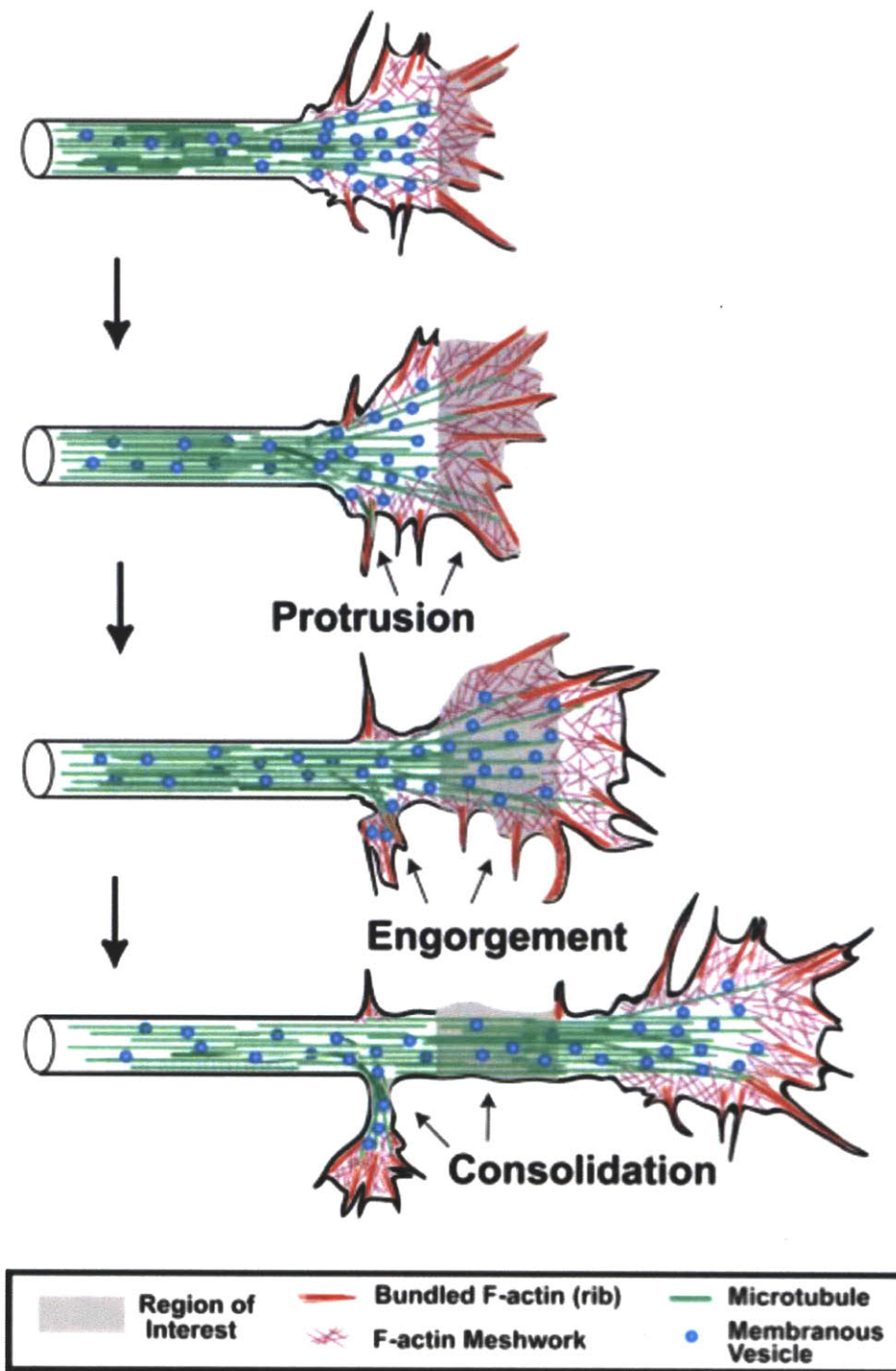
Figure 1-4: Stages of Axon and Branch Growth
From (Dent and Gertler, 2003).

1.2.3 Growth Cone Turning

In order for the growth cone to navigate according to spatial landmarks the cytoskeletal machinery must be capable of biased movement to achieve accurate turning toward a cue or away from a repellant (Figure 1-5). Attractive guidance cues, such as netrin, promote F-actin elongation and stabilization while dynamic microtubules explore the P domain potentially delivering cargo necessary to stabilize adhesions and recruiting key signaling components, such as actin-binding proteins or kinases (Lebrand et al., 2004; Suter et al., 2004). After the initial actin protrusion toward a cue, engorgement and consolidation locks the axon into growth in that direction. Alternatively, repulsive cues, like semaphorin, cause destabilization of actin filaments and microtubules, which then lead to partial growth cone collapse. Outgrowth then continues in the region of the growth cone not affected by the repulsive cue and repulsive turning occurs (Kalil and Dent, 2005).
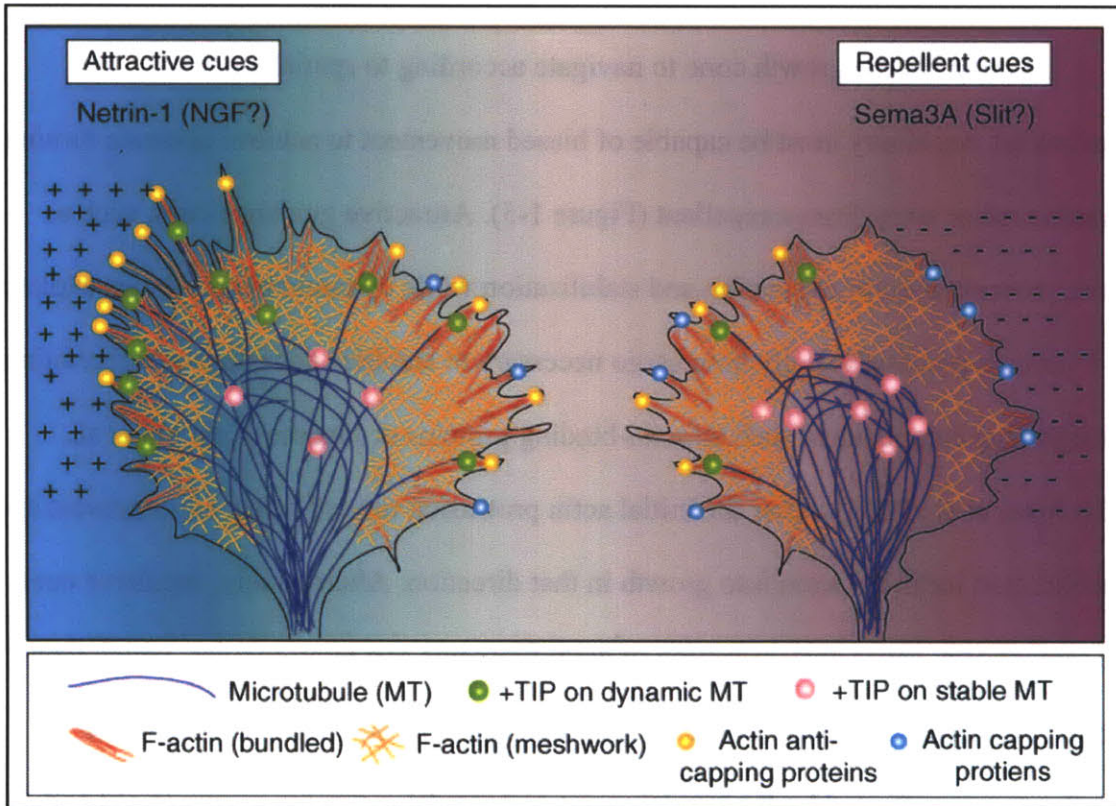
Figure 1-5: Growth Cone Turning
From (Kalil and Dent, 2005)

1.2.4 Axon Branching

A major focus of the axon guidance field has been to elucidate the mechanisms and signaling pathways employed by growth cone pathfinding during development. However, in many cases in the central nervous system, the primary axon bypasses the target with collateral branches subsequently forming in the region. These branches later form synaptic connections and the primary axon is lost in a process called axon pruning (Halloran and Kalil, 1994; Luo and O'Leary, 2005). Axon branching, common to almost every neuron, is crucial for the assembly of interconnected networks and many of the same mechanisms regulate growth cone behavior and branching (Hall and Lalli, 2010). Axon branching is characterized by dynamic reorganization of actin and microtubule cytoskeleton, influenced by guidance molecules, and regulated by similar signaling pathways (Dent et al., 2003). This being said, the mechanisms of axon guidance and axon branching are not identical and the resulting morphological complexity is the basis for the nervous system's intricate circuitry (Gallo, 2011; Gibson and Ma, 2011).

A growing axon can form a branch in two typical ways: growth cone splitting or outgrowth of a branch from the axon shaft. Both of these modes require stable microtubules to splay apart and invade local actin rich structures similar to axon growth and growth cone guidance. Growth cone splitting is not a major mechanism used during synaptic targeting but contributes to the basic organization of the nervous system (Gallo, 2011). Bifurcation of dorsal root ganglion growth cones as they enter the spinal cord is the archetypal example of this branching method (Schmidt et al., 2007). Growth cone splitting begins with suppression of protrusions at the leading edge of the growth cone, while cytoskeletal dynamics continue on each side. This continued asymmetrical growth

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leads to the formation of two growth cones that continue to advance as individual entities (Letourneau et al., 1986; Ma and Tessier-Lavigne, 2007).

Outgrowth of a branch from the axon shaft, called collateral branching, is the most common type of branching (O'Leary et al., 1990; Snider et al., 2010). The axon shaft is mostly composed of stable microtubules and exhibits minimal protrusive activity. Collateral branch formation requires the initiation of protrusive actin, filopodia or lamellipodia, from the axon shaft and is known to occur in two ways: local inhibition of cytoskeletal stabilizing factors or protrusions following growth cone pausing (Dent and Kalil, 2001). Corticospinal axons project collateral branches well after the primary growth cone has passed (Bastmeyer and O'Leary, 1996; Heffner et al., 1990). Pioneering live-imaging studies in cortical slice culture revealed the particular segments of the axon, which initiated multiple filopodia, some of these filopodia would stabilize and mature into collateral branches. Using the same live-imaging method, it was later shown that cortical colossal growth cones underwent extension and retraction in one region of the brain leading to little net growth, called growth cone pausing. Eventually the growth cone continues axon extension, but the segment of the axon in this particular region retains protrusive activity, resulting in branch formation and synaptic connections with the cortical target (Halloran and Kalil, 1994).

As mentioned previously, response to guidance cues in the growth cone and branching may not be regulated in the same way. Treatment with Slit-2 causes growth cone collapse but promotes axon branching (Brose and Tessier-Lavigne, 2000). The chemoattractant netrin-1 is a potent inducer of branching but had no effect on axon extension (Dent et al., 2004). Other groups have shown netrin-1 does stimulate axon

extension so the difference in branching and axon extension response may be specific to the neuron type or point in development (Bouchard et al., 2008; Bouchard et al., 2004; Rajasekharan et al., 2009). There are a growing number of extracellular and intracellular factors known to influence branching, such as classic axon guidance systems or extracellular matrix molecules, yet the molecular signaling pathways that underlie axonal branching are just beginning to be identified (Schmidt and Rathjen, 2010).

1.2.5 Synapse Formation

The 10^{10}-10^{11} developing neurons that undergo dramatic morphological changes to pattern the nervous system network must then receive and make 10,000 synaptic contacts in order to form a fully functioning brain (Ziv and Garner, 2004). Upon reaching their targets, axon growth cones interact with dendrites via cell-cell contacts and form cell adhesions. If the contacts are stable, presynaptic and postsynaptic proteins, vesicles and ion channels are recruited, and this site matures into a functional chemical synapse (Shen and Cowan, 2010).

Synaptic junctions are asymmetric structures composed of three compartments: the presynaptic terminal, the synaptic cleft and the postsynaptic density (Garner et al., 2002). Presynaptic terminals are characterized as protrusions along the axon that are filled with synaptic vesicles, which usually contain neurotransmitters. The postsynaptic density is a cluster of receptor and ion channels at high concentrations. Between these two structures is a 20nm wide gap called the synaptic cleft. The small volume of the cleft allows for signals released in the presynapse to be rapidly taken up by the postsynapse and tightly regulated (Lee and Sheng, 2000).

F-actin is highly concentrated at the presynaptic and postsynaptic terminals and actin dynamics are crucial for synapse formation (Ziv and Smith, 1996). At early stages of synapse formation, if immature neuronal cultures 5-6 days *in vitro* are treated with latrunculin A, which inhibits actin polymerization, there is a loss of presynaptic sites and dispersal of vesicles. However, as neurons mature, presynaptic sites become increasingly resistant to latrunculin treatment and by 18-20 days *in vitro* they are not affected at all (Zhang and Benson, 2001). There is increasing evidence that synapse formation is important not only early in development but throughout life. As the understanding of learning and memory grows, it may become clear that a dynamic cytoskeleton is necessary for synaptic plasticity (Shen and Cowan, 2010).

1.3 Axon Guidance Systems

In 1890, the Spanish neurologist Santiago Ramon y Cajal observed club-shaped structures at the end of processes emanating from nerve cells fixed in a histological section. He named these structures 'growth cones' and hypothesized that they might have 'chemical sensitivity' which would allow them to burrow through the embryo establishing contacts with distant targets through detection of environmental cues (Ramón y Cajal, 1995). These postulations were remarkably insightful and many hold true over one hundred years later. Recently, understanding of the cues, receptors and signaling events underlying axon guidance has grown dramatically. Yet as we learn more about particular systems it is clear many of these signaling pathways interact and are integrated in a spatial and temporal manner resulting in an even more complex guidance system than was imagined 20 years ago (Chilton, 2006; Dent et al., 2010).

1.3.1 Netrins and their receptors DCC and Unc5b

The first evidence of axon path-finding in response to extracellular cues came about in the 1980s. Explants of a rat dorsal spinal cord cultured alongside explants of a rat spinal floor plate caused commissural axons, in the spinal cord explants, to grow toward the floor plate explants and away from their typical dorsal-ventral trajectory (Tessier-Lavigne et al., 1988). Around the same time, studies in *C. elegans* characterized an uncoordinated phenotype, which was due to defects in nervous system development and identified several genes involved. One of these genes was named *unc-6* and encoded a secreted protein with sequence homology to the extracellular matrix molecule laminin

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(Hedgecock et al., 1990). These observations provided strong evidence for the existence of guidance molecules.

Later two proteins, named netrins, that mimicked the rat commissural axon guidance activities and were homologous to UNC-6, were purified (Kennedy et al., 1994). Netrins are a family of conserved, secreted guidance proteins that act through: DCC, UNC-5 homologs, and Dscam (Liu et al., 2009; Moore et al., 2007). The guidance effects of netrins are bifunctional; netrin signaling through DCC homodimers mediate axon attraction and outgrowth, while DCC/UNC-5 heterodimers mediate axon repulsion *in vitro* and *in vivo* (Tessier-Lavigne and Goodman, 1996). Signaling responses though UNC-40/DCC and UNC-5 are dependent on secondary messangers, such as the intracellular cAMP levels and Ca^{2+} (Hong et al., 1999; Ming et al., 1997b). Unc-34/Ena was recovered from as a suppressor of UNC-5 mediated repulsion and may be an important factor influences actin remodeling (Colavita and Culotti, 1998). However, it is still unclear how these and other secondary signals are regulated *in vivo*.

The UNC-40/DCC receptors have 3 conserved cytoplasmic motifs: P1, P2, and P3. Experiments in *C.elegans* suggest that parallel signaling pathways mediated by the P1 and P2 motifs link UNC-40/DCC to cytoskeletal remodeling. P1 acts via the Ena/VASP homolog UNC-34 while P2 acts via the Rho GTPase CED-10 and actin binding protein, UNC-115. Interestingly, it has been recently reported that the P1 of the DCC receptor is associated with protein synthesis machinery and regulates translation (Tcherkezian et al., 2010). The P3 domain is necessary for UNC-40/DCC homodimerization and UNC-40/UNC-5 heterodimerization (Gitai et al., 2003). It has been proposed that, netrin-

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induced dimerization of UNC-40/DCC acts as a scaffold for effector molecules to promote downstream signaling events.

1.3.2 Sema and their receptors Plexin and Neuropilin

Semaphorin is a member of a large family of signaling proteins, both secreted and membrane-bound, which are required for development of many organs, not just the nervous system (Raper, 2000). All members are characterized by a ~420 amino acid sequence termed the Sema motif at their N terminus. The first Semaphorins were identified from chicken embryos by purifying molecules that caused growth cone collapse *in vitro* (Luo et al., 1993). Vertebrate semaphorins are classified in 5 subfamilies based on the presence of particular motifs. Unlike other characterized guidance cues, semaphorins have no clear invertebrate orthologs. It appears that a number of semaphorin genes existed in metazoans and have duplicated and diverged throughout evolution while maintaining their role in development (Tessier-Lavigne and Goodman, 1996).

Semaphorins signal through mutimeric receptor complexes and although the composition of the complexes is not fully known, it is thought they all contain the receptor plexin. Plexins comprise a large family of transmembrane proteins that directly bind semaphorins and activate signaling cascades. A well-studied member of the semaphorin family, Sema3A, signals through a receptor complex of plexin and a co-factor neuropilin. Neuropilins do not seem to have signaling function but rather contribute to ligand specificity (Dickson, 2002).

The best characterized function for the semaphorins is the role of Sem3A in axon repulsion. Sema3A causes growth cone collapse and reduces cortical branching by depolymerizing actin filaments (Fan et al., 1993). This happens in part through the

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inactivation of the actin severing protein cofilin by the phosphorylation of LIM kinase (Aizawa et al., 2001). Microtubules that interact with and track along actin filaments also collapsed toward the central region of the growth cone after F-actin was lost in response to Sema3A. These changes in the growth cone cytoskeleton in response to Sema3A did not affect axon elongation but eventually did change the direction of growth and reduce the number of branches (Dent, 2004).

1.3.3 Slits and their receptor Robo

The Roundabout (Robo) receptor was first identified in a *Drosophila* genetic screen for midline guidance defects. The axons mutant for this transmembrane protein continually circled the midline, as if in a traffic roundabout. It was postulated this gene was a receptor for a midline repellant since loss of the protein results in axons aberrantly staying in this area (Seeger et al., 1993). Subsequently, the repulsive cue, Slit, was identified and, in parallel, found to be a factor that stimulates elongation and branching in sensory neurons in invertebrates and vertebrates (Brose et al., 1999; Li et al., 1999; Wang et al., 1999).

The role of the slit cue and robo receptor as a repellant in midline guidance has been well characterized. Spinal column commissural neurons that are attracted to the midline express no detectable robo before they cross. After crossing, the expression of robo receptor dramatically increases, preventing re-crossing (Kidd et al., 1998). In *Drosophila* this switch is facilitated by the activity of commissureless (comm), which is an endosomal surface receptor for robo that prevents the robo from reaching the cell surface when co-expressed (Keleman et al., 2002). Once the axons have crossed the midline, comm is degraded and the robo receptor can reach the cell surface, repelling

them from the midline and ensuring the axons only cross once. In vertebrates, Rig-1 is thought to play an analogous role to comm, except instead of sequestering the complexes away from the normal subcellular localization, Rig-1 and Robo form a non-functional receptor complex on the cell surface (Camurri et al., 2005; Dickson, 2002). After midline crossing, loss of Rig-1 results in a functional robo receptor.

Robo has several intracellular binding partners that modulate changes in the cytoskeleton leading to growth cone collapse, repulsive turning or branching. In *Drosophila,* the actin-binding protein Ena and its negative regulator Abelson kinase (Abl) directly bind the cytoplasmic tail of the receptor (Bashaw et al., 2000). Microtubule dynamics may be affected by signaling downstream of slit by the activation of Orbit/MAST, which binds the tips of dynamic microtubules and can cause looping, thereby slowing growth cone advance (Lee et al., 2004). Slit also signals through complex pathways to the Rho family of small GTPases in order to direct actin filament dynamics (Hall and Lalli, 2010).

1.3.4 Neurotrophins

Neurotrophic factors are target derived trophic molecules that regulate the growth and survival of developing neurons and maintenance of mature neurons (Gillespie, 2003). In the 1940s, it was shown that removal of a chick limb bud caused neuronal hyperplasia whereas transplanting an extra limb caused hypoplasia (Hamburger and Levi-Montalcini, 1949). These studies suggested the synaptic target plays an important role in neuronal development by determining the size of its neural center (Yuen et al., 1996). At the time it was hypothesized that one neuronal population had one neurotrophic factor, and this factor was present in the target tissue at a lower concentration than was needed to

maintain viability of all innervating neurons. It is now known that neurotrophic factors can act on many neuronal and non-neuronal cell types and not only potentiate survival but also influence the cytoskeleton to regulate axon guidance and branching (Gillespie, 2003; Kalil et al., 2000; Tucker, 2002).

BDNF is an extremely versatile neurotrophin that seems to play a role in every aspect of nervous system development. First discovered because it promotes survival and differentiation of select neuronal populations, now is known to be essential to axon guidance, adult synaptic plasticity, and learning and memory (Ji et al., 2005). BDNF binds the tyrosine kinase receptor TrkB (Rodriguez-Tebar et al., 1990). Gradients of BDNF stimulate turning toward high concentrations in Xenopus spinal neurons (Ming et al., 1997a) and this attractive turning requires cyclic nucleotides and PKA activity (Song et al., 1997). It recently has been shown that BDNF enhances cortical branching through the inhibition of MAPK signaling, which results in destabilized microtubules (Jeanneteau et al., 2010). This suggests a novel branching mechanism in the cortex in which BDNF aids in maturation of axonal arbors and synaptic connectivity by influencing microtubule stability.

Another neurotrophic factor, FGF-2, or bFGF, is the most abundant in the fibroblast growth factor central nervous system (Zechel et al., 2010). Pausing of cortical neurons was known to be a precursor to branch points (Szebenyi et al., 1998). Therefore target-derived growth factors expressed in the cortex were tested for their influence on the cytoskeleton. It was determined that FGF-2 was the most effective in promoting branches of cortical axons, likely signaling through FGF Receptor-1 or -3 (Szebenyi et al., 2001). Although FGF-2 remains one of the most potent inducers of collateral

branching, it was only recently determined that this effect on branching is through the upregulation of katanin and spastin, two microtubule severing proteins (Qiang et al., 2010). Similarly to BDNF, FGF-2 induced cortical branching predominantly remodels microtubules.

1.4 Downstream Signaling Mechanisms

The axon guidance cues and receptors described above act through a wide variety of signal transduction cascades. These pathways lead to a number of cell biological effects (Bashaw and Klein, 2010). This section focuses on the intracellular signals that lead to the reorganization of the cytoskeleton and ultimately shape neuronal morphology. Many of these signaling mechanisms are accessed by different receptors and there is significant crosstalk between downstream signals. The following overview will be limited to the effector pathways that are relevant to the systems and the experiments discussed in the subsequent chapters.

1.4.1 Rho GTPases

The Rho family of small GTPases acts as a molecular switch that controls cytoskeletal dynamic downstream of almost all guidance receptors (Koh, 2006). The Rho GTPases include Rac1, Cdc42, and Rho and their activity couples upstream directional cues with downstream cytoskeletal remodeling by either enhancing actin polymerization or promoting disassembly. When these proteins are active and facilitate the activation of effector proteins, GTP is bound. GTP binding is aided by guanine exchange factors (GEFs). GTPases are inactive when the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the protein or GTPase activating proteins (GAPs) (Watabe-Uchida et

al., 2006). Understanding the roles individual GTPases play in axon guidance has not been straightforward since multiple GTPases can have antagonist functions in a particular neuron and be activated in response to the same guidance cue. Recent data has suggested spatial localization and activation of GTPases by GAPs and inactivation by GEFs is required for distinct cellular functions (Lowery and Van Vactor, 2009).

As diagramed in Figure 1-6, the GEF Trio is an important regulator of axon guidance decisions downstream of the DCC receptor (Forsthoefel et al., 2005). Trio contains 2 Rho GEF domains, one activates Rac and RhoG and the second activates RhoA. *C. elegans* UNC-73/Trio is necessary for neuromuscular junction formation downstream of UNC-40/DCC (Alexander et al., 2010). Cortical neurons from Trio null mice have no Rac activation and display a variety of CNS guidance defects that overlap with defects seen in Netrin-1 and DCC null mice (Briancon-Marjollet et al., 2008). Dock180 is another RacGEF important to netrin-DCC attraction (Bashaw and Klein, 2010). Dock180 knockdown in commissural neurons blocked turning in an explant assay and reduced axon midline crossing (Li et al., 2008). It is still unclear if Trio and Dock180 function in the same pathway to activate Rac downstream of DCC.

Rho GTPase effectors affect a wide range of actin dynamics, from F-actin assembly to actin retrograde flow, actin disassembly, and recycling (Raftopoulou and Hall, 2004). Rho or Rac activation can lead to the phosphorylation and inactivation of cofilin, an actin depolymerizing protein, which is necessary for axon growth (Harrington et al., 2008; Ng and Luo, 2004). Other major effector proteins downstream of Rac and Cdc42 are nucleation factors, such as Arp2/3 and formins (Matusek et al., 2008). The Arp2/3 actin-nucleating complex is regulated by N-WASP/WAVE, which is activated by

cdc42 (Banzai et al., 2000; Strasser et al., 2004) Although Arp2/3 role in axon outgrowth

and guidance has been difficult to define, it is implicated in axon guidance and neuronal

morphology (Korobova and Svitkina, 2008; Strasser et al., 2004; Withee, 2004). Another

actin nucleator that plays a role in axon guidance downstream of the GTPase Rac is the

DAAM formins (Matusek et al., 2008). Where as Arp2/3 nucleates actin as a branch off

of an existing actin filament, DAAM formins nucleates, linear actin filaments through

processive elongation (Barko et al., 2010). Although much is unknown about the

regulation of DAAM formins, it will be interesting if these actin nucleators are

differentially activated or function in parallel in response to guidance cues.
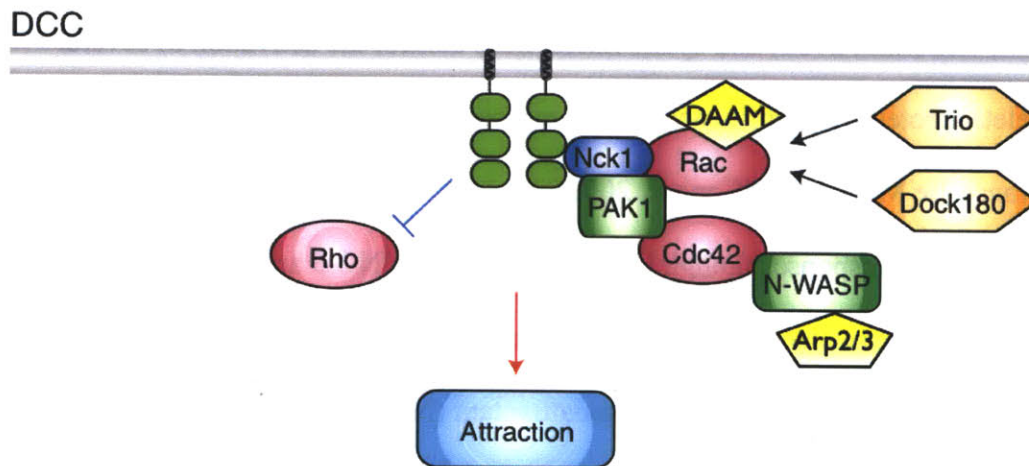
Figure 1-6: Rho GTPase signaling downstream of the DCC receptor
From (Hall and Lalli, 2010).

1.4.2 Cyclic Nucleotides and Calcium

Cyclic nucleotides and calcium can directly mediate guidance responses *in vitro,* although evidence *in vivo* suggest they are important messengers that modulate the strength of a guidance response (Bashaw and Klein, 2010). When growth cones approach a guidance factor gradient, higher calcium concentrations are on the side of the occupied guidance receptor, in both attractive and repulsive guidance, illustrating that this secondary message can act in either pathway (Gomez and Zheng, 2006). Activation of cyclic nucleotides on one side of the growth cone is sufficient to induce turning *in vitro* (Murray et al., 2009). Also, these secondary messengers play an important role in axon branching. Calcium transients regulate axon extension versus branching growth (Hutchins and Kalil, 2008) and cyclic nucleotides control the activation of several cytoskeletal proteins that influence branch morphology (Francisco et al., 2009; Mingorance-Le Meur and O'Connor, 2009).

Signaling responses though UNC-40/DCC and UNC-5 are dependent on the intracellular cyclic nucleotide levels. When the ratio of [cAMP] is in at least a threefold excess of [cGMP], Netrin acts as a chemoattractant (Nishiyama et al., 2003; Song and Poo, 1999). Conversely, when [cGMP] is in excess of [cAMP] the response to Netrin will be repulsion. However, a clear demonstration that this change in response occurs *in vivo* has not been shown. After bath application of Netrin, growth cones significantly increase the number and length of filopodia and show accelerated lamellipodial dynamics (Lebrand et al., 2004). Ena/VASP proteins are required for a filopodial response, and phosphorylation of Mena by PKA parallels this increase in filopodia. It is clear that other

parallel pathways (e.g. Rac) link DCC to the cytoskeleton independent of Ena/VASP (Shekarabi et al., 2005b).

Vertebrate Ena/VASP proteins are phosphorylated by Protein Kinase A (PKA) and Protein Kinase G (PKG) (Reinhard et al., 2001). Phosphorylation by PKA at a conserved site between Mena/VASP/EVL is required for Ena/VASP function in lamellipodial dynamics (Halbrugge and Walter, 1989; Lambrechts et al., 2000; Loureiro et al., 2002). Phosphorylation of VASP at a c-terminal site by PKG(also contained in Mena but not EVL) inhibits F-actin binding, bundling and anti-capping activity *in vitro* (Barzik et al., 2005).
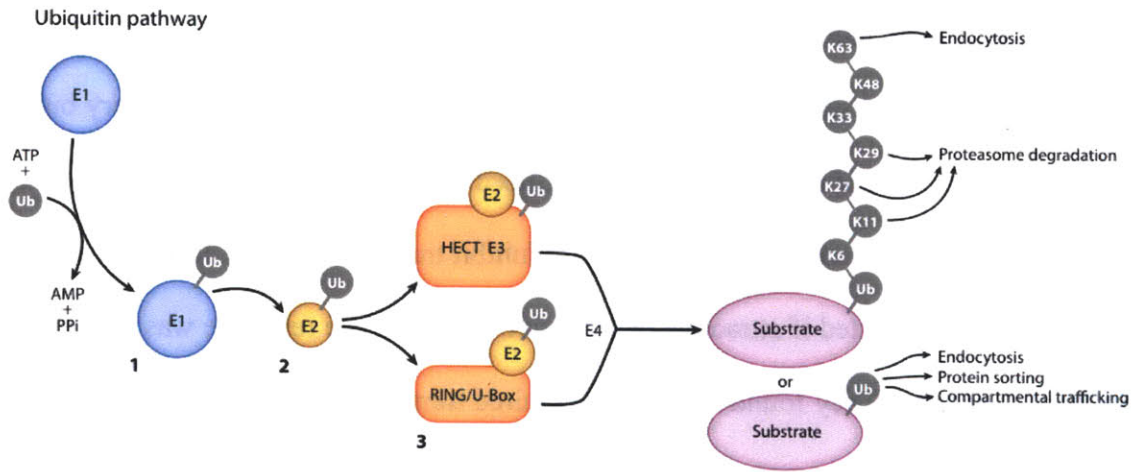
1.4.3 Protein Synthesis and Degradation

Some of the most conserved mechanisms regulating developmental processes are those that mediate the production and elimination of proteins. Recent work has shown the regulation of protein synthesis and degradation is indispensable for the fine-tuning of neuronal morphology (Fulga and Van Vactor, 2008; Lin and Holt, 2008; Yi and Ehlers, 2007).

The neuron is a highly polarized cell and it is increasingly clear that the growth cone makes response decisions independently from the cell body. This 'decentralization' involves selective localization and translation of subsets of mRNAs (Holt and Bullock, 2009). There are many advantages to controlling gene expression at the transcriptional level, there can be distinct properties to newly synthesized proteins, and in a highly polarized cell the new protein products can be spatially localized. Also it more efficient to store mRNA and synthesize protein as needed at such a distance from the cell body (Lin and Holt, 2008; Tcherkezian et al., 2010).

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Protein synthesis in response to guidance cues has emerged as a critical mechanism mediating cytoskeletal remodeling. The guidance cues Netrin and BDNF induce asymmetric localization and translation of β-actin mRNA in the growth cone. If this asymmetric translation is blocked, attractive turning in response is lost. However, repulsive turning is not lost if β-actin translation is blocked. It was found that translation of β-actin is repressed during repulsive turning and if this repression of β-actin translation is blocked repulsive turning is lost (Leung et al., 2006; Yao et al., 2006). The cytoplasmic tail of DCC also associates with translation machinery and given netrin induces asymmetric translation suggests the activated receptor may scaffold the necessary machinery to carry out this response (Tcherkezian et al., 2010).

Protein degradation has important roles in neuronal development and long-term plasticity. Ubiquitin proteasome activity is prevalent throughout the life of a neuron and many neurodegenerative disorders are associated with defects in the ubiquitin proteasome system (UPS) (Yi and Ehlers, 2007). Proteins targeted for UPS-mediated degradation are covalently tagged with a polypeptide of ubiquitin moieties. As seen in Figure 1-7, this process is mediated by a cascade of enzymes starting with the ubiquitin-activating enzyme (E1), followed by the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3). The assembly of a chain of at least four ubiquitin groups marks proteins for degradation by the 26S proteasome. Importantly, substrate recognition and specificity is mediated by the specificity of the E3 ligase and of these three enzymes, the E3 has the most diverse protein sequence and expression patterns (Deshaies and Joazeiro, 2009; Segref and Hoppe, 2009).

Figure 1-7: The Ubiquitin Conjugation Machinery
From (Mabb and Ehlers, 2010).

The Holt lab carried out innovative work in order to understand the role of protein synthesis and degradation during guidance of the *Xenopus* retinal ganglion cells (RGC). It was known that growth cones contained translation and degradation machinery but it was unclear how and if these systems were utilized during axon guidance (Campbell and Holt, 2001). Using pharmacological means to inhibit translation and proteolysis in the growth cone, they tested if attractive or repulsive guidance cue response was dependent on these processes. They found some cues required translation or proteolysis but only Netrin-1 required both in order to respond properly (Campbell and Holt, 2001). The Netrin-1 receptor DCC undergoes ubiquitination and degradation, possibly by the ubiquitin ligase Siah-1, and down regulation of the receptor attenuates downstream signaling pathways (Hu et al., 1997; Kim et al., 2005).

Later this group was able to show ubiquitin dependent regulation was not necessary for long-range pathfinding in the RGCs but is required for terminal branching (Drinjakovic et al., 2010). They found terminal branching was regulated by the E3 ubiquitin ligase Nedd-4, through marking PTEN, a PI3K phosphatase, for degradation. At the same time, another group studying the Nedd-4 knockout mouse described a role for this protein in dendritic branch formation. In this system Nedd-4 mono-ubiquitinated Rap2 and inactivated its ability to bind and activate TINK, a kinase that regulates actin dynamics, therefore inhibiting branch formation (Kawabe et al., 2010). The Nedd-4 E3 ligase highlights the capability of ubiquitin tagging not only to regulate the abundance or subcellular localization of proteins but also regulate their activity (DiAntonio, 2010).

1.5 Cytoskeletal associated Effector Proteins

The neuron's distinct cell morphology is maintained by the control of actin

nucleation, depolymerization, bundling and contraction (Dent et al., 2010). The

regulation of actin dynamics have been well characterized in many cell types and it is

thought that more than 100 actin accessory proteins exist (Pollard and Cooper, 2009).

Many of the actin accessory proteins have been identified in neurons but given a neurons

unique shape, even among different classes of neurons, it is likely that the localization

and quantity of these proteins plays an important part in their morphology (Kalil and

Dent, 2005).

1.5.1 Actin Associated Effector Proteins

Ena/VASP The Ena/VASP family of proteins binds actin and influences the geometry of

growing actin filament networks. Vertebrates have highly-conserved Ena/VASP paralogs

with overlapping function and expression: Mena, Evl, and VASP. In invertebrates, there

is a single ortholog in *Drosophila* Enabled (Ena) and in *C. elegans* UNC-34 (Krause et

al., 2003). Ena/VASP proteins are highly expressed in the nervous system and were the

first example of barbed-end binding proteins implicated in axon guidance (Drees and

Gertler, 2008).

Ena/VASP promotes long sparsley branched actin filaments by binding the barbed

ends of F-actin and also binding G-actin, enhancing the rate of polymerization and

delaying termination filament elongation by capping protein (Barzik et al., 2005; Bear et

al., 2002; Hansen and Mullins, 2010). The activity of these proteins results in the

formation of quickly growing filopodia and lamellipodia. During guidance, Ena/VASP

are required for filopodia formation downstream of the guidance cue Netrin *in vivo* (Chang et al., 2006) and *in vitro* (Lebrand et al., 2004).

Ena/VASP proteins do not bind the cytoplasmic tail of DCC and no cytoplasmic tail adaptor proteins have been identified that could mediate this interaction. One mechanism of Ena/VASP activation downstream of DCC involves PKA signaling. As previously mentioned, cyclic nucleotide signaling plays a considerable role in response to guidance cues. When neurons are stimulated with netrin or a PKA activator there is a significant increase in the number of filopodia in the growth cone and along the axon shaft. This response is dependent on Ena/VASP proteins, which are phosphorylated immediately following netrin or PKA treatment (Lebrand et al., 2004; Shekarabi et al., 2005a). Vertebrate Ena/VASP proteins are phosphorylated by Protein Kinase A (PKA) and Protein Kinase G (PKG) (Reinhard et al., 2001). Phosphorylation by PKA at a conserved site between Mena/VASP/EVL is required for Ena/VASP function in lamellipodial dynamics and cell motility in fibroblasts, and causes a bandshift in mobility on an SDS-PAGE gel (Halbrugge and Walter, 1989; Lambrechts et al., 2000; Loureiro et al., 2002). Phosphorylation of VASP at a c-terminal site by PKG (also contained in Mena but not EVL) inhibits F-actin binding, bundling, and anti-capping activity *in vitro* (Barzik et al., 2005).

Netrin treatment does not stimulate cAMP or PKA activation in growth cones and PKA is dispensable for axon extension toward netrin *in vivo* (Bashaw and Klein, 2010; Bouchard et al., 2004). Therefore the exact mechanism of Ena/VASP phosphorylation and regulation downstream of netrin stimulation remains unclear.

MRL (Mig-10, RIAM, Lamellipodin) This family of actin binding proteins interact with

to Ena/VASP through the EVH1 domain and is required for lamellipodia formation and

dynamics (Krause et al., 2004). MIG-10 is one of the first molecules to asymmetrically

polarize in response to Netrin-1 in the *C. elegans* HSN neuron (Chang et al., 2006). MRL

proteins are linked to many regulatory pathways by directly binding the Ras superfamily

proteins, phosphoinositides PI (3,4)P2, and tyrosine kinase abl (Michael et al., 2010;

Quinn et al., 2006). Ena/VASP proteins are required for full MRL function yet there is

evidence to suggest other actin regulators act downstream of this family of proteins

(Quinn et al., 2008).

Arp2/3 This complex is a seven-subunit actin nucleating protein that binds to the sides of

actin filaments and initiates a new F-actin branch. The role of Arp2/3 in axon outgrowth

and guidance has been difficult to define. Invertebrates require activated Arp2/3 for

nervous system development but when these proteins are inactivated in individual

neurons there are no obvious growth or morphological defects (Ng and Luo, 2004; Shakir

et al., 2008; Zallen et al., 2002). Vertebrates systems have also produced contradictory

results regarding the function of Arp2/3 in neuronal development. The data suggests

Arp2/3 is not essential for axon formation and outgrowth but is necessary for specific

axon guidance pathways (Korobova and Svitkina, 2008; Pinyol et al., 2007; Strasser et

al., 2004). In response to netrin stimulation, inhibition of Arp2/3's activator N-WASP in

hippocampal neurons leads to a block in the increase of filopodia formation and growth

cone area (Shekarabi et al., 2005a).

Formins: DAAM1 and mDia Formins could represent an alternate or complement to Arp2/3 nucleation in the nervous system and until recently little was known about their function or expression. Drosophila DAAM1 is highly expressed in the nervous system, loss of the protein reduces growth cone filopodia and causes a range of CNS phenotypes. Reductions in levels of DAAM exacerbates phenotypes caused by loss of Ena, in addition they partially co-localize suggesting they may act together (Matusek et al., 2008). Diaphanous Related formin mDia2 nucleates linear filaments by staying processively associated with the barbed end of the actin filament while supporting rapid insertion of new G-actin subunits (Zigmond et al., 2003). mDia2 binds Ena/VASP proteins and is an important factor in neurite outgrowth (Arakawa et al., 2003; Pellegrin and Mellor, 2005). It will be interesting to determine why proteins that can independently drive filopodia would exist in the same structure and if this feature allows growth cones greater plasticity in their response.

Myosin-X This myosin is an unconventional molecular motor that undergoes a novel form of movement into and out of filopodia, termed intrafilopodial motility. Myosin-X motor domains bind actin filaments and have potent filopodia assembly activity (Zhang et al., 2004). This unique myosin is expressed in the developing nervous system and plays an essential role in trafficking the guidance receptor DCC to the membrane in response to Netrin (Zhu et al., 2007). Myosin-X binds Ena/VASP proteins, β-integrins, and microtubules and is, therefore, poised to assimilate external cues for coordinated cytoskeletal reorganization (Tokuo and Ikebe, 2004; Weber et al., 2004; Zhang et al., 2004).

Capping protein This barbed end associated protein blocks access of monomer addition to the barbed end of the filament thus halting filament polymerization and reducing the length of F-actin (Xu et al., 1999). Antagonism between filament elongation and capping proteins contribute to filopodial dynamics (Dent et al., 2010). Deletion of the multifunctional capping protein EPS8 results in an increase in filopodia in the growth cone after BDNF treatment suggesting a regulatory role in this signaling pathway (Menna et al., 2009).

1.5.2 Microtubule Associated Effector Proteins

Map1b Neuronal specific microtubule stabilizing proteins (MAPs) are likely effectors of axon guidance. Phenotypes of a MAP1b knockout mouse suggested a role in neuronal development, as the mouse was deficient for many brain structures known to form in response to axon guidance cues (Meixner et al., 2000). Both Netrin and BDNF modify the phosphorylation of MAP1b via CDK5 and GS3Kβ, which influences the stability of microtubules (Del Río et al., 2004). The phosphorylation state of MAP1b may act as a regulator of microtubule dynamics or act as a scaffold for additional MAPs (Tymanskyj et al., 2010).

MAP1b is a microtubule-associated protein that also binds actin (Noiges et al., 2002; Togel et al., 1998). In response to netrin, MAP1b is phosphorylated by GSK3β, resulting in a lower affinity for MTs leading to their destabilization and slower polymerization (Pedrotti and Islam, 1996). MAP1b is also in a complex with the Rho

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GTPase, Tiam1, which activates Rac and cdc42 activity resulting in the remodeling of the actin cytoskeleton (Montenegro-Venegas et al., 2010).

Katinin/Spastin Microtubule severing proteins are important for neuronal development. Overexpression or loss of these enzymes results in diminished outgrowth (Karabay et al., 2004; Riano et al., 2009). While overexpression of spastin alone results in an increase in collateral branches (Yu et al., 2008) and the guidance cue FGF-2 increases the expression of katinin and spastin prior to branch formation (Qiang et al., 2010).

APC Adenomatous polyposis coli is a microtubule plus end tracking proteins, concentrated at the growing end of a microtubule. APC is a large protein that can bind microtubules and regulators the actin cytoskeleton, such as IQGAP1 and mDia2. During guidance and branching APC localization is regulated by GSK3β (Koester et al., 2007).

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Chapter 2

The Role of Trim9, a neuronal specific ubiquitin ligase, in signaling

from Netrin/DCC during cortical axon branching.

The results from Figure 2-3 contributed to the following manuscript:

Hao, J.C., Adler, C.E., Mebane, L., Gertler, F.B., Bargmann, C.I., Tessier-Lavigne, M., 2010. The tripartite motif protein MADD-2 functions with the receptor UNC-40 (DCC) in Netrin-mediated axon attraction and branching. Developmental Cell 18, 950-960.

Note: Figure 1 was generated by D. Rubinson and R. Jagannathan and Figure 2 was generated by J. Hao. The remaining figures, and data contained within, were produced by the author.

Chapter 2 Table of Contents

2.1 Abstract

During central nervous system development cortical axons extend collateral branches to connect to multiple targets. Many guidance cues and their receptors have a characterized role during cortical branching but the pathways that link these signals to cytoskeletal remodeling remain poorly understood. Trim9 is a brain specific ubiquitin ligase and its *C. elegans* ortholog has recently been shown to be a cytoplasmic cofactor to UNC-40 (DCC) during axon attraction and branching in response to UNC-6 (Netrin). In mice, Trim9 binds the cytoplasmic tail of DCC and also binds Ena/VASP proteins and Myosin-X, which are cytoskeletal effectors downstream of Netrin-1. We show inhibition of Trim9 ubiquitin ligase activity blocks Netrin-1 induced cortical branching, but these axons are still competent to branch in response to FGF-2 without Trim9 activity. The results of this study show Trim9 is necessary for Netrin-1 induced branching through its interaction with the cytoplasmic tail of DCC and cytoskeletal-associated proteins.

2.2 Introduction

The goal of studying the nervous system development is not only to understand the complexity of an individual neuron, but also to understand how this cell integrates into an even more complex circuitry. A fundamental feature of this complicated system is the ability for one neuron to establish contacts with multiple synaptic targets (Gallo, 2011). Assembly of this network replies on proper neuronal outgrowth, guidance, and branching to achieve accurate connectivity. As it navigates, a growth cone must respond to a complex set of extracellular signals, which promote growth cone attraction or repulsion. In addition, extracellular signals must also concurrently affect axon branching or branch pruning in order to make multiple contacts between neurons (Bashaw and Klein, 2010; Schmidt and Rathjen, 2010).

2.2.1 Mechanisms of axon guidance and branching

Cortical neurons navigate long distances, for example the cortico-callosal neurons extend across the corpus collosum and the cortio-spinal neurons extend into the spinal column, by interpreting cues through their primary growth cone. One of the final steps to proper neuronal wiring is target innervation. In the cortical navigation systems mentioned above, the final steps in target innervation are executed by collateral branches instead of the primary axon (Halloran and Kalil, 1994; Kuang and Kalil, 1994; O'Leary et al., 1990). Guidance of the initial axon is critical for proper patterning of the nervous system, and axon branching provides the framework for the distribution and integration of complex signals (Schmidt and Rathjen, 2010).

The outgrowth of axons and the extension of branches are regulated independently *in vivo* and *in vitro*, as branches can continue to grow while an axon stalls and retracts (Bastmeyer and O'Leary, 1996; Dent, 2004). Although they are regulated separately there are many parallels between growth cone guidance and axon branching. Similar to the steps of axon outgrowth, collateral branches are initiated by actin protrusions, followed by microtubule splaying and polymerizing into the periphery. Subsequently, the microtubules stabilize and bundle in the actin rich extension allowing the branch to mature and continue to extend (Dent and Kalil, 2001; Gallo, 2011). In both instances, guidance cues can simultaneously affect both axon guidance and axon branching by promoting or inhibiting changes in the cytoskeleton (Dent, 2004).

It is still unknown how multiple processes from a single neuron can have different rates of outgrowth or responses to guidance cues, but there is data to suggest several mechanisms. In mushroom body neurons of *Drosophila*, it was found that loss of one Rac isoform affected branching but not axon outgrowth or guidance, loss of two isoforms only affected guidance, and loss of all three Rac isoforms blocked outgrowth. This experiment illustrates that these processes are mechanistically linked but require different levels of Rac GTPase activity to properly form (Ng et al., 2002). In addition, it has been shown that the ubiquitin proteasome system was required for growth cone turning of the retinal ganglion cells (RGC) of *Xenopus*. However, when a dominant negative ubiquitin was expressed in the RGC cells in the context of a developing embryo, axon guidance proceeded normally while axon branching was defective (Drinjakovic et al., 2010). This result demonstrates that the targeted degradation of some proteins is an essential mechanism for branching but dispensable for axon guidance. Moving forward, it will be

necessary to define signaling pathways that directly regulate the axonal cytoskeleton during the formation of collateral branches to determine the difference between axon guidance and branching.

In the previous chapter, several downstream effector proteins were mentioned where localization, phosphorylation state or protein levels changed in response to a netrin cue, though none have been implicated in axon branching. Bath application of cortical neurons with netrin-1 results in a burst of F-actin dynamics and increased splaying of microtubules in the growth cone and, within a few hours, an increase in collateral branching without axon outgrowth. Netrin-1 applied locally to the axon shaft, quickly results in filopodia protrusions which stabilize into collateral branches with no increase in axon length (Dent, 2004). The branching promoting effects of FGF-2, however, are mediated through pausing of the growth cone and not through protrusions along the axon shaft. In response to this cue, branches form approximately 24 hours later (Szebenyi et al., 2001). The rapid growth of branches in response to netrin-1 treatment, as opposed to branching over the period of a day with FGF-2, suggests different signaling mechanisms are involved (Dent, 2004).

2.2.2 Ena/VASP, a downstream effector of Netrin/DCC signaling

The Ena/VASP protein family is implicated in regulation of actin filament dynamics, cell migration, and growth cone guidance. Vertebrates contain three highly-conserved Ena/VASP proteins that are functionally interchangeable: Mena, EVL, and VASP (Gertler et al., 1996; Krause et al., 2003). Single orthologs are found in

Drosophila (Ena), *C. elegans* (UNC-34), and *Dictyostelium* (dVASP). Ena/VASP

proteins are concentrated at the tips of extending filopodia and lamellipodia, and

associate with growing actin filaments at or near the barbed end (Lanier et al., 1999). In

addition, Ena/VASP proteins function to antagonize the binding of actin filament capping

proteins and reduce actin branching, thereby promoting the formation of long,

unbranched actin filament networks (Barzik et al., 2005).

Drosophila Enabled (Ena) was identified by its genetic interactions with the

Abelson tyrosine kinase (Gertler et al., 1995; Gertler et al., 1990). *Ena* mutants exhibit

defects in axon outgrowth and axon guidance (Gertler et al., 1995). *Ena* also plays a role

in motor axon branching in the ISNb neuron by promoting actin assembly in concert with

the phosphatase Dlar (Wills et al., 1999). In Xenopus, it was found that inactivation of

Ena in retinal ganglion cells (RGCs) resulted in a defect in terminal arborization but not

axon pathfinding (Dwivedy et al., 2007).

Genetic evidence in flies and *C. elegans* implicates Ena/UNC-34 function in both

the repulsive Slit/Robo and attractive netrin/DCC axon guidance pathways (Gitai et al.,

2003; Yu et al., 2002). In addition, Ena/UNC-34 function is required for netrin-induced

repulsion mediated by the UNC-5 receptor (Colavita and Culotti, 1998). Purified Ena has

been shown to bind the cytoplasmic tail of Robo, but not DCC or UNC-5. In mice,

deletion of Mena causes midline defects in the brain, including failure of the corpus

collosum and hippocampal commissures to form, phenotypes reminiscent of the axon

guidance defects observed in netrin/DCC mutants (Lanier et al., 1999) (Fazeli et al.,

1997). In vertebrates there is no evidence that there is a direct interaction between Mena

and the DCC receptor. Therefore it has been postulated that Ena/VASP proteins act downstream of netrin and DCC through signaling cascades.

2.2.3 MADD-2, in axon guidance and branching

The model organism *C. elegans* has been a powerful tool to identify new genes and pathways involved in nervous system development. A directed screen, in the ADL neuron of *C. elegans* that has a stereotyped dorsal and ventral branch, was used to identify novel mechanisms that control branching *in vivo*. Mutants defective in ADL branching mapped to three genes: *unc-6*, *unc-40* and *madd-2* (Hao et al., 2010). This data is consistent with previous reports that showed UNC-6/netrin and the receptor UNC-40/DCC had a role in axon branching while revealing a new mechanism of regulation through MADD-2 (Dent, 2004).

MADD-2 is a putative ubiquitin ligase, which strongly affects branching and had a moderate effect on ventral attractive guidance, while having no effect on repulsive guidance or growth. MADD-2 is a cytoplasmic protein that binds the intracellular tail of UNC-40, and in this system plays an analogous role to the receptor UNC-5. Ectopically expressed UNC-5 receptor, when in complex with DCC, mediates repulsion to a netrin cue in the ADL neuron, which usually has no response to this signal (Hamelin et al., 1993). Conversely, Ectopic expression of MADD-2 in the ADL neuron results in an attraction to netrin (Hao et al., 2010). It is thought that MADD-2 mediates this response through asymmetric recruitment of downstream effectors. MIG-10/MRL is one of the first cytoskeletal effector proteins to asymmetrically polarize in response to netrin in the HSN neuron (Adler et al., 2006). In MADD-2 null HSN neurons, asymmetric

polarization of the receptor UNC-40 is unaffected while MIG-10 is no longer

asymmetrically polarized in response to netrin (Hao et al., 2010).

Another group studying midline patterning and the extension of body wall muscle

(BWMs) arms found a role for MADD-2 in this developmental process (Alexander et al.,

2010). Each BWM has a muscle arm, which extends membrane and docks postsynaptic

machinery at this site, similarly to the vertebrate neuromuscular junction (White et al.,

1986). The muscle arm, like a growth cone, responds to the guidance cue UNC-6/netrin

through the receptor UNC-40/DCC (Alexander et al., 2009). In this system, MADD-2

binds the Rho GEF UNC-73/Trio and UNC-40/DCC, and MADD-2 enhances the ability

of these two proteins to interact and form a functioning signaling complex (Alexander et

al., 2010).

MADD-2 contains a RING domain which can mediate ubiquitin transfer to

substrate binding proteins (Deshaies and Joazeiro, 2009). In both studies, no binding

partners of MADD-2 were degraded through the ubiquitin proteasome system (Alexander

et al., 2010; Hao et al., 2010). It is possible that MADD-2 does not target proteins for

degradation however, disruption of the activity of the RING domain alone was sufficient

to phenocopy the null mutants suggesting ubiquitin ligase activity is necessary for its

function (Alexander et al., 2010).

2.2.4 Tripartite Motif Protein 9

The ortholog to *C. elegans* MADD-2 is mammalian Tripartite Motif Protein 9

(Trim9), which is specifically expressed in the nervous system (Berti et al., 2002). *Madd-2* has more sequence similarity to Trim9 than any of the other TRIM superfamily

proteins. Trim9 is a member of the TRIM protein family, which share an overall N-terminal architecture, including: a RING domain followed by two B-Boxes and a coiled-coil domain (Reymond et al., 2001). There are more than seventy members of the mammalian TRIM family and they can be further divided into nine sub-classifications based on their C-terminal organization. Trim9 is one of six proteins that belongs to the CI subfamily, defined by a COS motif, fibronectin type III domain and a SPRY domain (Short and Cox, 2006). The most extensively characterized CI-TRIM is MID1/Trim18 for its role in the human disease, Opitz Syndrome. In this disease, MID1/Trim18 ubiquitin ligase activity is impaired, resulting in a accumulation of its substrate protein phosphatase 2a (PP2a), and this leads to defects in brain development (Schweiger and Schneider, 2003).

Analysis of Trim9 function in *Drosophila* also uncovered a role for this protein in axon guidance. Loss of dTrim9 in flies results in a droopy wing phenotype. dTrim9 is expressed in the class IVda neurons of the peripheral nervous system and loss of dTrim9 in these neurons leads to a failure of these neurons to cross the midline and thickening of the longitudinal tracts. These phenotypes were enhanced in a *netrin* or *frazzled*/DCC mutant background and overexpression of *netrin* or *frazzled*/DCC suppressed the mutant dTrim9 phenotype (Song et al., 2011). Similarly to worms, dTrim9 functions in axon guidance in the netrin/DCC pathway but its role in branching was not tested.

Less is known about the function of Trim9 in vertebrate development. It's been shown that human Trim9 is an ubiquitin ligase that acts through the E2 enzyme UbcH5b and localizes to cortical and brainstem Lewy bodies, but is repressed in Lewy bodies of Parkinson patients, suggesting Trim9's ubiquitin ligase activity may be defective in

certain pathologies (Tanji et al., 2010). No reports have analyzed the role of Trim9 in vertebrate nervous system development.

We found that Trim9 is a novel Ena/VASP binding partner. Ena/VASP proteins play key roles in the formation of proper actin architecture necessary to promote neurite outgrowth and accurate axon guidance downstream of netrin-1. Our results demonstrate that Trim9 is required for axon branching in response to DCC signaling during, potentially by linking DCC to Ena/VASP-mediated cytoskeletal remodeling.

2.3 Results

2.3.1 Trim9 is a novel Ena/VASP binding partner

A yeast two-hybrid screen was performed to identify new Ena/VASP binding partners in order to gain insight into how Ena/VASP proteins function during nervous system development. Of the three vertebrate family members, Evl is the least complex member that contains all of the major domains. Full length Evl was used as bait in a mouse embryonic cDNA library. Five independent positive clones contained sequences corresponding to Trim9, an E3 ubiquitin ligase (Figure 2-1). Trim9 is a member of the tripartite family of proteins and all members have similar N-terminal domain organization. TRIM proteins have diverse expression patterns, tissue specificity and functions. Trim9 is a neuronal specific ubiquitin ligase. The positive Trim9 clones from this screen included aa45-532, which did not include the RING domain. Inactivation or removal of the RING domain of E3 ligases blocks addition of ubiquitin to substrates and increase the duration of interaction with a substrate (Tursun et al., 2005).This is a likely explanation for the stable interaction observed in this assay and the reason this ubiquitin ligase did not target binding partners for degradation.
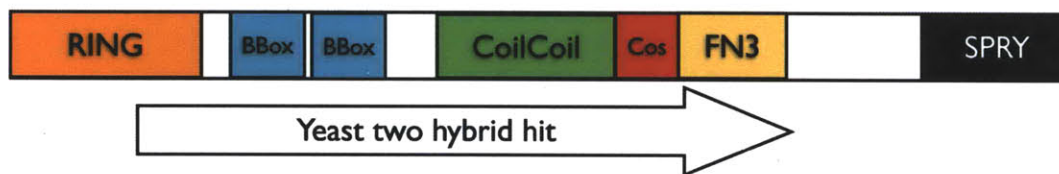

Figure 2-1: Trim9 is a novel Ena/VASP binding partner.
Schematic of full length Trim9 domain organization. The arrow indicates the sum of the Trim9 partial clones isolated in the yeast two-hybrid screen.

2.3.2 Madd-2, Trim9 ortholog, is an important signaling molecule in *C. elegans*

At the same time Trim9 was identified as a binding partner to Ena/VASP proteins, our collaborators also found the *C.elegans* gene *madd-2* in a screen for proteins involved in axon branching. Mutant worms with a branching defect were mapped to only three genes: *unc-6* (netrin guidance cue), *unc-40* (DCC receptor) and *madd-2* (putative E3 ubiquitin ligase). Further analysis indicated *madd-2* functions with *unc-6* and *unc-40* in axon branch formation and attractive axon guidance (Figure 2-2).
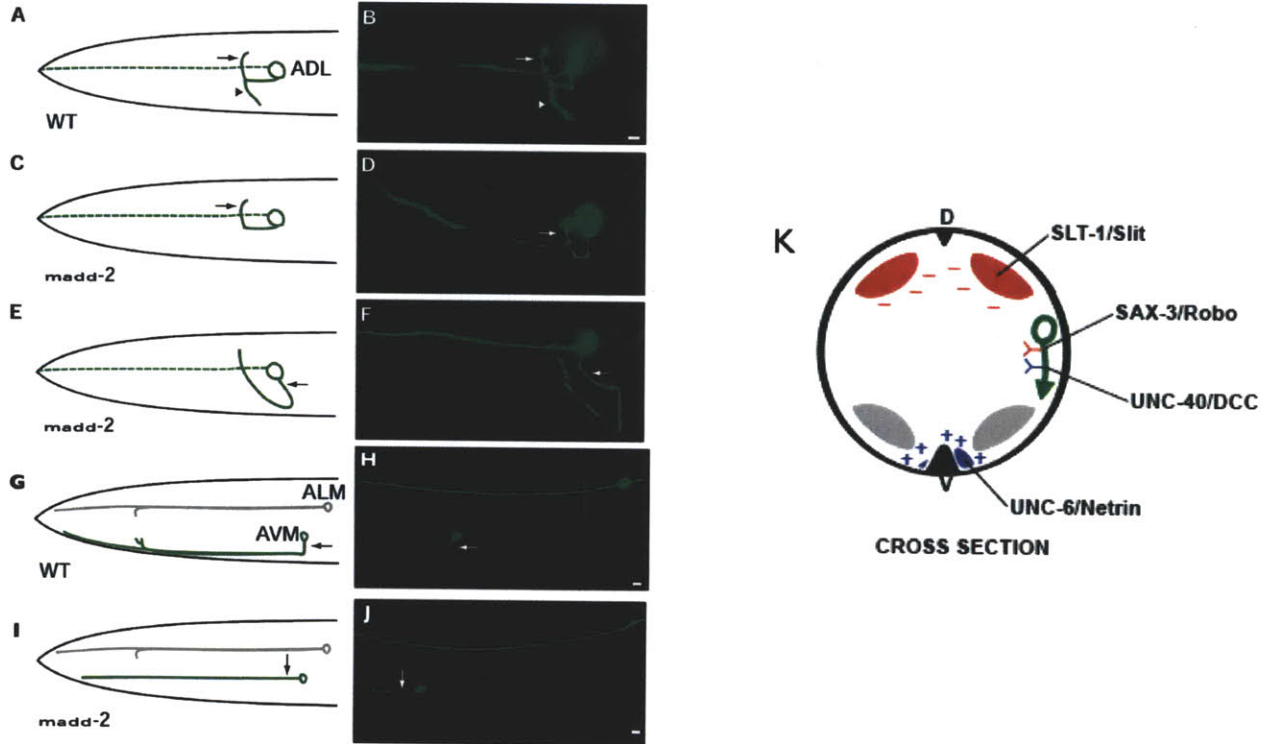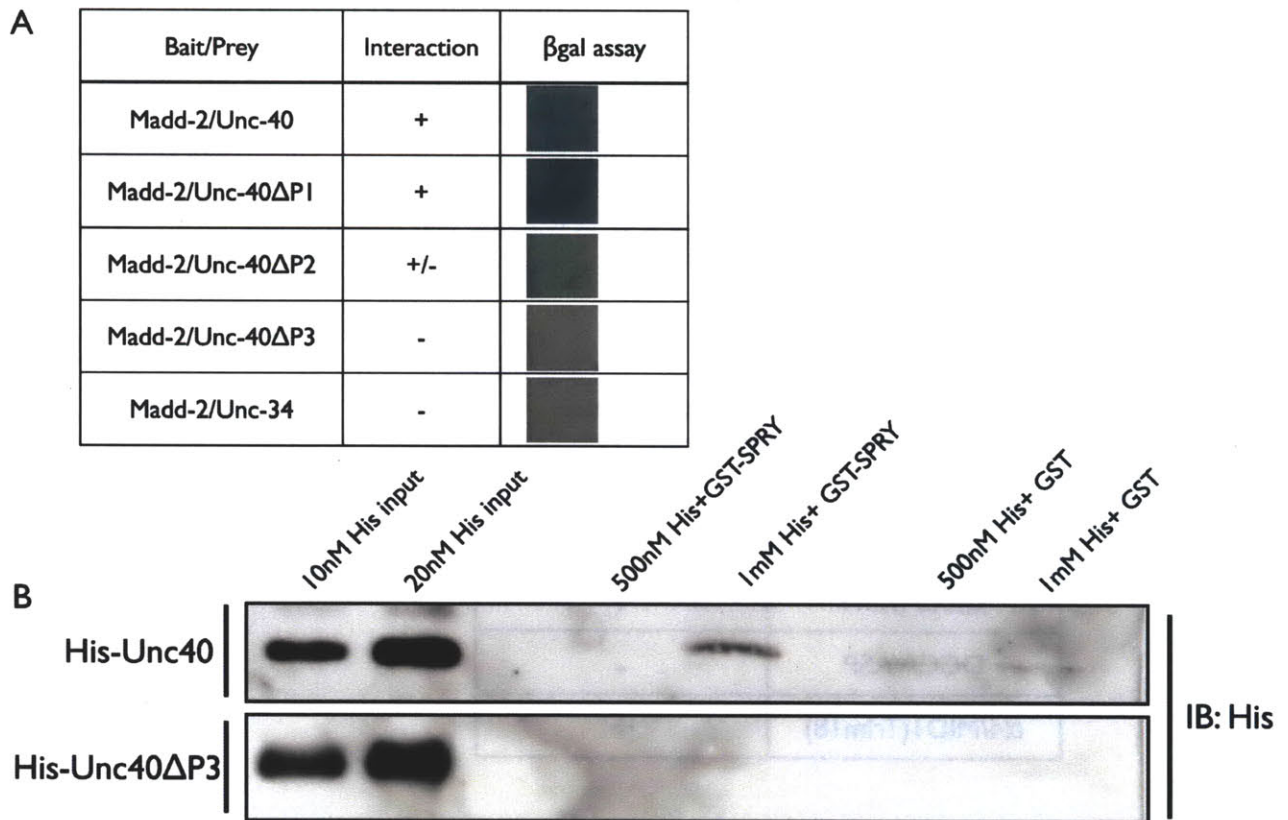

Figure 2-2: MADD-2 mutants result in branching defects in the ADL neuron and guidance defects in AVM neuron.
A-F: ADL neurons were visualized using the srh-220::gfp transgene and shown in the left panel as a schematic. G-H: AVM visualized with mec-4::gfp and schematics. K: AVM ventral guidance. Expression of Unc-40 and Sax-3 allow the AVM axon to extend towards Unc-6 and away from Slt-1. Adapted from (Hao et al., 2010).

Signaling downstream of *unc-40* is mediated in part by *unc-34*. We used the yeast two-hybrid system to determine if *C. elegans* MADD-2 could be a molecular link between these proteins. We showed that MADD-2 interacts with the cytoplasmic tail of UNC-40 and this interaction was weakened with a deletion of the P2 motif and completely lost with a deletion of the P3 motif (Figure 2-3A). UNC-34 protein was toxic to yeast, so we could not determine if there was an interaction between MADD-2 and UNC-34 in this assay.

The crystal structure of a *Drosophila* SPRY domain revealed a discreet binding pocket that recognizes a linear peptide motif (Woo et al., 2006). The SPRY domain of MADD-2 showed high similarity to this crystallized structure suggesting it may bind this motif. A sequence analogous to the SPRY binding motif is present in the P3 motif of UNC-40. Using a direct binding assay we demonstrated the MADD-2 SPRY domain alone could bind the P3 motif of UNC-40. When the P3 motif of UNC-40 was deleted, binding no longer occurred (Figure 2-3B). In *C. elegans* MADD-2 SPRY domain binds the P3 motif in UNC-40, but it is still unclear how effector proteins are regulated downstream of a netrin cue through MADD-2.

A

Bait/Prey	Interaction	βgal assay
Madd-2/Unc-40	+	
Madd-2/Unc-40ΔP1	+	
Madd-2/Unc-40ΔP2	+/-	
Madd-2/Unc-40ΔP3	-	
Madd-2/Unc-34	-	

B

His-Unc40

His-Unc40ΔP3

IB: His

10nM His input   20nM His input   500nM His+GST-SPRY   1mM His+ GST-SPRY   500nM His+ GST   1mM His+ GST

Figure 2-3: Unc-40 P3 motif binds Madd-2 SPRY domain.
A: Directed yeast two hybrid shows a requirement for the P3 motif of Unc-40 for an interaction with Madd-2. No interaction between Madd-2 and Unc-34 was found in yeast. (+) interaction, (-) no interaction, (+/-) weak interaction. B: Madd-2 SPRY domain associated in vitro with Unc-40 but not Unc-40 with a P3 deletion.

2.3.3 Trim9 binds DCC, Trim9 E/V interaction specific not true to all TRIMs

Given our results in *C. elegans,* we expanded our initial yeast two-hybrid

screen as depicted in Figure 2-4. Full length Trim9 did bind to Evl and Mena though

the interaction was much weaker than the clones that did not include the RING

domain. This is likely due to the transient interactions full-length ubiquitin ligases

have with substrates and binding partners. Trim9 also interacts with the

cytoplasmic tail of murine DCC. We were concerned that other TRIM family

members expressed in the brain could interact with Ena/VASP proteins. MID1

(Trim18) has high sequence similarity to Trim9. MID1 and Evl did not interact

whereas, as expected, MID1 and its binding partner alpha4 did. These data suggests the

interaction between Trim9 and Ena/VASP is specific and not a general characteristic of

TRIM family ubiquitin ligases.

Bait/Prey:	Interaction
Evl/Trim9	+
Evl/MID I	-
DCC/Trim9	+
DCC/VASP	-
α4/MID I (Trim I8)	+

Figure 2-4: Full length Trim9 binds Evl and DCC.
Directed yeast two hybrid shows Trim9 can bind Evl and the cytoplasmic tail of DCC. Related MID1 does not bind Evl. (+) interaction, (-) no interaction.

To study endogenous Trim9 protein, we raised a rabbit polyclonal against the

BBox domains which are the most divergent among the TRIM protein family. Published

in situ hybridization and protein immunoblot data indicates that Trim9 is specifically

expressed in the murine nervous system throughout development and in adults. The

Trim9 polyclonal recognizes three predicted isoforms in cortical and hippocampal

neurons (Figure 2-5). After testing our antibody on Trim9 null tissue, we found it was

specific for immunoblots but not immunocytochemistry.
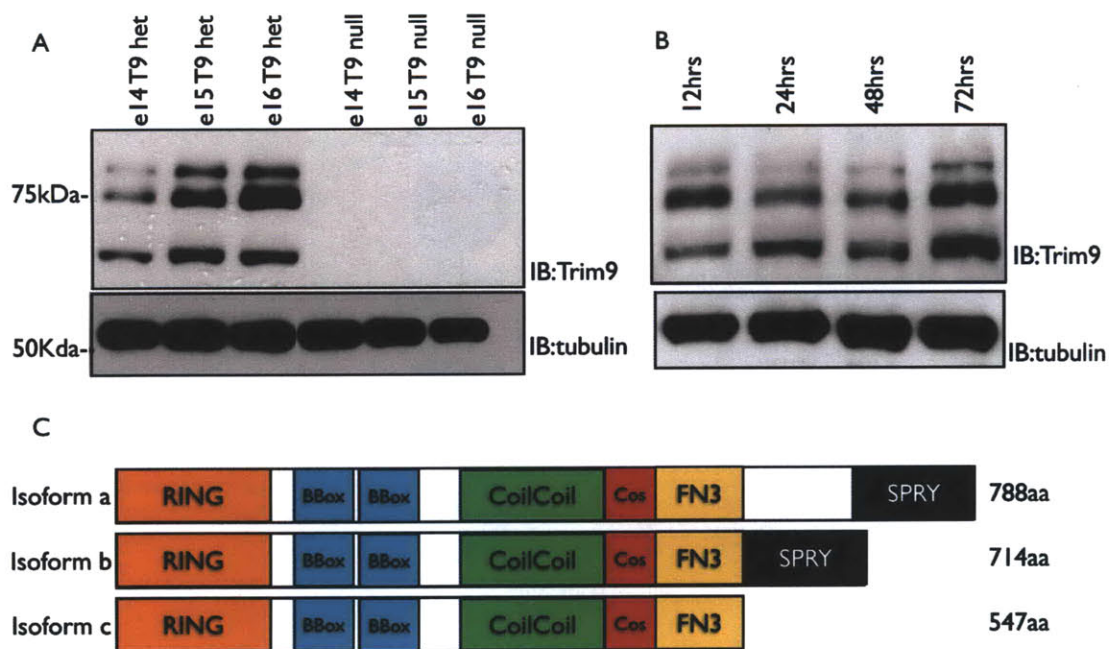
Figure 2-5: Three Trim9 isoforms are expressed in cortical neurons.
A: Rabbit polyclonal raised against the BBox domains shows Trim9 expression in whole brain lysate. Trim9 antibody recognizes no bands in brain lysate of the Trim9 null mouse. B: Trim9 is expressed during cortical axon specification and branching. C: Schematic showing domains of Trim9 isoforms.

To confirm the interactions found in yeast, we performed a GST-pulldown assay from mouse embryonic brain lysate. Similarly to *C. elegans*, we found that Trim9 GST-SPRY could bind DCC from mouse embryonic brain lysate (Figure 2-6A). A peptide array was used to map the sequence of DCC that bound SPRY. Staggered 25 amino acid peptides that included the entire sequence of the cytoplasmic tail of DCC were attached to a nitrocellulose membrane and this membrane was overlayed with GST-SPRY. Consistent with our *C. elegans* data, the SPRY domain bound to the predicted peptides in the P3 motif and had a shorter binding site in the P2 motif (Figure 2-6B).
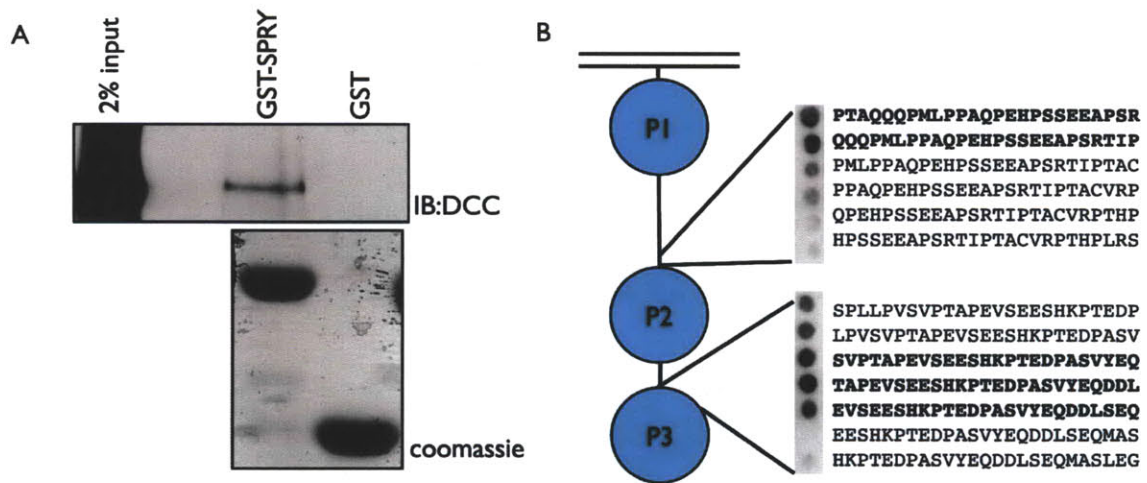
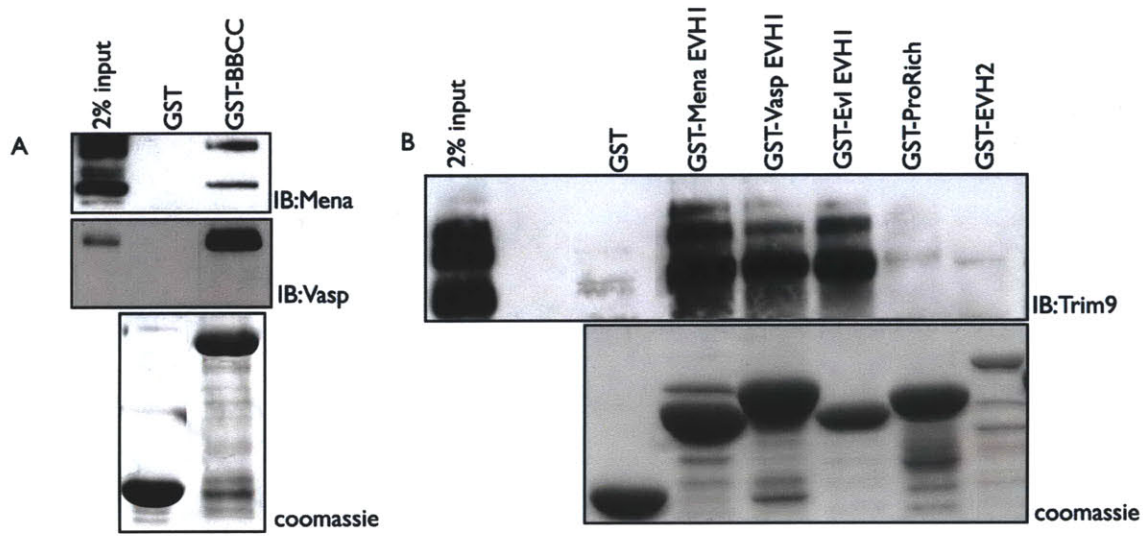**Figure 2-6: Mapping Trim9 binding sites in DCC.**
A: Cortical lysate was incubated with GST-SPRY fusion protein shown in the coomassie staining in the lower panel. Beads were washed and bound DCC was visualized by immunoblotting. B: Peptide array of the cytoplasmic tail of murine DCC overlaid with GST-SPRY and spots were visualized by immunoblotting.

To determine the regions of Trim9 necessary to interact with Ena/VASP proteins, we purified the smallest region of Trim9 that could properly fold into a tertiary structure, the BBox Coiled Coil domains. We chose to only look for an interaction with endogenous Mena and VASP because the expression of Evl in the brain is low. GST-BBox Coiled Coil, but not GST alone, could bind Mena and VASP from mouse embryonic brain lysate (Figure 2-7A).

The reciprocal experiment, mapping the binding site in Ena/VASP for Trim9, gave unexpected results. Ena/VASP proteins have three conserved domains, EVH1, Proline Rich and EVH2. EVH1 domain binds a proline-rich motif with the consensus (D/E)FPPPPX(D/E)(D/E) abbreviated FPPPP. EVH1 binding proteins include the axon guidance receptor Robo, actin associated proteins zyxin/vinculin and lamellipodin. The proline rich region binds SH3 and WW domains containing proteins and also profilin.

80

The EVH2 binds G- and F-actin and mediates tetramerization of Ena/VASP(Bear and

Gertler, 2009). We used GST tagged version of these domains to determine binding to

endogenous Trim9. Interestingly, Ena/VASP EVH1 binds Trim9 even though Trim9

does not contain the canonical FPPPP binding motif (Figure 2-7B). Currently only one

other protein, Tes, is known to bind the EVH1 domain through its non-FPPPP containing

LIM3 domain. Trim9 BBox domains have some similarity to a LIM3 domain and may

bind the EVH1 domain in this atypical way.

Figure 2-7: Trim9 unconventional Ena/VASP EVH1 binding partner.
A and B: Cortical lysate was incubated with GST fusion proteins shown in the coomassie staining
in the lower panel. Beads were washed and bound proteins were visualized using a rabbit
polyclonal antibody specific to the protein of interest.

**2.3.4 Trim9 localizes to dynamic puncta**

Trim9 has a potential microtubule binding motif (COS motif) but no actin binding

domains. Given that this protein binds the DCC receptor tail and Ena/VASP proteins we

sought to determine its localization in primary neurons. We used fluorescent and epitope

tagged versions of Trim9 cDNA. Trim9 localizes to puncta throughout the neuron and in

the tips of filopodia along the axon shaft and growth cone of primary cortical neurons (Figure 2-8A). It is unexpected to find a protein that does not have an actin-binding domain localized to filopodia tips, which are mostly actin rich structures. Trim9 co-localizes Ena/VASP in these filopodia tips (Figure2-8B). When Trim9 is expressed in neurons null for Ena/VASP protein, Trim9 no longer localizes to filopodia and are diffuse throughout the cell (Figure 2-9). Therefore Trim9 is dependent on Ena/VASP for localization to filopodia tips.
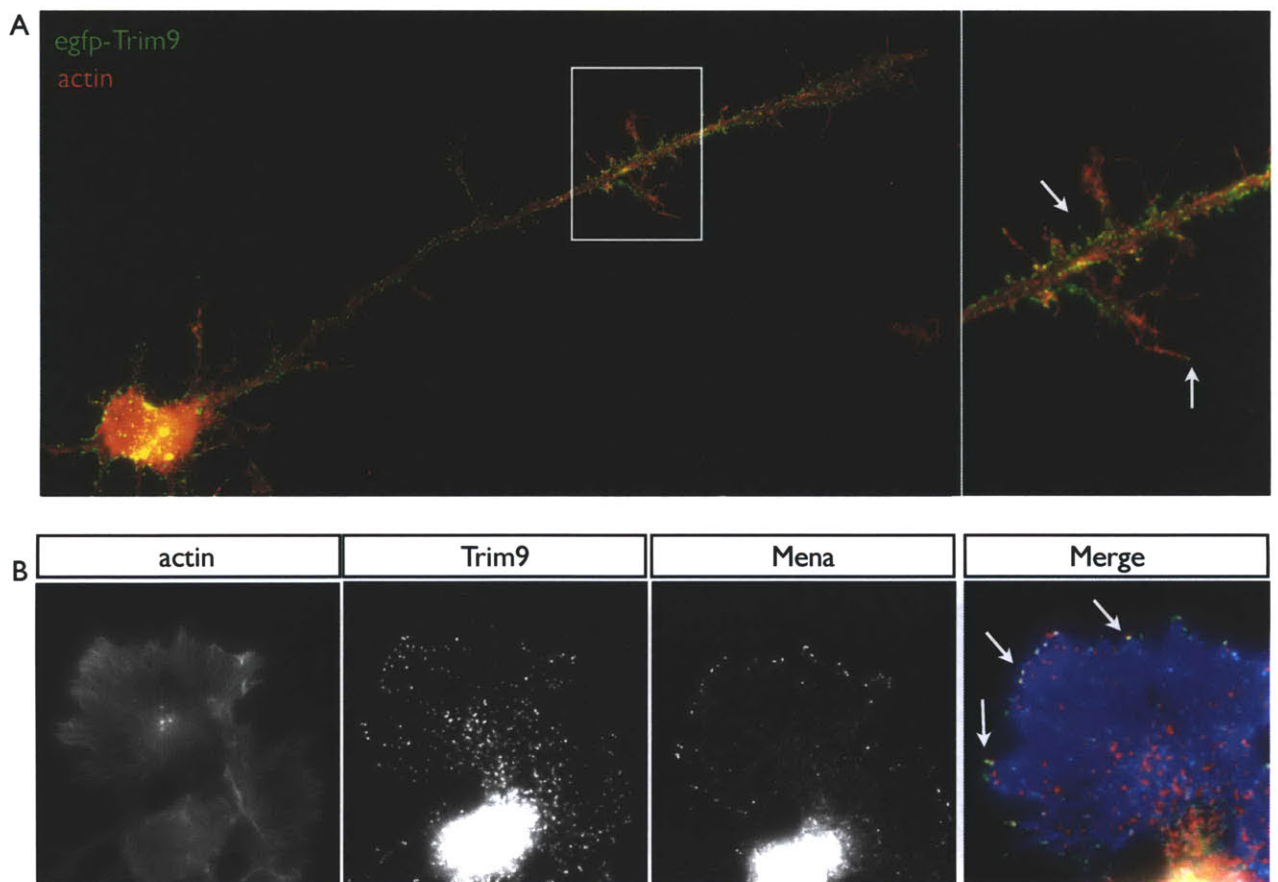
Figure 2-8: EGFP-Trim9 localizes to puncta and colocalizes with Mena.
A: Tagged Trim9 localizes to puncta along the axon shaft and the tips of filopodia. B: MYC-Trim9 colocalizes with endogenous Mena in filopodia tips.
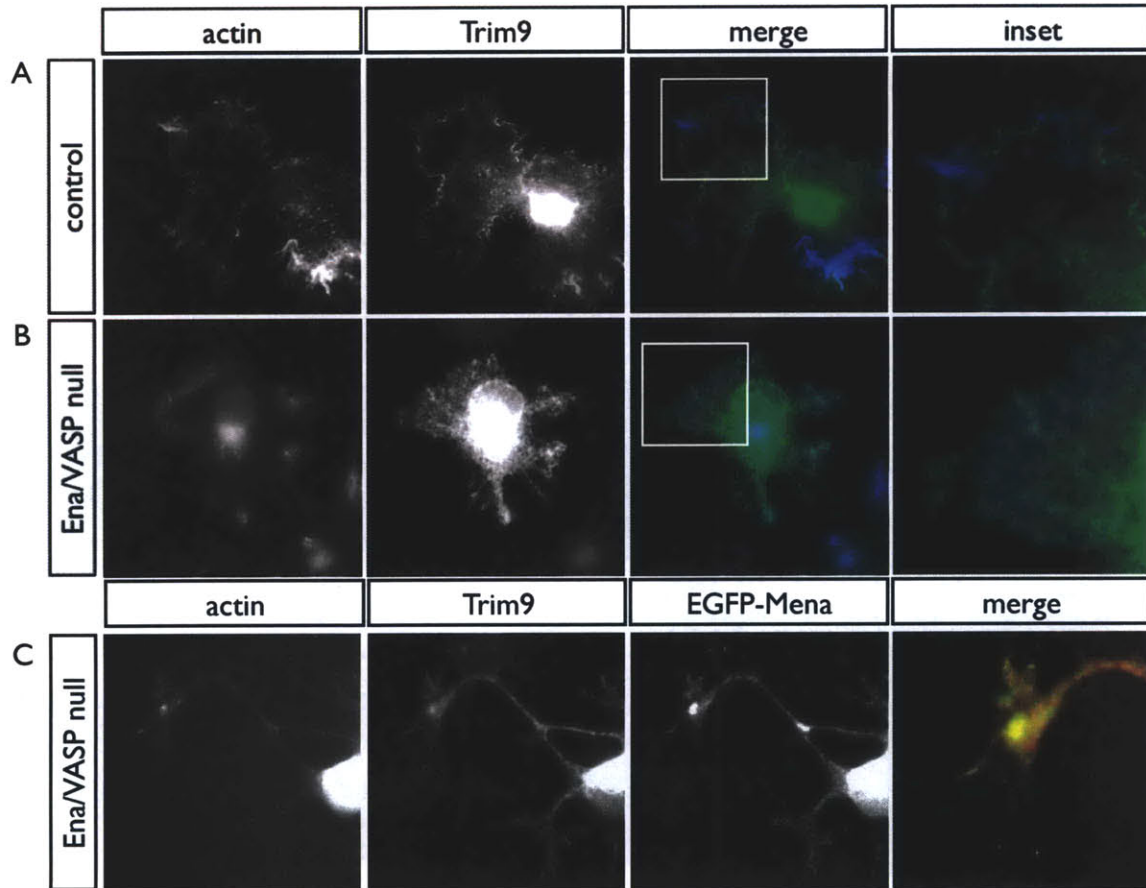
Figure 2-9: Trim9 localization depends on Ena/VASP
A: MYC-Trim9 localizes to the periphery of a stage one neuron. B: MYC-Trim9 localization is diffuse in a stage one neuron null for Ena/VASP. C. EGFP-Mena expressed in an Ena/VASP null neuron restores Trim9 localization to filopodia tips.

EGFP tagged Trim9 forms protein aggregates in most cell types but in cortical neurons these aggregates resulted in a large amount of neuronal cell death, which makes live cell imaging difficult. We found a promoter that would express Trim9 at low levels and allowed us to follow its localization over time. It was clear from time lapse imaging that Trim9 puncta are dynamic and move throughout the cell. It was also surprising to observe that the Trim9 puncta display intra-filopodial motility (Figure 2-10A). Only one molecule, Myosin X, has been documented to move into and out of a filopodia before the structure retracts back to the cell (Berg and Cheney, 2002). However, expressing both

Myosin X and Trim9 in neurons was extremely toxic to neurons. Instead we expressed

both of these proteins in COS cells. Myosin-X and Trim9 co-localize and co-

immunoprecipitate. Myosin X also binds the P3 motif of DCC and is essential for

receptor trafficking in response to netrin-1. Expression of Myosin X, Trim9 and DCC in

COS cells shows a distinct co-localization in filopodia tips.
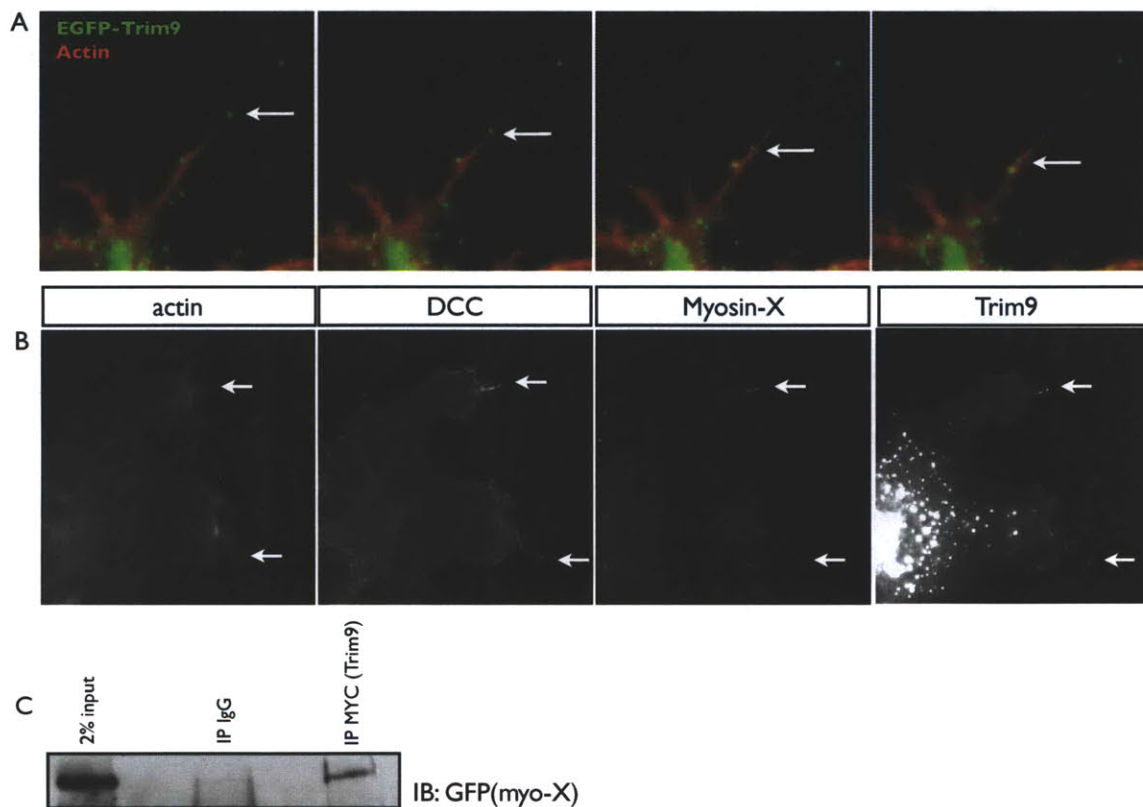
Figure 2-10: Trim9 interacts with Myosin-X.
A: Time lapse imaging of EGFP-Trim9 showing retrograde movement of puncta within a filopodia. B: Trim9, Myosin-X and DCC co-localize in filopodia tips. C: MYC-Trim9 and GFP Myosin-X co-immunoprecipitate when co-expressed in COS cells.

2.3.5 Trim9 functions specifically downstream of Netrin-1/DCC signaling

Previous work has shown that inhibition of proteasome function blocks netrin-1

growth cone turning in *Xenopus*(Campbell and Holt, 2001). Our genetic analysis in *C.*

elegans and biochemical data in mice, suggests Trim9 is an important molecule in this signaling pathway(Hao et al., 2010). We sought to characterize the functional role of Trim9 in response to netrin-1. We first attempted to use RNAi to deplete Trim9 protein in cortical neurons without success. To test Trim9's stability, we used cyclohexamide to block new protein synthesis in cortical neurons in culture and found that after 72 hours close to half of the Trim9 protein was still present. By this point in time, neurons have already formed an axon and initiated branch formation. Therefore Trim9 is a stable protein whose half-life precludes the use of RNAi for evaluating axon guidance or branching defects in cortical cultures. Also while working on Trim9, a highly related gene, Trim67, was annotated in the NCBI database. Trim67 (also called Trim9-like protein) which shares 76% sequence similarity with Trim9. Trim67 is predicted to heterodimerize with Trim9 due to its 91% similarity across the coiled coil domain and may have a similar function (Reymond et al., 2001).

We focused our efforts on methods that would block or inhibit Trim9's (and Trim67's) function. Studies of several ubiquitin ligases have shown expression of a deletion of the RING domain (ΔRING) in the same cell as the endogenous ubiquitin ligase protein blocks normal E3 ligase function and acts as a dominant-negative (Tursun et al., 2005). In *C. elegans*, expression of a ΔRING Madd-2 construct in wild type worms phenocopied the null defects, suggesting that a ΔRING-TRIM9 might be an effective and specific method to block the function of both Trim9 and Trim67.

In mice, expression of the ΔRING Trim9 construct led to morphological changes in cortical neurons and impaired morphological differentiation. To determine how Trim9 may function in nervous system development we evaluated systems were Ena/VASP

proteins have a prominent role. Ena/VASP proteins play an important role in neurite formation (Dent et al., 2007; Kwiatkowski et al., 2007). ΔRingTrim9 transfected neurons had a delay in progression from stage1 to stage 3 morphology, but this phenotype was not as severe as a loss of Ena/VASP proteins (Figure 2-11).
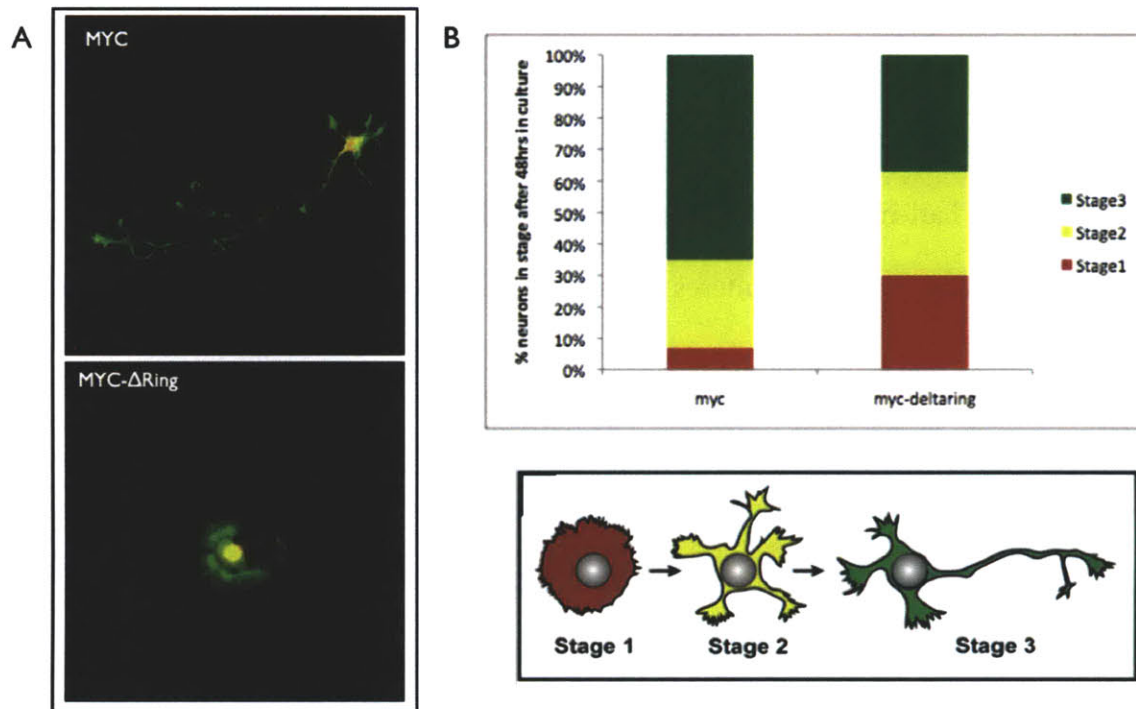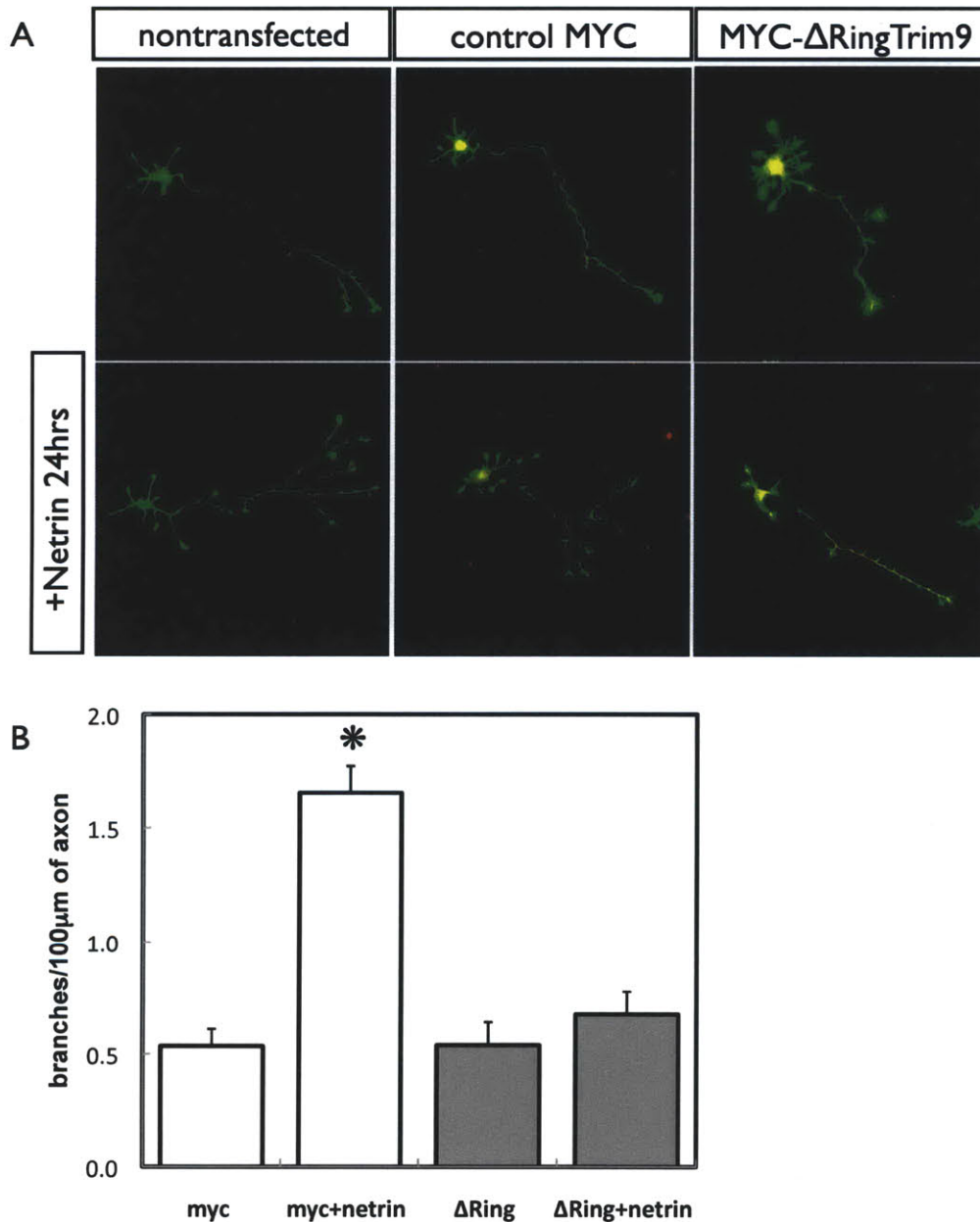

Figure 2-11: Inhibition of Trim9 Ubiquitin ligase activity causes a delay in neuritogenesis.
A: Representative images of cortical neurons transfected with ΔRingTrim9 fixed after 48hrs. B: Quantitation of neurons found in each developmental stage. Developmental stages diagramed below the graph.

The neuronal cell types that express Trim9 respond to netrin stimulation *in vitro*. Cortical neurons initiate new branches from the primary axon in response to netrin bath application. Since *C. elegans* madd-2 mutants fail to branch properly in response to a netrin cue, we examined the effect of expressing ΔRingTrim9 on morphological responses of mammalian cortical neurons to netrin stimulation (Figure 2-12). We quantified branching number in ΔRingTrim9 mutants and controls, and found a

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significant reduction in the number of branches produced after netrin bath application.

The ΔRing Trim9 transfected neurons did not form collateral branches in response to the

netrin-1 cue.
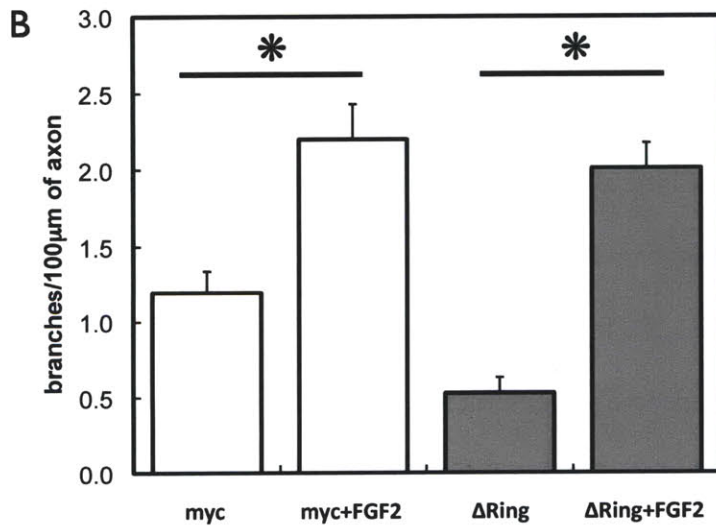

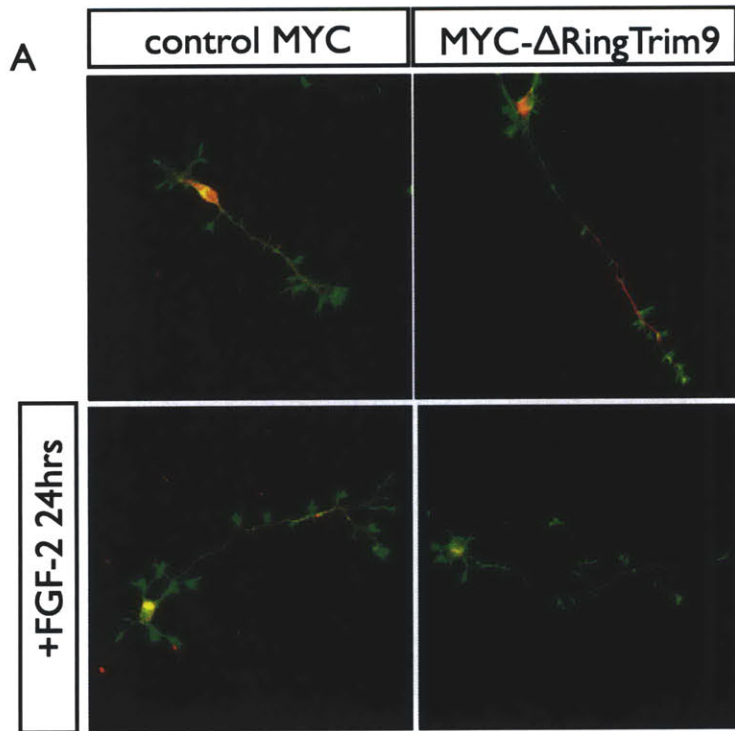**Figure 2-12: Inhibition of Trim9 Ubiquitin ligase activity causes a defect in Netrin-1 branching.**
A: Neurons transfected with ΔRingTrim9 did not form branches in response to netrin-1
B: Quantitation of number of branches per 100um of axon.

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We sought to determine if this was a defect in a general branching mechanism or if this was specific to the netrin-1 signaling pathway. Many guidance cues can influence branching (Schmidt and Rathjen, 2010). One example, FGF-2 causes a significant increase in the number of branches in cortical neurons (Szebenyi et al., 2001). FGF-2 acts through the fibroblast growth receptor 1 or 3 and likely activates the MAP kinase pathway (Reuss and von Bohlen und Halbach, 2003; Zechel et al., 2010). Neurons expressing the ΔRing Trim9 construct can branch in response to FGF-2 indicating the defect in branching is specific to signaling downstream of the netrin-1 cue (Figure 2-13).

Figure 2-13: Inhibition of Trim9 Ubiquitin ligase activity has no effect on FGF2 branching.

A: Neurons transfected with ΔRingTrim9 did form branches in response to FGF-2.

B: Quantitation of branches in each treatment per 100uM of axon length.

2.3.6 Trim9 binds Map1b and may control microtubule stability

Even though Trim9 binds DCC, Ena/VASP and MyosinX we have found no evidence that it facilitates ubiquitin transfer onto these proteins or targets them for proteasomal degradation. To identify candidate substrates for Trim9's ubiquitin ligase activity we used immunoprecipitation of ΔRingTrim9 followed by mass spectrometry. As mentioned before, deletion of the RING domain increases the binding affinity of an ubiquitin ligase and its substrate by blocking transfer of the ubiquitin tag and therefore degradation through the proteasome. The herpes simplex virus infects close to 100% of neurons *in vitro* and yields high protein expression within a few hours, we used this method to infect cortical neurons in culture. We designed a construct that expresses MYC tagged ΔRing Trim9 and infected cortical neurons in culture. ΔRing Trim9 was immunoprecipitated using the MYC monoclonal 9E10 and bound proteins were identified by mass spectrometry.

Several interesting binding partners such as, secretory proteins, actin and tubulin were found. One binding partner of interest was Map1b, an actin and microtubule binding protein involved in signaling downstream of netrin and regulating microtubule stability (Bouquet et al., 2007; Del Río et al., 2004; Pedrotti and Islam, 1996). Map1b levels can be controlled by the ubiquitin proteasome system but the E3 ligase that is involved is not clear (Allen et al., 2005; Cleveland et al., 2009). This data lead us to hypothesize that Trim9 may be an E3 ubiquitin ligase for Map1b.

Protein identified	# of peptides
Trim9	7
Sec23 Interacting Protein	7
βIII tubulin	3
Trim67	2
Mapl b	2
actin	1
α tubulin	1

Figure 2-14: Proteins Identified in mass spectrometry screen
Table listed proteins identified and the number of peptides that were recovered for immunoprecipitation of MYC-Trim9.

2.4 Conclusion

Axon branching largely influences cortical circuitry. Extracellular signals that influence many aspects of neuronal development also influence collateral branching (Gibson and Ma, 2011). Netrin-1 can increase the number of collateral branches in cortical neurons without the cooperation of the growth cone, unlike FGF-2 which requires growth cone pausing to form branches (Dent, 2004). Trim9 is an E3 ubiquitin ligase whose activity is necessary downstream of netrin-1 branching but is dispensable for collateral branching downstream of FGF-2. As we have shown, Trim9 coordinates cytoskeletal dynamics downstream of the netrin/DCC pathway and this specificity to a particular cue suggests this protein is important for the differential responses of neuronal processes to branching factors.

Trim9 functions downstream of DCC at least in part as a protein scaffold that recruits effector proteins to the receptor. Prior to this study it was unknown how Ena/VASP might function in the response to netrin. We suggest Trim9 brings Ena/VASP and DCC into a complex. Myosin-X can induce filopodia, bind to Ena/VASP proteins and the cytoplasmic tail of DCC (Bohil et al., 2006; Tokuo and Ikebe, 2004; Wei et al., 2011). This unconventional myosin is necessary for proper DCC localization in response to a netrin cue (Zhu et al., 2007). Myosin-X and Trim9 both have intrafilopodial motility, while Ena/VASP proteins do not (Figure 2-9). Our data shows Myosin-X, DCC and Trim9 co-localize in filopodia tips presumably along with Ena/VASP proteins. By binding Ena/VASP proteins and DCC, Trim9 organizes a macromolecular complex that is competent to quickly form filopodia in response to netrin.

Axonal branching relies on precise control of proteins levels along the axon shaft through the UPS (Schmidt and Rathjen, 2010). Several E3 ubiquitin ligases have been identified for their role in branching, such as Nedd-4 in RCG neurons (described in chapter1) or Zebrafish *esrom* in retinal neurons (D'Souza et al., 2005; Drinjakovic et al., 2010; Kawabe et al., 2010). Trim9 is another E3 ubiquitin ligase involved in cytoskeletal remodeling leading to branch formation suggesting that selective degradation of signaling components is a mechanism necessary for collateral branching in many classes of neurons.

There are six members of CI subfamily of TRIM proteins and only MID1 (Trim18, closely related to Trim1) has been well characterized. MID1 functions as a E3 ligase specific for protein phosphatase 2a (PP2a). Opitz syndrome patients have mutations in MID1 that prevent the protein from localizing to microtubules where PP2a accumulates, resulting in a hyperphosphorylation of microtubule associated proteins and mutiple, severe midline defects (Trockenbacher et al., 2001). Recently a study of another CI family member, Trim36, found that this protein is assymetricaly localized in the developing Xenopus embryo and its activity was necessay for microtubule polymerization and it is associated with kinetechore proteins (Miyajima et al., 2009) (Cuykendall and Houston, 2009). These data and our results with Trim9 suggest that the CI subfamily could have a general role in regulating microtubule stability.

It was known that branches and axons can be selectively guided but the mechanism of differential control was not well understood. Our study of Trim9 may provide an explanation for how this mechanism can occur. MADD-2 does not have strong effect in guidance but does in branching; suggesting that this protein could

stimulate branch formation in response to netrin without significantly influencing axon guidance.

This differential effect may be through Trim9's potential substrate, Map1b. This protein is enriched in the distal axon and growth cone during guidance, but depending on its phosphorylation state, can also localize to only stable microtubules (Tymanskyj et al., 2010). Loss of Map1b results in excess branching and defects in axon guidance (Bouquet et al., 2007; Meixner et al., 2000). Netrin can modify the Map1b phosphorylation state and therefore restrict its localization to the axon or growth cone (Del Río et al., 2004). Trim9 is not an essential component within the structure of a filopodia and does not evenly localize to each actin protrusion in the axon shaft or growth cone (Figure 2-8). It is possible that Trim9 complexes key molecules needed for branching in response to netrin-1 and removes inhibitors that could block branching, like microtubule stabilizing Map1b. These results provide evidence that localization of Trim9 to areas along an axon can confer differential responsiveness that results in accurate branch patterning during development.

2.5 Materials and Methods

Antibodies and Reagents

The following antibodies were used in this study: Trim9 polyclonal was generated by injecting rabbits with murine Trim9 recombinant protein amino acids 158-271, c-MYC (clone 9E10 from Santa Cruz), anti-Mena Monoclonal (generated in Gertler lab), anti-VASP rabbit polyclonal (generated in Gertler lab), anti-DCC (clone G97-449 from BD Pharmingen), anti-GST (clone G1417 Sigma), anti-His(clone H1029 Sigma), anti-map1b (clone13725 from Lifespan Biosviences). Recombinant netrin-1 (R&D systems), Recombinant FGF-2 (MBL International) human trim9 cDNA (gift from T. Cox, UW, Seattle, WA), GST constructs subcloned into pGEX6P1(GE Healthcare), rat DCC cDNA (gift from MTL), pC2-egfp vector containing bovine myosin X cDNA (gift from R. Cheney, UNC Chapel Hill,NC), MYC tag vector pCs2+ (gift from U. Philipar, MIT)

Yeast two hybrid

LexA two-hybrid system selection and beta-Galactosidase assays were performed according to the manufacturer's protocol (Clontech).

Binding assays

MADD- 2 cDNA was cloned into pGEX6P1 and GST-fusion proteins were prepared as described (Yu et al., 2002). UNC-40 and UNC-40ΔP3 were cloned into pQE80L His-tag expression vector (Qiagen, Valencia, CA); expressed proteins were purified using Talon resin, eluted with 100mM imizadole and dialyzed. For binding assays, 2ug of immobilized GST- fusion protein was mixed with 500mM or 1uM of purified his-tagged protein in 10mM Tris pH 7.5, 150mM NaCl, 0.1% NP40. Samples were incubated at 25C for 1 hour and washed 4 times in the same buffer. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-6xHis antibodies (Qiagen).

Cortical neuron culture and transfection

Cortical neuron cultures were prepared from embryonic E14.5-E16.5 mice as previously described (Kwiatkowski et al., 2007). Cortices were dissected, trypsinized and dissociated. Neurons were initially cultured in 5% FBS (Hyclone) in Neurobasal Medium (GIBCO) and switched to serum free media supplemented with B27 (GIBCO) after 2 hrs in culture. Neurons plated on glass and plastic were coated with 0.2-1mg/ml poly-D-lysine (Sigma) at 5000 cells/cm^2. For transfection, after dissociation neurons were resuspended in Amaxa Nucleofector solution (mouse neuron kit; Amaxa Biosystems, Cologne, Germany) and transfected with Amaxa Nucleofector according to manufacture's instructions.

GST pulldown

GST pulldown was carried out as previously described (). E15 embryonic mouse cortex was lysed in 1%NP40 or RIPA buffer. Lysate was precleared with GST immobilized on glutathione-sepharose beads (manufacturer) and then incubated with incubated with 5-10ug of GST fusion protein or GST immobilized on glutathione-sepharose beads at 4C

for 4 hours. The beads were wahed three times with lysis buffer and bound proteins were resolved by SDS-PAGE and immunoblotting.

Immunofluorescence

Cortical neurons and COS cells were cultured as previously described (Kwiatkowski et al., 2007). Cells were fixed in 4% paraformaldehyde-PHEM (Strasser et al., 2004), rinsed with PBS, permeabilized with 0.2% Triton-X100, and blocked with 10%BSA/PBS. The samples were then incubated with primary antibody for one hour, washed 3 times with PBS and incubated with fluorescently labeled secondary antibody at 1:500 (Millipore) and fluorescently coupled phalloidin for one hour. Images were collected on a Deltavision microscope and deconvolved using Softworxs software (Applied Precision).

Time lapse fluorescent imaging

Imaging was performed on gridded or nongrided glass bottom dishes (Mattek) coated with poly-D-lysine (sigma). Time lapse images were taken on a Nikon TE300 microscope or Deltavision microscope. During imaging cells were kept at 37°C and supplemented with 5% CO_2 (Solent, Segensworth, UK).

Peptide Array

Peptides were generated that corresponded to 25 amino acid stretches in the murine DCC cytoplasmic tail (aa 1012-1447), overlapping by 3 amino acids in each progressive spot. These peptide were covalently linked to a nitrocellulose membrane (Koch Institute biopolymers facility). The membrane was then overlayed with recombinant GST-SPRY or GST alone and protein was visualized using a mouse monoclonal antibody to GST, mouse secondary HRP antibody and ECL Plus Detection Reagents (Amersham).

Branching assay

Netrin-1 or FGF-2 were bath applied to mouse cortical neurons from E14-E16 embryos which had been in culture for 48hrs. After 24hrs of treatment the cells were fixed and branches were scored. The primary axon length was measured from the cell body to the central region of the growth cone. Branches were defined as processes extending from the primary axon that were at least 20um long. We normalized the numbers of branches as a function of branches per 100um of axon length as previously denscribed (Dent and Kalil, 2001).

MassSpec IP

Primary cortical neurons were dissected and cultured as described above. HSV Myc-ΔRing Trim9 was thawed at 37°C immediately before addition to supernatant of dissociated cortical cultures at a MOI of 2. 6 hours post infection the neurons were lysed in 1% Trition-X100, 50mM Tris pH7.5 and 150mM NaCl. Soluble protein was immunoprecipitation by Myc mononclonal 9E10 (santa cruz) covalently attachted to proteinA bead (Thermo scientific) and Isotype control IgG (sigma). After extensive washing, bound proteins were eluted with 8M Urea for 30min. This sample was analyzed by LC-MS on a LTQ ion trap Mass Spectrometer (Koch Institute biopolymers facility).

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Chapter 3

Regulation of Actin Architecture by Barbed-End Binding Proteins

The results described in this chapter contributed to two manuscripts:

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Kwiatkowski, A.V., Rubinson, D.A., Dent, E.W., Edward van Veen, J., Leslie, J.D., Zhang, J., Mebane, L.M., Philippar, U., Pinheiro, E.M., Burds, A.A., Bronson, R.T., Mori, S., Fässler, R., Gertler, F.B., 2007. Ena/VASP Is Required for neuritogenesis in the developing cortex. Neuron 56, 441-455.

Note: The electron micrographs were produced by the author. Accompanying data in the study of Ena/VASP null neurons was produced by Erik Dent and in the study of mDia filopodia was produced by Melanie Barzik.

Chapter 3 Table of Contents

3.1 Abstract

Filopodia are thin actin, rich protrusions that play a central role in cell motility, guidance, cell-cell signaling, and adhesion. These finger-like extensions act as directional sensors, potentially through the enrichment of guidance receptors and cell adhesion molecules at their tips. Many different actin-binding proteins are essential for the formation, maintenance, and dynamics of these structures. The Ena/VASP family of proteins binds the growing end of actin filaments and promotes long, unbranched structures. Ena/VASP proteins are implicated in many developmental processes and serve as important signaling molecules that influence the structure of actin. In this study we sought to understand how changes in barbed-end associated proteins affect the actin cytoskeleton and how these changes influence cell morphology and developmental processes.

3.2 Introduction

Directed cell movement is necessary for embryonic patterning, tissue development, wound healing, and immune response (Pollard and Borisy, 2003). Individual motile cells must adhere to substrates, remain in contact with neighboring cells, migrate through tissues, and respond to extracellular cues. Polymerization of actin filaments is essential for cell movement and these filaments can be assembled into a variety of structures that give a cell shape and provide the mechanical force behind cell motility. (Pollard and Cooper, 2009).

Spontaneous assembly of actin is an unfavorable reaction and under physiological conditions requires initiation by an actin nucleator, such as Arp2/3 or formins (Chesarone and Goode, 2009). However, actin filaments polymerize quickly once the assembly process begins. Close to 100 proteins bind actin, maintain pools of actin monomer, regulate assembly and structure of filaments, and crosslink filaments into networks or bundles (Pollard and Cooper, 2009). F-actin can be assembled into more complex structures, such as lamellipodia, filopodia stress fibers, and podosomes (Gupton and Gertler, 2007).

Lamellipodial protrusions are thin, densely branched F-actin meshworks that comprise the leading edge of a cell (Chhabra and Higgs, 2007), the molecular mechanism behind the formation of this structure is well defined (Blanchoin et al., 2000; Pollard et al., 2000; Svitkina and Borisy, 1999). As mentioned previously, filopodia are bundled, finger-like protrusions of F-actin. In both lamellipodia and filopodia, the growing end of actin filaments are oriented outward and generate the forces that efficiently push the membrane forward (Chhabra and Higgs, 2007; Pollard and Borisy, 2003).

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There are many regulators of filopodia formation and dynamics, therefore in different systems, many distinct mechanisms of filopodia formation have been proposed (Gupton and Gertler, 2007). The convergent elongation model of filopodia formation suggests that filopodia arise from the dendritic actin array in the lamellipodium, by the elongation of privileged filaments. The growing barbed end of these privileged filaments is protected from capping protein by association with Ena/VASP proteins, which promotes rapid polymerization and clustering. This subset of filaments then becomes bundled by the actin cross-linker fascin and a filopodia is formed (Svitkina et al., 2003).
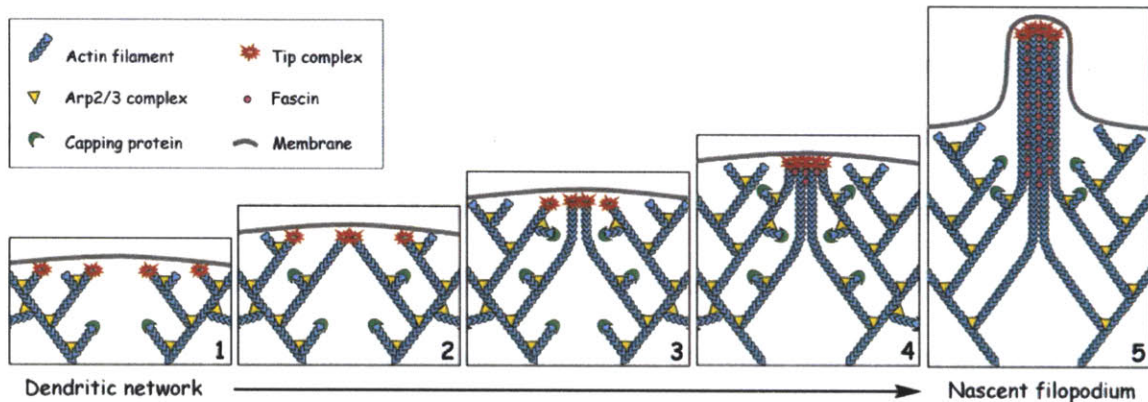
Figure 3-1: Filopodia formation by convergent elongation.
Figure from (Svitkina et al., 2003).

Interestingly, filopodia can also form in the absence of Arp2/3 or its activators, suggesting that other mechanisms of filopodia formation exist (Sigal et al., 2007; Steffen et al., 2006). An alternate hypothesis, referred to as de novo filament elongation, suggests that a formin, such as mDia, can nucleate actin filaments, polymerize rapid elongation, and stabilize the structure into a filopodia without the assistance of other actin-binding proteins (Pellegrin and Mellor, 2005; Zigmond et al., 2003). It is likely that both of these

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models of filopodia formation exist in various cell types and structures during development.

Filopodia are essential for physiological processes such as embryo development, wound healing, and cell signaling. In general, filopodia are thought to be sensors of the extracellular environment (Davenport et al., 1993) because they reorient toward attractive cues (Zheng et al., 1996). Guidance receptors, integrins, and cadherins can localize to filopodia tips and the extension of filopodia can activate signaling pathways farther back within the cell (Letourneau and Shattuck, 1989; Shekarabi, 2002). Filopodia may also function to establish nervous system circuitry, as they are necessary for the formation of neurites, which are the precursors of axons and dendrites (Dent et al., 2007). Filopodia are also essential for dorsal closure of drosophila embryos, an early stage in development of the embryo (Jacinto et al., 2000). Lastly, filopodia act as sensors for cell migration because adhesion molecules are associated with filopodia tips, this structure extend into the extracellular matrix, and adhere to permissive substrates (Galbraith et al., 2007).

3.3 Ena/VASP in nervous system development

Ena/VASP proteins localize to areas of dynamic actin reorganization, including the tips of filopodia and the leading edge of lamellipodia. They have a well-established role in filopodia formation and maintenance but the precise role Ena/VASP proteins play during filopodia formation has remained controversial (Applewhite et al., 2007; Han et al., 2002; Lebrand et al., 2004). In this section, we will focus on the role Ena/VASP proteins play in nervous system development. Cortical neurons are born in the subventricular zone, migrate to occupy more superficial layers of the cortex, and send out multiple processes to establish the proper framework of the forebrain (Marin and

Rubenstein, 2003). However, little is known about the mechanisms that modulate

cytoskeletal dynamics during the initiation of neurites (Luo, 2002).

Loss of function analysis was made difficult due to the presence of three

Ena/VASP family members with similar functions and overlapping expression patterns in

the nervous system. In order to overcome this problem, all three family members in mice

were knocked out and the phenotypes were analyzed. Although a single allele of Mena

(Mmvvee) was sufficient to produce viable fertile mice, animals null for all three

Ena/VASP genes (mmvvee) died between E16.5 and P0. When embryos in this age range

were examined they had many defects including intra-amniotic hemorrhaging, vascular

defects, hydros fetalis, and frequent exencephaly (Furman et al., 2007; Kwiatkowski et

al., 2007). In addition, chimeric mice made using ES cells in which all three genes were

deleted and marked with GFP positive cells to allow for analysis of cell autonomous

defects (Kwiatkowski et al., 2007) .

### 3.3.1 Results: The role of Ena/VASP in cortical neuritogenesis

Although some of the defects seen in the triple knockout embryos were outside

the nervous system, the major phenotype of the mmvvee mice was a disorganization of

the central and peripheral nervous system. Sections of the cortex were analyzed for axon

fiber tracts using the antibody Tau-1. Tau-1 staining should be enriched in the IZ where

cortical axon fiber tracts are located. In control animals normal staining was observed,

while in mutant animals little-to-no Tau-1 staining was observed, suggesting Ena/VASP

null neurons could not properly form axons (Kwiatkowski et al., 2007).

To study this defect at higher resolution, cortical neurons from littermate controls and triple null embroys (mmvvee) were cultured *in vitro* and imaged. Cortical morphogenesis follows a stereotyped set of stages in culture. Stage 1 morphology occurs a few hours after attachment to the substrate *in vitro*, and the neuron is round with filopodia extending around the periphery of the cell. After approximately 24 hours, protrusions around the cell coalesce and form multiple neurites, a process called neuritogenesis that is characteristic of stage 2 morphology. After 48 hours in culture one of these neurites extends and becomes the axon while the remainder of the neurites become dendrites at this point the neuron is in stage 3 morphology (Fukata et al., 2002). Strikingly after 48hrs in culture, neurons null for Ena/VASP proteins (mmvvee) remained in a stage 1 morphology, while close to 80% of control neurons had a defined axon (Figure 3-3A) (Dent et al., 2007).
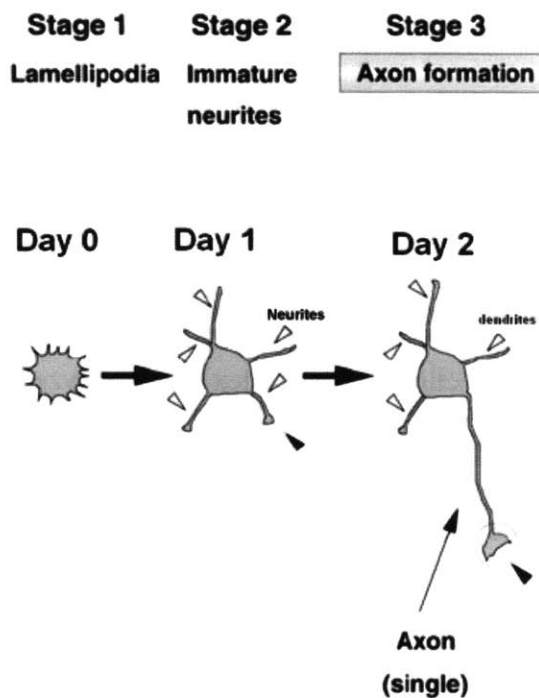
Figure 3-2: Stereotyped stages of cortical development in culture.
Adapted from (Fukata et al., 2002)

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When chimeric embryos containing Ena/VASP-deficient ES cells labeled with GFP were isolated and cultured for 12 hours we found that Ena/VASP deficient neurons could not elaborate neurites even if they had differentiated within an overall normal microenvironment. Immunofluorescent staining for major cytoskeletal components demonstrated that the mutant-derived neurons had roughly the same amount of actin and microtubule content and similar localization, but lacked bundled F-actin and filopodia (Figure 3-3B). We examined the differences in these structures at a higher resolution using platinum replica electron microscopy. This method allows for the visualization of the architecture of the actin cytoskeleton at the resolution of a single 7nm actin filament (Svitkina et al., 1995). We simultaneously analyzed both GFP positive (mmvvee) and GFP negative (wild type control) neurons from the same chimeric animal via correlative electron microscopy. After plating for 12 hours and ensuring stage 1 morphology, we took fluorescent images and immediately fixed the cells for EM processing. As seen in Figure 3-3C, GFP negative neurons contain actin arcs oriented perpendicular to the membrane, bundled into filopodia, and incorporating actin filaments from both sides of the structure. In contrast, GFP positive neurons (Figure 3-3D) have a general disorganization of F-actin, with most filaments incorporated into actin arcs oriented parallel to the membrane.
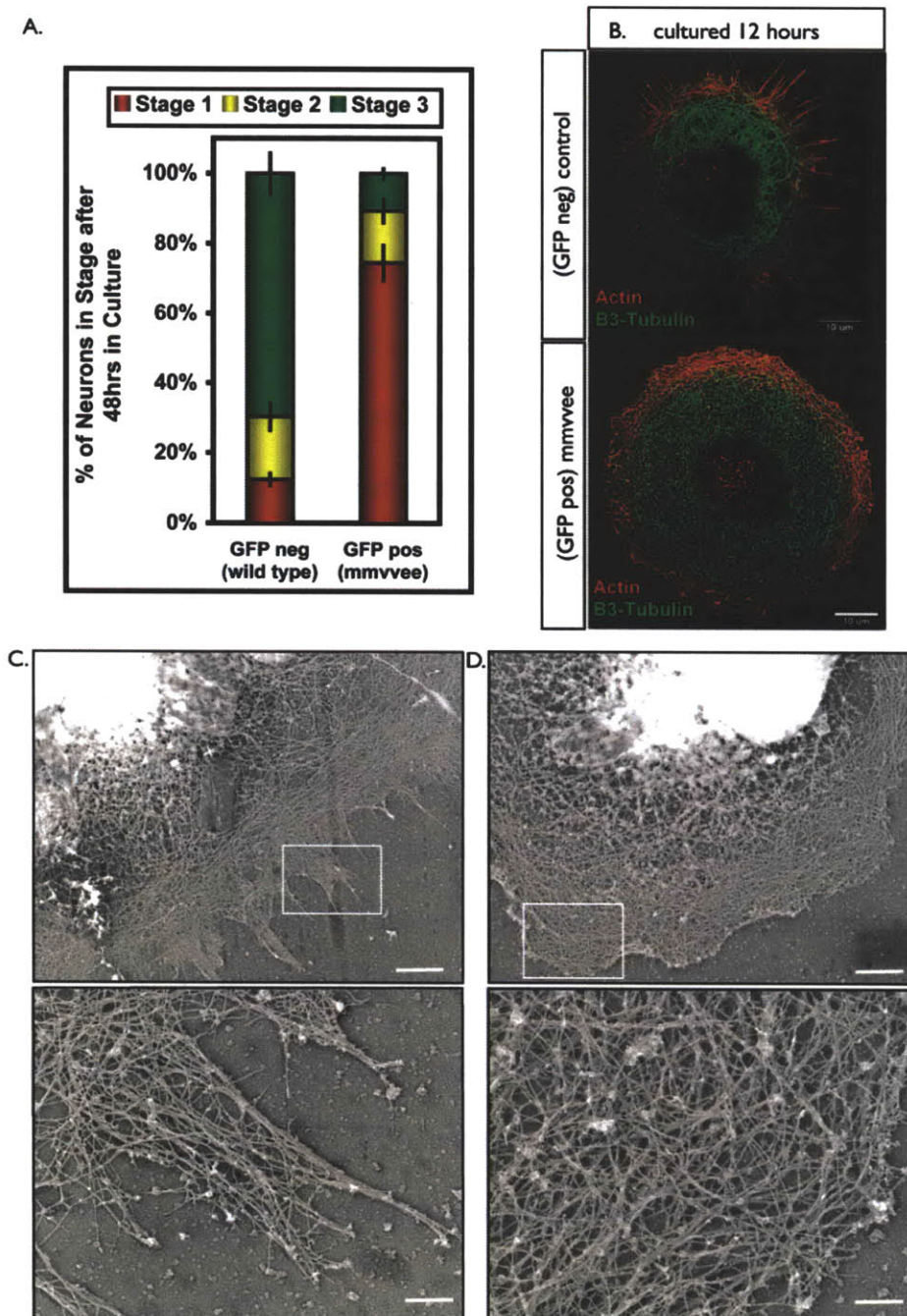
Figure 3-3: Ena/VASP are required for filopodia formation in stage 1 neuron.
A: Scoring for developmental stage after 48 hours in culture revealed the majority of GFP positive (wild type) neurons had progressed to stage 3 morphology, while close to 80% of mmvvee null neurons were still in stage1. B: Two stage 1 neurons from a chimeric brain stained for βIII tubulin and actin. WT and mmvvee neurons contained approximately equal amounts of actin and tubulin but mmvvee neurons lacked filopodia. C and D: Platinum replica electron microscopy of GFP negative wildtype (C) and GFP positive mmvvee null (D). Wildtype neurons showed bundled actin perpendicular to the membrane protruded from the cell edge and mmvvee neurons have a general disorganization of actin cytoskeleton resulting in circumferential rings of actin archs. Scale bar for C and D top panel is 1um and bottom panel is 150nm.

In the mmvvee embryos, there was a marked decrease in axon tract formation. However, a few mmvvee embroys had cobblestone cortex, where cortical neurons migrate through breaks in the pial membrane and form ectopias outside of the brain (Olson and Walsh, 2002). Even though mmvvee neurons rarely had Tau-1 positive staining, these cobblestone cortex ectopias were positive for Tau-1 (Kwiatkowski et al., 2007). This suggests that some difference between the ectopia and cortical environments rescued the ability of mmvvee neurons to form axons. One major difference between the ectopic environment and the cortex is that laminin is abundant in the pial membrane but largely absent from the cortex. Remarkably, plating the mmvvee neurons on laminin rescued neuritogenesis (Figure 3-4A). However, the number of neurite-like and filopodia-like extensions in mmvvee neurons plated on laminin were less than in control neurons (Dent et al., 2007).

Using time-lapse imaging, and immunofluorescent staining, we observed that laminin induced filopodia-like structures that contained actin but were unstable and kinked. To study these structures at a higher resolution, we again used platinum replica electron microscopy. As seen in Figure 3-4D, mmvvee neurons plated on laminin contained bundled actin filaments, but these filaments were not supported by actin filaments emerging from the actin dendritic network and, instead, splayed off the side of the parallel bundles. Although these filopodia-like structures had an unusual architecture, they contained canonical filopodial markers (e.g. fascin) and retained the ability to rescue neuritogenesis (Dent et al., 2007).
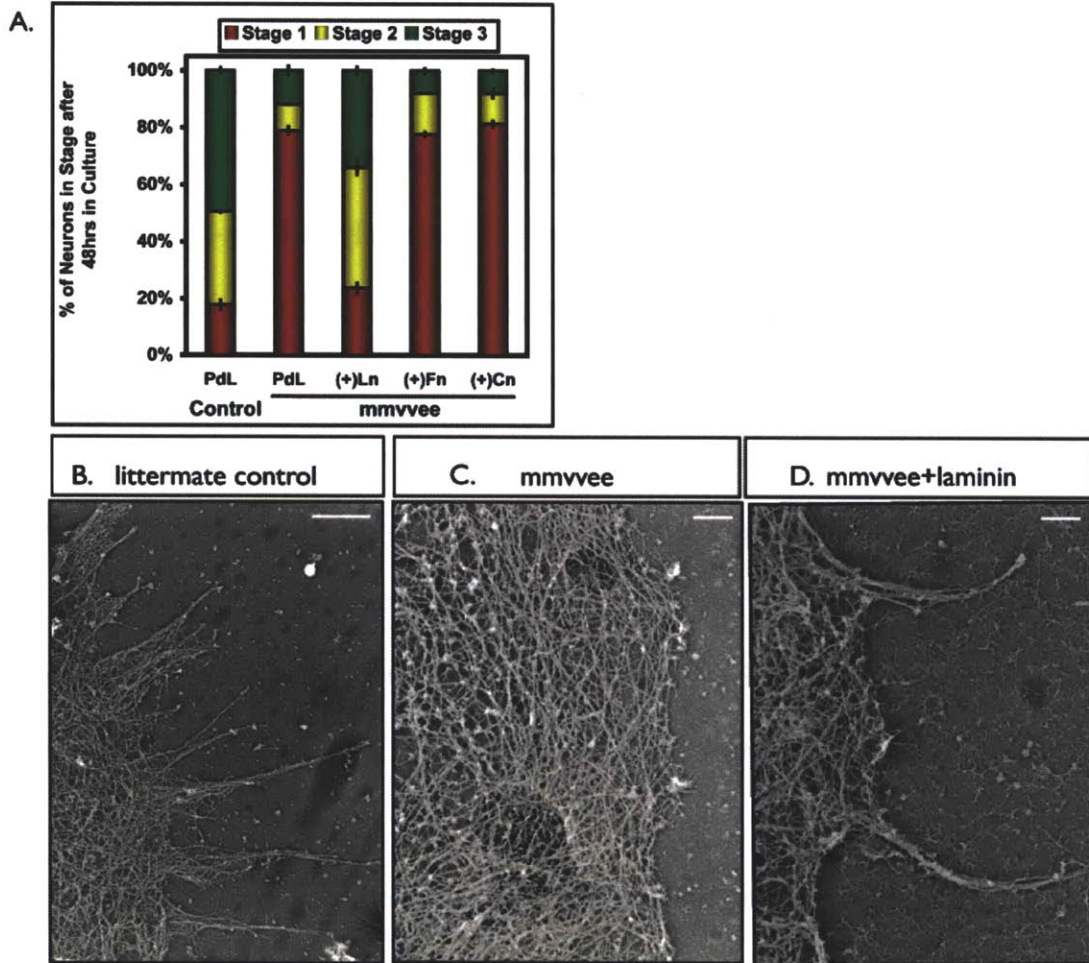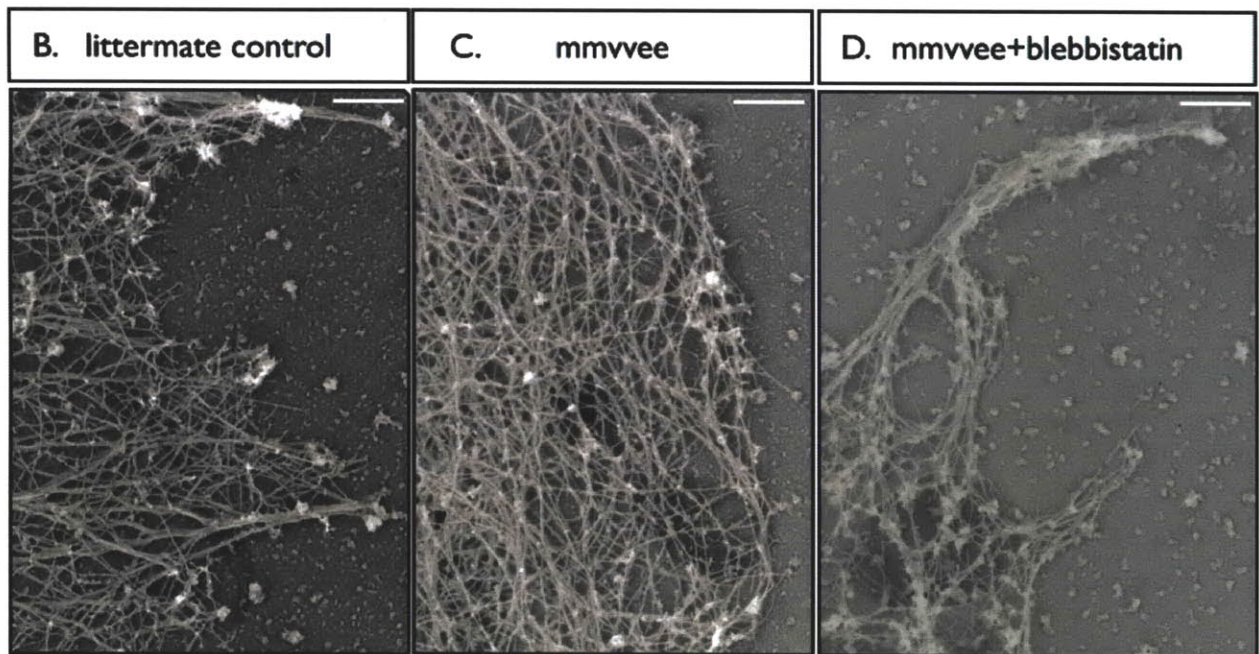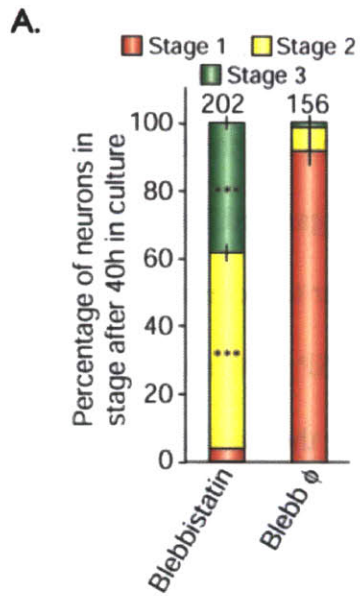
Figure 3-4: Laminin rescues neurite formation through the formation of filopodia-like structures

A: Scoring for developmental stage of littermate control and mmvvee null neurons plated on poly-D-lysine (PdL) or PdL supplemented with 20 ug/ml laminin-1(+Ln), 20 ug/ml fibronectin (+Fn), or 20 ug/ml of collagen-1 (+Cn) after 48 hours in culture. Laminin was the only extracellular matrix molecule that can restore stage development to levels of control neurons. B,C and D: platinum replica electron microscopy of control and mmvvee neurons plated on (C) PdL or (D) PdL with 20 ug/ml laminin-1. Laminin induced curved filopodia-like extensions that contained bundled actin supported from only one side. Scale bar represent 0.5um in B-D.

Studies have suggested that myosin II works downstream of laminin-induced outgrowth (Turney and Bridgman, 2005). Similarly to laminin, inhibiting myosin II gave rise to filopodia-like extensions. At high resoution, we observed that the bundled actin filaments induced by myosin-II inhibition, although very disorganized, had actin filaments incorporated from both sides of the protrusion similar to a classical filopodia (Figure 3-5D). We hypothesize that in the absence of Ena/VASP proteins, myosin II actin crosslinking activity inhibits filopodia formation by increasing the stability of the actin arcs. When myosin II is inhibited, actin crosslinking is disrupted allowing actin to bundle perpendicularly to the membrane, driving protrusion.

Figure 3-5. MyosinII inhibition rescues neurite formation through the formation of filopodia-like actin protrusions

A: Scoring for developmental stage of littermate control and mmvvee null neurons treated with 50uM of blebbistatin or 50uM of the inactive enatiomer (φ). Blebbistain addition rescues nerite formation and progression to stage 3. B, C and D: Platinum replica electron microscopy of control, mmvvee and mmvvee after 50uM blebbistatin addition. Blebbistatin results in actin rich bundles forming at the periphery of the neuron. Scale bar is 1um.

3.4 The role of Ena/VASP in metastatic breast cancer migration

The leading cause of death in cancer patients is metastasis (Chaffer and Weinberg, 2011). Malignant transformation is sustained by a rearrangement of the cytoskeleton resulting in altered cell-cell adhesions, migratory, and invasive behavior. Metastasis, the process that leads to cancer cells escaping from the primary tumor and colonizing distant secondary sites in the body, is the leading cause of death in cancer patients. Cell motility and the regulation of the actin cytoskeleton are critical components of cancer metastasis, and identification of factors that can predict tumor cells' migratory potential *in vivo* could greatly impact clinical practice (Philippar et al., 2008; Roussos et al., 2010).

In order to identify factors that give rise to motile, invasive tumor cells capable of enhanced motility, the Condeelis lab developed an assay that allows for the isolation of invasive cells directly from a rat primary tumor (Wang et al., 2004). Among the sets of genes identified as upregulated in invasive cells compared to non-motile cells *in vivo*, Mena was one of the key genes upregulated in the "invasive signature" and is thought to be a central switch point in the regulation of cancer cell motility during metastasis. Mena is expressed at low levels in normal mammary epithelium yet is overexpressed in high-risk lesions and primary metastatic tumors (Di Modugno et al., 2004). Furthermore, It was discovered that a specific splice isoform of Mena is expressed in invasive tumor cells, and is called Mena[INV] (Figure 3-6) (Goswami et al., 2009). Mena[INV] sensitizes tumor cells to the growth factor EGF, allowing them to respond to otherwise undetectable levels of the pro-migratory signal (Philippar et al., 2008).
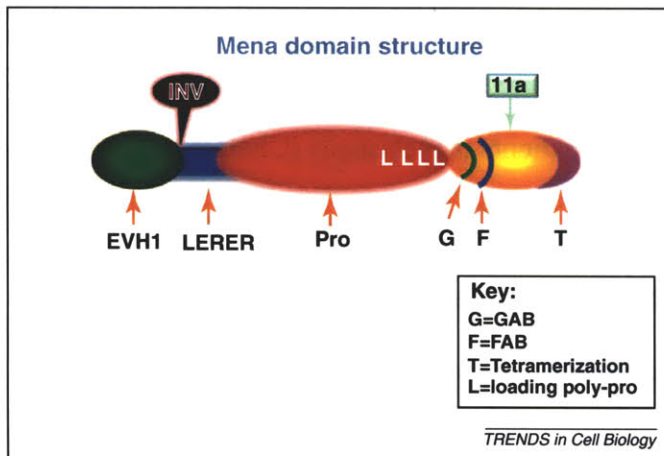
Figure 3-6. Mena domain structure and sites of alternatively included sequence
From (Gertler and Condeelis, 2011)

Another Mena splice isoform was identified that contained a 21aa insertion in the EVH2 domain and was called Mena11a (Figure 3-6)(Di Modugno et al., 2007). This 21aa insertion is next to the F-actin binding motif, has a putative phosphorylation site, and may influence F-actin binding. Mena has multiple alternatively spliced products, and Mena11a is an isoform expressed in normal epithelia tissues. Mena11a expression is present in epithelial human ductal pancreatic cancer cells, and is lost in more aggressive cancer cells expressing mesenchymal markers (Pino et al., 2008). As a result, this gene splice variants may act valuable cancer biomarkers. To determine the functional consequence of changes in Mena splice isoform expression, Mena and Mena11a were studied in carcinoma cells. It was found that when compared with Mena, Mena 11a damped protrusion in response to EGF. In *in vitro* studies, Mena11a had similar F-actin binding and bundling activity when compared to other Mena isoforms, but it showed an impaired ability to capture F-actin barbed ends (Balsamo et al, in preparation).

3.4.1 Results: Splice isoform Mena 11a disrupts actin organization

The Mena splice isoform, Mena11a, is exclusively expressed in epithelial mouse tissues and is not expressed in invasive, migrating carcinoma populations (Goswami et al., 2009). We chose to study the effects of Mena11a expression on the actin cytoskeleton ultrastructure in the Ena/VASP deficient murine fibroblast cell line MVD7. These cells are genetically deficient in endogenous Mena and VASP and were screened for the absence of Evl (Bear et al., 2000). We compared lamellipodia protrusion in PDGF stimulated MVD7 fibroblasts after serum starvation and found that the expression of Mena11a reduced lamellipodia F-actin density and branching, as seen in Figure 3-7.
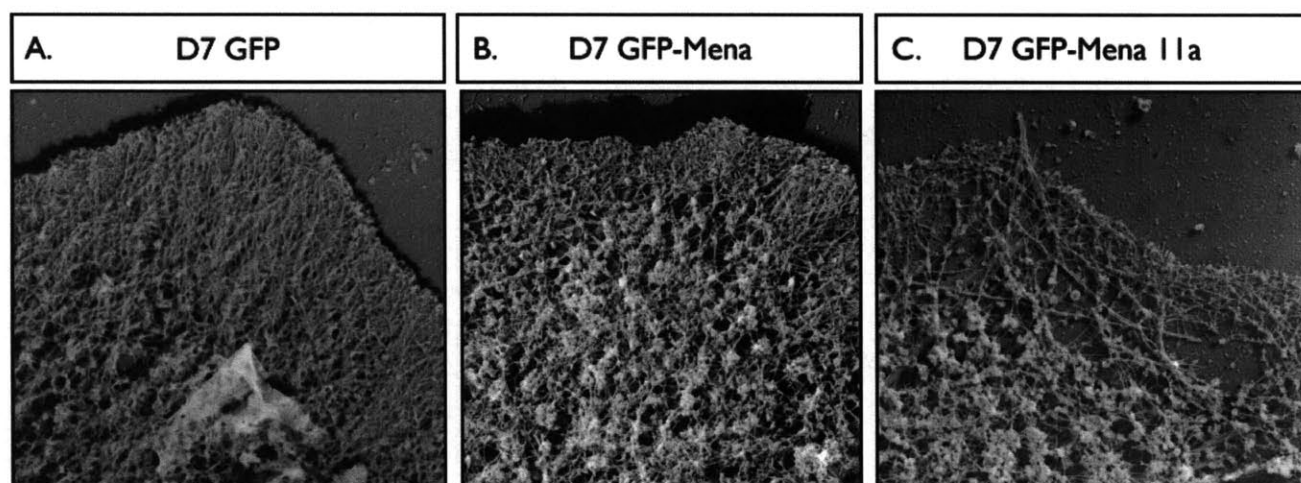

Figure 3-7: Expression of Mena11a results in defects in lamellipodia architecture
A-C: Platinum replica electron microscopy of MVD7 cells, fixed 5mins after PDGF stimulation. A and B: MVD7 cells expressing GFP or GFP-Mena formed highly branched F-actin rich lamellipodia. C: Lamellipodia of MVD7 cells expressing Mena11a was thin and the area behind the membrane edge was abnormally sparse.

At higher magnifications, we observed a reduction in F-actin density immediately behind the leading edge, which was also associated with a reduction in F-actin branching (Figure 3-8). Super-resolution immunofluorescence analysis has also shown that MVD7 cells expressing Mena11a have less Arp2/3 complex localized to the leading edge when

compared with Mena expressing cells and control cells. Total Internal Reflection

Fluorescence microscopy experiments have also shown that purified Mena11a has

impaired barbed end capture of growing F-actin filaments when compared with Mena and

VASP (Balsamo et al, in preparation). This suggests that the initial barbed end capture of

F-actin filaments plays an important role in the Mena-dependent organization of

lamellipodial protrusions and, possibly, recruitment of Arp2/3 complex.
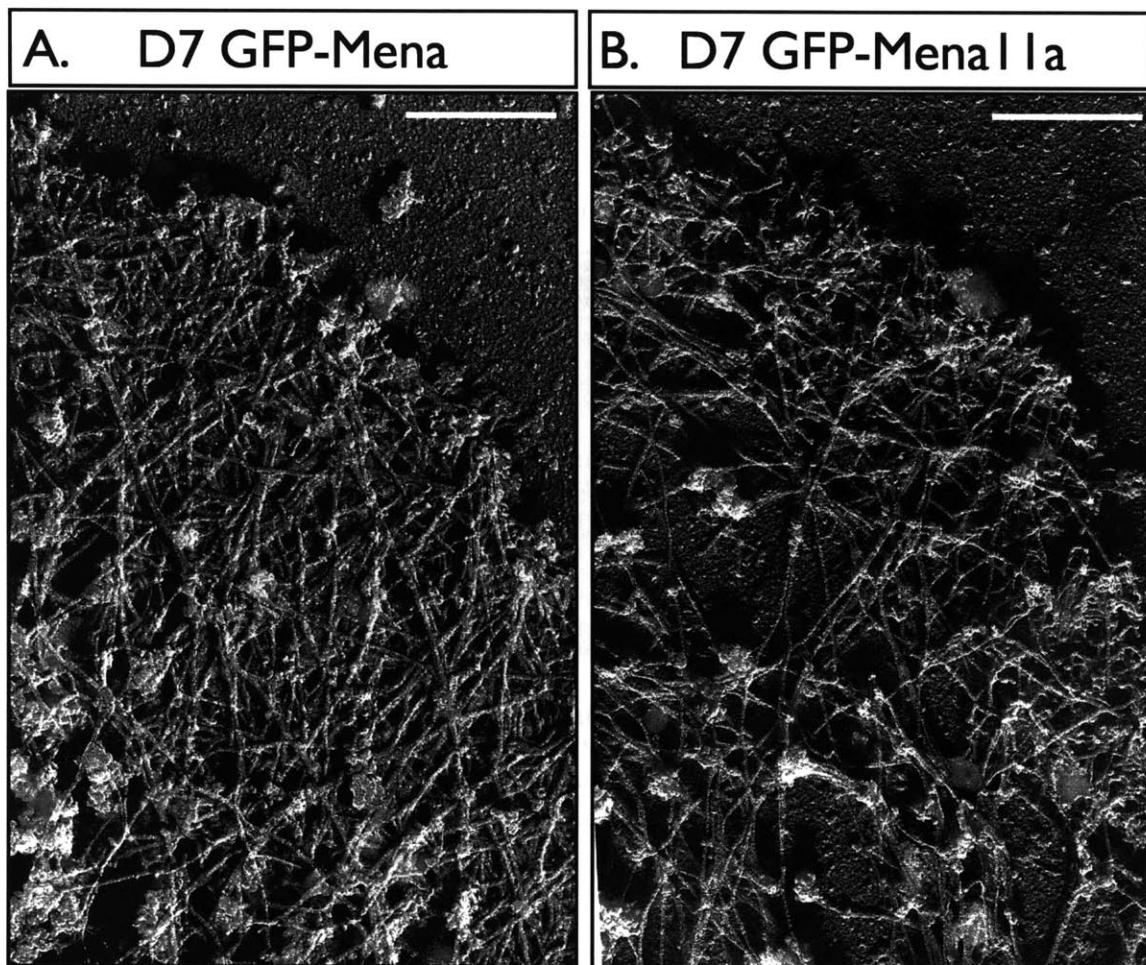
Figure 3-8: Mena11a expression reduces F-actin branching and density
A: MVD7 expressing GFP-Mena B: MVD7 expressing GFP-Mena11a has abnormal gaps in F-actin immediately following the leading edge of actin and lacks F-actin branching. Scale bar 100nm.

3.5 mDia and Ena/VASP in filopodia formation and dynamics

Despite the number of well-characterized molecules that play a role in filopodia formation and maintenance, their biological functions and mechanisms of assembly are still unclear (Mattila and Lappalainen, 2008). Ena/VASP proteins promote filopodia formation by antagonizing the activity of capping proteins and enhancing filament polymerization (Barzik et al., 2005; Bear et al., 2002). In addition, formins have recently emerged as potent inducers of filopodia formation and important regulators of the actin cytoskeleton. Formins are characterized by the presence of the formin homology domains FH1 and FH2. Diaphanous related formin (DRF) mDia2, and related mDia1, also have FH1 and FH2 domains, which are responsible for actin nucleation, barbed-end binding, and anti-capping protection *in vitro* (Pruyne et al., 2002; Sagot et al., 2002; Zigmond et al., 2003). mDia2 initiates filopodia formation by nucleating actin filament formation while remaining associated with the barbed end, which permits the rapid addition of actin subunits and promotes elongation (Kovar et al., 2006; Zigmond et al., 2003).

It is possible that different filopodia structures, dynamics, and functions are suited to meet the needs of particular cell types. There are a diverse set of proteins that are localized to filopodia tips and known to be important for their formation and maintenance. The morphology and dynamics of membrane protrusions can be altered by regulation of actin filament length and filament stability, density, and organization (Bear and Gertler, 2009). However, the effect of individual actin-associated proteins on the characteristics of filopodia is unknown. In this study we sought to determine how the

differential expression of Ena/VASP or mDia2 affects characteristics of filopodia formed by these proteins.

3.5.1 Results: VASP and mDia2 induce filopodia with distinct characteristics

To analyze features of filopodia assembly through distinct mechanisms, we chose to analyze VASP and mDia2 induced filopodia in the MVD7 fibroblast cell line. These cells lack Ena/VASP proteins and also express no detectable mDia1 or mDia2 (Barzik et al., in preparation).

Formins are maintained in an inactive conformation and activated by Rho GTPases which can disrupt this intramolecular interaction resulting in activation (Rose et al., 2005). mDia2 is autoinhibited between the N-terminal diaphanous inhibitory domain (DID) and the C-terminal diaphanous autoregulatory domain (DAD). Expression of full-length mDia2 does not induce filopodia formation in MVD7 cells, likely because it is not activated (Li and Higgs, 2005). A constitutively active mDia2 mutant in which is a Met-Ala mutation in the DAD relieves the intramolecular inhibition without activation by RhoGTPases induces numerous filopodia in MVD7 cells (Wallar et al., 2006). To simplify the system, all of the experiments in the following section were carried out with the constitutively active isoform of mDia2.

Although, MVD7 cells rarely form filopodia, transfection of VASP or mDia2 resulted in an increase in the number of filopodia formed. mDia2 expression resulted in a significant increase in the number and length of filopodia when compared with expression of VASP. The actin bundling protein fascin is used as a canonical marker for filopodia, distinguishing them from other structures such as retraction fibers. As seen in Figure 3-9, filopodia formed in MVD7 cells, MVD7 cells expressing GFP-VASP, or

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GFP-mDia2 all contained fascin (Barzik et al, in preparation). This result indicates that VASP and mDia2 can independently form filopodia and do not need to synergize for filopodia initiation.

Although the filopodia from all three conditions contained fascin, they had very different morphologies and dynamics. VASP filopodia protruded steadily from the lamellipodium leading edge, and were straight and rarely kinked. In contrast, mDia2 filopodia dynamics were highly irregular, protruding from all sides of the cell and not just from the leading edge. These filopodia often bent and collapsed backwards onto the lamellipodia. Differences in filopodia number, length, and dynamics between GFP-VASP and GFP-mDia expressing cells prompted us to analyze the actin ultrastructure. Platinum-replica electron microscopy analysis showed that filopodia in MVD7 cells or GFP-VASP expressing cells contained thick bundles of actin filaments deeply embedded within the lamellipodial network (Figure 3-9A,B'). In contrast, mDia2 filopodia were formed from bundled actin incorporated from the periphery of the actin cytoskeleton and were not anchored into the lamellipodium network (Figure 3-9C'). These differences in filopodia architecture may explain why VASP filopodia are steady and straight while mDia filopodia are highly unstable and often bent.
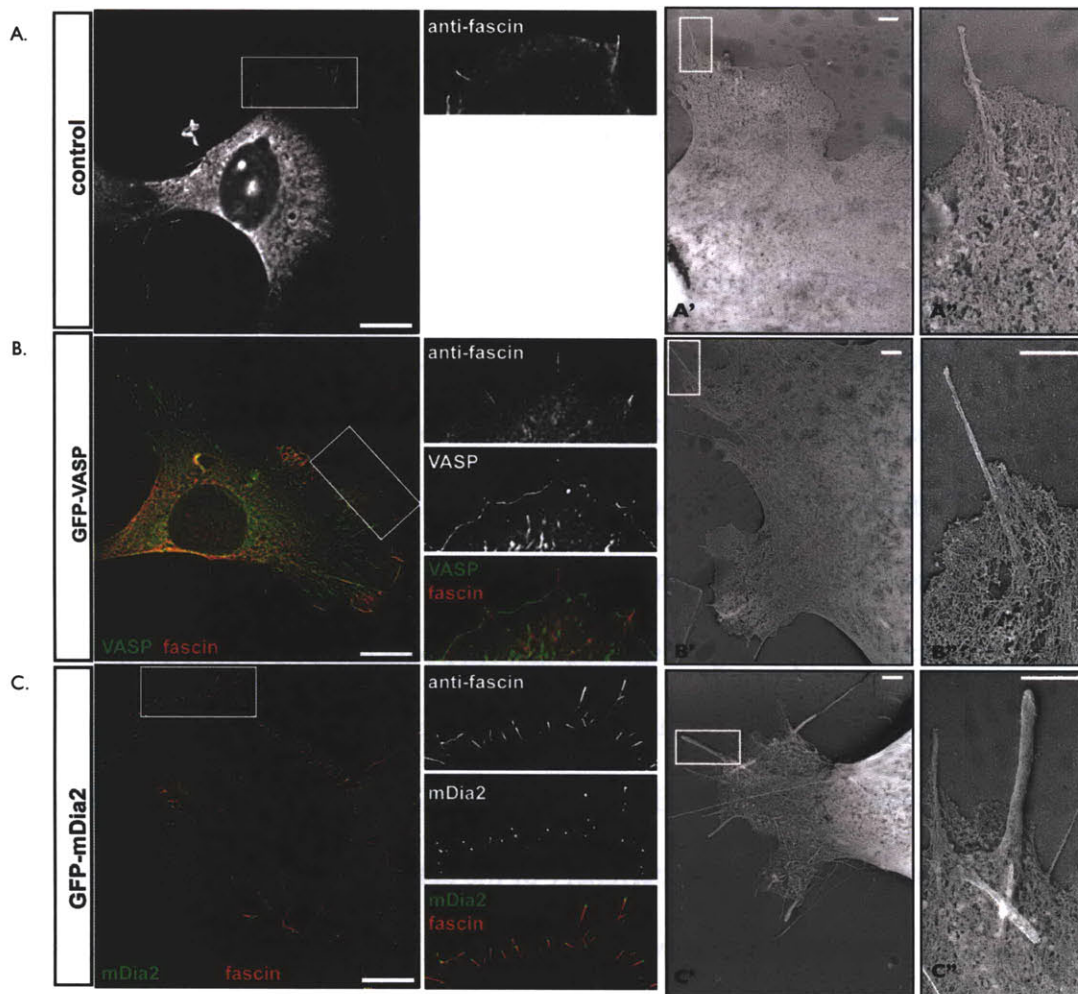
Figure 3-9: Filopodia formed by VASP or mDia2 contain physiological markers while filopodia initiated by mDia2 are not anchored in the lamellipodium actin networks.

A-C: MVD7 cells or MVD7 cells expressing GFP-VASP or GFP-mDia contain filopodia that are bundled by fascin. Platinum-replica electron microscopy analysis of actin cytoskeletons and filopodia. A: Filopodia in control MVD7 cells or B: cells expressing GFP-VASP contained filopodia that were deeply embedded within the lamellipodial network. C: Filopodia formed in MVD7 cells expressing GFP-mDia2 were characterized by thick actin bundles that were not anchored in the deeper lamellipodium. Scale bar in left panel represent 15um. Scale bars on right represent 1um.

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Filopodia are known to promote initial focal contacts with the substrate and, therefore, play a substantial role in cell spreading (Partridge and Marcantonio, 2006). Since VASP and mDia2 filopodia differed in their dynamics, we evaluated their ability to initiate cell spreading on laminin. MVD7 cells and cells expressing GFP-VASP exhibited comparable spreading efficiency after 30 minutes and had a similar morphology (Figure 3-10A,B), while MVD7 cells expressing mDia2 cells had a significantly reduced surface area (Figure 3-10D). After 30 minutes of spreading mDia expressing cells had a large number of long filopodia but no lamellipodia (Figure 3-10C). The high number of filopodia did not delay spreading, though spreading was hindered by the inability of mDia2 filopodia to support the formation of focal adhesions contacts during cell spreading (Barzik et al, in preparation).
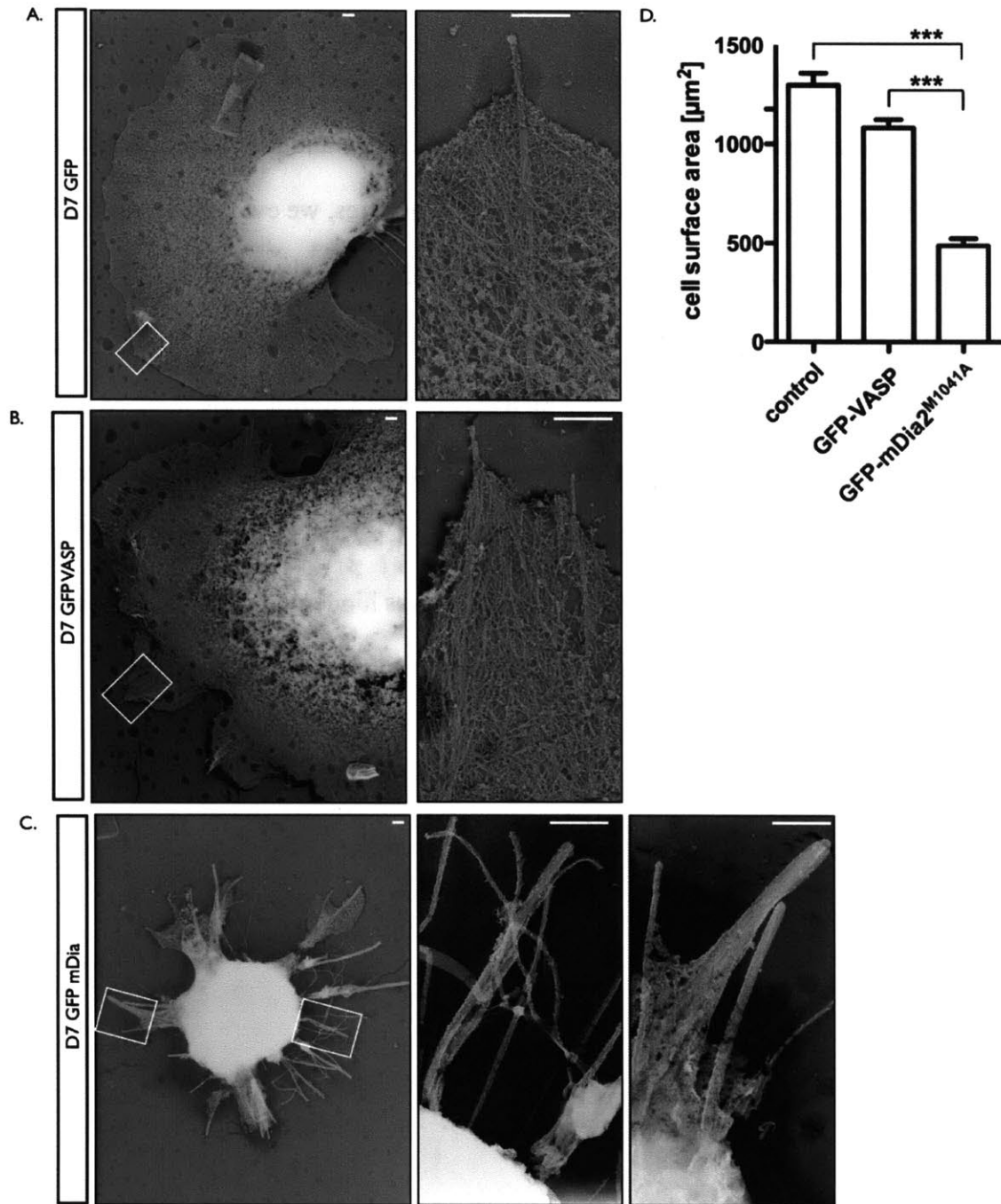
Figure 3-10: GFP-mDia2 expression delays initial cell spreading events.
A-C: Cells were allowed to spread on laminin-coated coverslips for 30min and fixed. A and B: MVD7 or MVD7 expressing GFP-VASP form circumferential lamellipodium with some filopodia. C: MVD7 expressing GFP-mDia2 form only a few lamellipodia-like structures and many filopodia that are not anchored in an actin network. D: Quantitation of cell surface area after 30 minutes of spreading. Scale bars represent 1um.

3.6 Conclusions

Currently we have a broad outline of the mechanisms various cell types use to produce asymmetry and motility, but gaining a deeper understanding of coordinated movement will depend on new insights into the integration of the various signals used to produce directional persistence. In this chapter, several actin-binding proteins that influence the actin ultrastructure were characterized by their ability to support physiological processes through the regulation F-actin structures.

Ena/VASP null neurons do not form axon tracts due to a lack of filopodia and a failure to form neurites. In cortical neurons, actin bundling and filopodia formation are critical early steps in neurite formation that can be regulated by many factors. We have shown that filopodia actin-bundle architecture itself is an essential component of neurite initiation, rather than some other Ena/VASP function, because other pathways that form filopodia-like extensions can rescue a block in neuritogenesis. It has been previously suggested that filopodia extensions are the structural basis for neurite formation, and our work directly supports this hypothesis (da Silva and Dotti, 2002; Dehmelt and Halpain, 2003)

High numbers of filopodia are considered characteristic of an invasive cancer cell (Vignjevic et al., 2007). Mena is upregulated in human breast cancer, as well as pancreatic, colon, gastric and cervical cancer (Gertler and Condeelis, 2011). The Mena splice isoform, Mena11a, is only expressed in epithelial tissues. By analyzing the molecular differences in Mena11a compared with other Mena isoforms, we have gained insight into the control of cell motility in cancer. Since Mena11a appears to negatively

regulate the formation of F-actin structures, our results may be useful in the development of new methods to reduce the severity of metastatic cancers.

This study of VASP filopodia and mDia filopodia has shown that distinct mechanisms of filopodia formation give rise to structures with different properties, dynamics, and functions. This is supported by the EM micrographs, which provide strong evidence that VASP induced filopodia form through the convergent elongation model, and mDia induced filopodia form by de novo filopodia nucleation and elongation. Filopodia formed through different mechanism and by different molecules can have different biological functions. While mDia filopodia hindered early cell spreading events, VASP filopodia supported this process. Although filopodia assembly can be achieved through a limited number of actin-binding proteins, their biological function likely requires the coordinate regulation of many molecules in this structure.

3.7 Materials and Methods

Antibodies and Reagents
The following antibodies and reagents were used in ths study: β-III tubulin rabbit polyclonal (Promega), AlexaFluor Phalloidin (Invitrogen), mouse clone 55K-2 anti-human fascin (DakoCytomation), mDia2 and constitutively active mDia2M1041A were a gift from A. Alberts (Van Andel Research Institute, Grand Rapids, MI)

Cortical neuron culture and transfection
Cortical neuron cultures were prepared from embryonic E14.5-E16.5 mice as previously described (Kwiatkowski et al., 2007). Cortices were dissected, trypsinized and dissociated. Neurons were initially cultured in 5% FBS (Hyclone) in Neurobasal Medium (GIBCO) and switched to serum free media supplemented with B27 (GIBCO) after 2 hrs in culture. Neurons plated on glass and plastic were coated with 0.2-1mg/ml poly-D-lysine (Sigma) at 5000 cells/cm^2.

Immunofluorescence
Cortical neurons and MVD7 cells were cultured as previously described (Kwiatkowski et al., 2007) and (Bear et al., 2000). Cells were fixed in 4% paraformaldehyde-PHEM (Strasser et al., 2004), rinsed with PBS, permeabilized with 0.2% Triton-X100, and blocked with 10%BSA/PBS. The samples were then incubated with primary antibody for one hour, washed 3 times with PBS and incubated with fluorescently labeled secondary antibody at 1:500 (Millipore) and fluorescently coupled phalloidin for one hour. Images were collected on a Deltavision microscope and deconvolved using Softworxs software (Applied Precision).

Platinum Electron Microscopy
Correlative Electron Microscopy was performed as previously described (Svitkina et al., 1995). Cortical neurons or D7 cells were cultured, as described previously, on coverslips coated with a gold locator grid. EGFP-positive cells were located by live cell florescence microscopy then immediately extracted for 4.5 min with 1% Triton X-100 in PEM buffer (100 mM PIPES, pH6.8, 1 mM EGTA, 1 mM MgC12) containing 10 uM phalloidin, 0.2% gluteraldehyde, and 4.2% sucrose as an osmotic buffer. Coverslips were washed with PEM containing 1 uM phalloidin, and 1% sucrose, fixed in 0.1 M Na-cacodylate buffer (pH 7.3), 2% gluteraldehyde, 1% sucrose and processed for electron microscopy. Cells previously identified as EGFP-positive were relocated using the gold grid for micrographs.

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Chapter 4

Conclusions and Future Directions

Conclusions Table of Contents

4.1 Trim9 regulates Netrin-1 induced branching

During central nervous system development cortical axons extend collateral branches to connect to multiple synaptic targets. Once formed, axon branches can extend independently of the primary axon, suggesting that individual branches respond differently to guidance cues (Dent, 2004). Over the past 20 years our knowledge of guidance cues, receptors, and downstream signaling cascades has greatly advanced. Most of the guidance signaling studies have focused on better understanding of axon outgrowth and pathfinding, while axon branching, an essential step in nervous system development, is not well defined.

At the onset of my research into the role of Trim9 in response to netrin, many components of the DCC signaling pathway had been discovered. It was known that Ena/VASP proteins were necessary for filopodia induction in response to netrin (Lebrand et al., 2004) and the same year, Map1b was identified as an important microtubule associated protein required for chemoattraction in response to netrin (Del Río et al., 2004) but the pathways that regulated both of the responses were unidentified. A few years later, it was demonstrated that Myosin-X was also needed for netrin induced outgrowth and this molecular motor redistributed the DCC receptor in response to this cue (Zhu et al., 2007). I found that the novel E3 ubiquitin ligase Trim9, binds the cytoplasmic tail of DCC, and Ena/VASP proteins. This work is the first description of a molecular link between the DCC receptor and Ena/VASP proteins, and describes a novel signaling complex that we hypothesize coordinates actin and microtubule dynamics during branching in response to netrin.

In *C. elegans*, the Trim9 ortholog MADD-2 was identified as a cytoplasmic co-factor necessary for branching and attractive axon guidance in response to UNC-6/netrin through the receptor UNC-40/DCC and had no effect on repulsion from SLT-2/slit through its receptor SAX-3/Robo. Ectopic expression of MADD-2 in neurons that express UNC-40/DCC, but do not usually respond to UNC-6/Netrin results in axons turning toward UNC-6/Netrin (Hao et al., 2010). Using an *in vitro* branching assay in mouse cortical neurons, I found that Trim9 has a conserved role in branching in response to netrin. Assessing Trim9's role in branching *in vivo* proved technically challenging, therefore, future work using *in utero* electroporation of the ΔRingTrim9 construct into cortical neurons is needed to test whether blocking Trim9 function could cause defects in branching in the context of an animal. We are also in the process of creating Trim9/Trim67 double knockout mice. My *in vitro* branching defect should reflect phenotypes in these mice.

In another *C. elegans* system, MADD-2 is essential for the formation of membrane protrusions in response to UNC-6 through UNC-40 during the formation of muscle arms, the postsynaptic membrane of the neuromuscular junction (Alexander et al., 2010). The authors hypothesized that the major role for MADD-2 is to facilitate an interaction between UNC-40 and the guanine nucleotide exchange factor (GEF) UNC-73/Trio. Interestingly, MADD-2 acts as a scaffold between UNC-40 and UNC-73/Trio and its ubiquitin ligase activity is necessary for proper muscle arm formation (Alexander et al., 2010). Trio knockout mice have impaired Rac activation and cortical neurons fail to extend neurite in response to netrin, suggesting this GEF is also important in mammalian nervous system development (Briançon-Marjollet et al., 2008). Since Trim9

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plays an analogous role to MADD-2 during neuronal branching, it would be interesting to determine if Trim9 plays an analogous role to MADD-2 in formation of the neuromuscular junction. Since it is not known whether Trim9 binds Trio in vertebrates, an interaction between Trim9 and Trio could be assessed experimentally through a direct binding assay, using purified proteins or a co-immunoprecipitation for cortical lysate.

Using a yeast two hybrid assay, several biochemical assays and immunocytochemistry, I have shown Trim9 interacts with Ena/VASP proteins and the cytoplasmic tail of DCC. Although it has been previously established that Ena/VASP proteins are downstream regulators of actin remodeling in the Netrin/DCC pathway, a direct interaction between Ena/VASP proteins and the cytoplasmic tail of DCC has not been demonstrated (Gitai et al., 2003; Lebrand et al., 2004; Yu et al., 2002). My work on Trim9 provides the first evidence for a direct molecular link between Ena/VASP proteins and DCC. I have found that Trim9 binds DCC via its C-terminal SPRY domain while binding Ena/VASP EVH1 via its BBox-Coiledcoil domain. It is still unclear how this protein complex is regulated before and after netrin stimulation. To assess a change in complex formation, I will use a parallel approach of quantitative immunoblotting and immunolabeling, before and after netrin treatment to assess the status of the complex of proteins associated with the cytoplasmic tail of DCC.

Myosin-X is an unconventional molecular motor that can induce filopodia, traffic netrin receptors and β-integrins (Divito and Cheney, 2008). Through co-immunoprecipitation and immunocytochemistry I have demonstrated that Trim9 binds Myosin-X. This unconventional myosin had been previously shown to bind Ena/VASP proteins and the cytoplasmic tail of DCC. Myosin-X is the only identified protein that

displays intrafilopodial motility. Interestingly, I found Trim9 also displays intrafilopodial

motility. It has been previously shown that Myosin-X is expressed at very low levels

early in cortical development *in vitro* and *in vivo* (Dent et al., 2007; Zhu et al., 2007).

Although expressed at low levels, this motor protein is required for outgrowth in response

to netrin by trafficking the DCC receptor to the membrane. Currently it is unclear if

Myosin-X plays a role in branch formation in concert with Trim9. To address this one

could deplete the protein or express a dominant negative form of the motor protein that

would block Mysin-X receptor trafficking to determine if netrin induced branching is

blocked.

The earliest steps in branching involves focal activation of signaling pathway with

in the axon (Gallo, 2011). Given the low levels of Myosin-X in cortical neurons, it is

possible that the complex of Trim9, Myosin-X, Ena/VASP and DCC is only present in a

subset of filopodia along the axon shaft and netrin stimulation results in branch formation

only in these areas. It will be interesting to overexpress Myosin-X in cortical neurons to

determine if there is an increase in branching.

Map1b is a microtubule-associated protein (MAP) that confers microtubule

stability and can also bind actin. This MAP is necessary for outgrowth in response to

netrin and Map1b knockout mice have several developmental defects similar to netrin

knockout mice (Bouquet et al., 2007; Del Río et al., 2004). It has been published that

Map1b is regulated by the ubiquitin proteasome system but the E3 ligase that mediates

Map1b degradation is unclear (Allen et al., 2005). I recovered Map1b in a mass

spectroscopy screen for proteins that would bind ΔRingTrim9. Deletion of the RING

domain of E3 ubiquitin ligases allows for a stable interaction with substrates that would

normally be degraded (Tursun et al., 2005). The substrate or substrates of Trim9's ubiquitin ligase activity are unknown and I will determine if Map1b is substrate of Trim9. Recently, the specific E1 enzyme and E2 enzyme Trim9 couples its E3 activity with have been determined (Tanji et al., 2010). An *in vitro* ubiquitin transfer assay can be used to determine if purified E1, E2, and Trim9 E3 facilitates the transfer of ubiquitin to purified Map1b. In addition, overexpression of full length Trim9 should reduce Map1b levels in cultured cortical neurons. If Trim9 is targeting Map1b to the proteasome in neurons where Trim9 has been ectopically expressed, treatment with proteasome inhibitors will rescue a reduction in Map1b protein levels. Expression of ΔRingTrim9 should also lead to an accumulation of Map1b protein. Since focal destabilization of the microtubule along the axon shaft is necessary for branch formation, abnormal accumulation of Map1b in ΔRingTrim9 expressing neurons potentially explains the cause of the branching defect in response to netrin.

It has been reported that in mouse dorsal root ganglia, loss of Map1b results in excessive branching suggesting that the levels of this protein must be regulated for proper neuron morphology to develop. In the central nervous system, Map1b null mice have defects in major commissures, such as the corpus callosum, similar to the netrin knockout mice. The Map1b null mice also have a reduced response to netrin chemoattraction (Meixner et al., 2000). We hypothesize that Trim9 is an ubiquitin ligase for Map1b, and therefore Trim9/Trim67 double knockout mice will have phenotypes similar to Map1b null mice.

Several guidance cues stimulate axon branching in cortical neurons. Expression of ΔRingTrim9 results in a block of branching in response to netrin but not to FGF-2. This

suggests that Trim9 plays a specific role in netrin induced branching. It has been previously shown that netrin can induce branches from filopodial protrusions along the axon shaft but not from the growth cone cytoskeleton (Dent, 2004). In contrast, FGF-2 treatment leads to branch formation from a paused growth cone (Szebenyi et al., 2001). Both netrin and FGF-2 induced branching require dynamic remodeling of the actin and microtubule cytoskeleton, and it is likely achieved by two different mechanisms. Actin remodeling downstream of FGF-2 signaling involves activation of the secondary messenger PKC and GAP-43, while netrin signals through the secondary messenger PKA and Ena/VASP (Lebrand et al., 2004; Leu et al., 2010). After FGF-2 treatment, there is an upregulation of microtubule severing proteins Spastin and Katanin, which results in short, dynamic microtubules (Qiang et al., 2010). We hypothesize that in response to netrin Trim9 targets Map1b for proteasomal degradation and thus influences microtubule stability in the axon shaft.

Branch formation requires microtubule exploration into regions of dynamic actin protrusions (Dent and Kalil, 2001). Filopodia formation along the axon shaft and requires Ena/VASP activity in response to netrin (Lebrand et al., 2004). Myosin-X is required for transport of DCC to the membrane and possibly for stabilizing filopodia or extracellular matrix adhesions (Zhang et al., 2004). Since Trim9 binds Ena/VASP, Myosin-X and the cytoplasmic tail of DCC, I hypothesize that Trim9 might act as a scaffold for these proteins. In addition, to accomplish branch formation, Trim9, may also function as an ubiquitin ligase and target Map1b to the proteosome, resulting in the degradation of the microtubule stabilizing protein allowing dynamic microtubules to explore actin protrusions and ultimately stabilize into a branch.
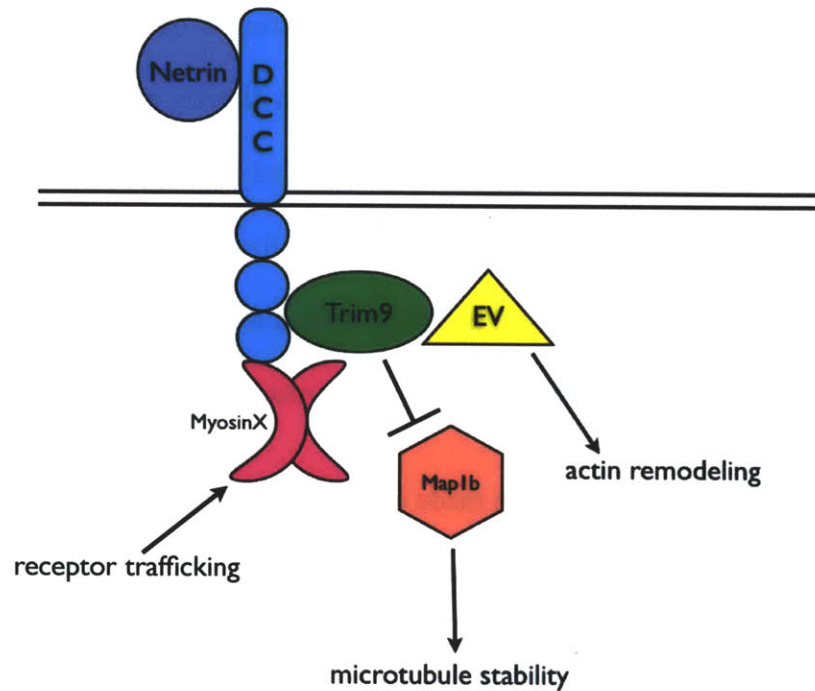
Figure 4-1 Hypothetical Model for Trim9 Signaling
Trim9 acts as a scaffold for Ena/VASP and potentially Myosin-X binding the cytoplasmic tail of DCC resulting in proper receptor localization and actin protrusions in response to netrin. Trim9 also poly-ubiquitin tags Map1b resulting in a reduction in Map1b levels and an increase in microtubule dynamics into the actin protrusions.

4.2 Trim9, a novel Ena/VASP binding partner

I found that Trim9 is a novel Ena/VASP EVH1 binding partner that surprisingly does not contain the proline-rich motif with the consensus sequence (D/E)FPPPPX(D/E)(D/E) (abbreviated FP4), a conventional Ena/VASP binding motif (Krause et al., 2003). Tes, a tumor suppressor that localizes to focal adhesions is the only one other unconventional Ena/VASP binding partner known to bind EVH1 without an FP4 motif (Boëda et al., 2007). The Tes LIM3 domain completes for EVH1 binding and therefore regulates the cellular localization of Mena but not VASP or Evl. The LIM3

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domain has several conserved cysteine/histidine residues coordinated by 2 zinc atoms much like a BBox domain of Trim9. It will be interesting to determine using a direct binding assay if the purified Trim9 BBox domain can directly bind the Ena/VASP EVH1 domain and if this interaction completes with a purified FP4 motif. The affinity of this interaction could result in changes in Ena/VASP localization and could potentially provide a mechanism by which focal points of branch formation are occurring.

4.3 Regulation of neuronal cytoskeleton via the ubiquitin proteasome system

In the past decade it has been made clear that the ubiquitin proteasome system is a powerful means of regulating circuit development (Yi and Ehlers, 2007). MID1(Trim18) is a closely related TRIM protein to Trim9 and mutations in this gene result in a human disorder called Optiz Syndrome, which is characterized by defects in midline structures throughout the body (Schweiger and Schneider, 2003). MID1 targets Protein Phosphtase 2a (PP2a) for degradation, and loss of MID1 ubiquitin ligase activity or aberrant localization of MID1 results in hypophosphorylation of microtubule MAPs and defects in nervous system development (Trockenbacher et al., 2001). Similarly, the ubiquitin ligase Nedd-4 binds the phosphatase PTEN (phosphatase and tensin homolog) and regulates PTEN protein levels through the ubiquitin proteasome system. A reduction in PTEN results in increased PI3K signaling and axon branching. However, without the activity of Nedd-4 PTEN blocks branching pathways (Drinjakovic et al., 2010). Nedd-4 also influences dendrite branching formation through the mono-ubiqutination and inactivation Rap2. In our model, the ubiquitin ligase Trim9 links guidance receptor activation with the proteins necessary for actin protrusions, and potentially regulates Map1b levels to influence microtubule stability in the axon shaft.

4.4 Role of Ena/VASP in the developing cortex

Ena/VASP proteins are necessary for neurite formation in cortical neurons and a loss of Ena/VASP proteins results in a block in neurite formation leading to a failure in axon formation (Dent et al., 2007; Kwiatkowski et al., 2007). We demonstrated that filopodia were necessary for neurite formation and that pathways resulting in filopodia-like F-actin bundles can bypass the requirement for Ena/VASP proteins. In a later study, it was determined that neurite formation requires both actin dynamics and exocytosis. During neurite formation and depending on the extracellular matrix, one of two independent exocytosis pathways was used to drive membrane delivery. One pathway worked through Ena/VASP proteins and Vamp2, while the second occurred in the presence of laminin through the coordination of Arp2/3 and Vamp7 (Gupton and Gertler, 2010). The underlying actin cytoskeleton in these two modes of exocytosis presumably has very different actin architecture based on the differences in Ena/VASP and Arp2/3 activity. Future work is needed to determine if the architecture of the actin cytoskeleton can directly influence vesicle trafficking and if proteins that modulate actin structures also regulate membrane or membrane cargo delivery.

Neurite initiation requires microtubule extension into filopodia and blocking of microtubule dynamics blocks neurite formation (Dent et al., 2007). I found a novel Ena/VASP localization at microtubules. I hypothesize that Ena/VASP directly binds microtubule associated proteins, such as Map1b, to can help coordinate actin and microtubule dynamics. In addition, Ena/VASP only localizes to microtubules within large paused growth cones. In cortical slice culture large paused growth cones were observed in the corpus collosum, where axons respond to multiple guidance cues and initiate

branches. It is likely there is a greater need for cross-talk between the actin and microtubule cytoskeleton in these situations. It would be interesting to determine what binding partners localize Ena/VASP to microtubules and in the case it is Map1b to determine the purpose of this interaction.

4.5 Filopodia formation and function

Decades of research into actin dynamics has resulted in the identification of key set of proteins important for formation and maintenance of filopodia across multiple cell types (Mattila and Lappalainen, 2008). However, the relative significance of each of these proteins has on filopodia formation varies, depending on the context. Recent progress has uncovered many proteins associated with filopodia formation resulting in new mechanistic models of filopodia formation and elongation (Gupton and Gertler, 2007). Filopodia formed downstream of Ena/VASP proteins and mDia differ in their dynamics and structure, partly due to the mechanism by which the filopodia are formed. In MVD7 cells, Ena/VASP filopodia are rooted deep in the network of lamellipodia, which is characteristic of filopodia formed by convergent elongation. mDia filopodia, however, appear to initiate directly out of the peripheral actin filaments suggesting *de novo* filopodia formation. Future work will be needed to identify all filopodia components and further characterization of these molecules will provide insight into the mechanism of filopodia formation, their capacity as cell sensors, and their ability to initiate adhesions.

4.6 References

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Appendix 1

Ena/VASP proteins at microtubules

Appendix Table of Contents

## A.2 Introduction

Ena/VASP proteins are found in many cell types, however, they are highly enriched in the nervous system. In neurons, Ena/VASP proteins are most prominent in filopodia tips though they are also at the leading edge of lamellipodial veils. Ena/VASP localization can also depend on the presence of EVH1 binding partners, such as the Robo guidance receptor or Lamellipodin Ras effector protein (Michael et al., 2010; Yu et al., 2002). Lamellipodin is known to recruit Ena/VASP proteins to specific sites in the cell through its EVH1 binding motifs (Krause et al., 2003).

Localization of cytoskeletal binding proteins influences the structure and dynamics of cell protrusions. It is increasingly clear that actin and microtubules cannot act alone and many studies have provided strong evidence of cross-talk between the two cytoskeletal components (Dent and Kalil, 2001; Schaefer et al., 2002). There are a number of proteins known to link actin and microtubules reorganization but the exact mechanisms that regulate these interactions are just beginning to be determined (Rodriguez et al., 2003).

A.3 Results

Trim9 contains putative microtubule binding motifs and we sought to determine if Trim9 localized to microtubules in cortical neurons. Using a fixation protocol specific for visualizing microtubule-associated proteins, we instead discovered that a population of neurons had actin-binding Ena/VASP proteins localized to microtubules. Interestingly fibroblasts on the same coverslip did not have Ena/VASP localized at the microtubules but rather at the leading edge of filopodia and lamellipodia (Figure A-1). This suggests that fundamental differences in these cells lead to differences in Ena/VASP localization.
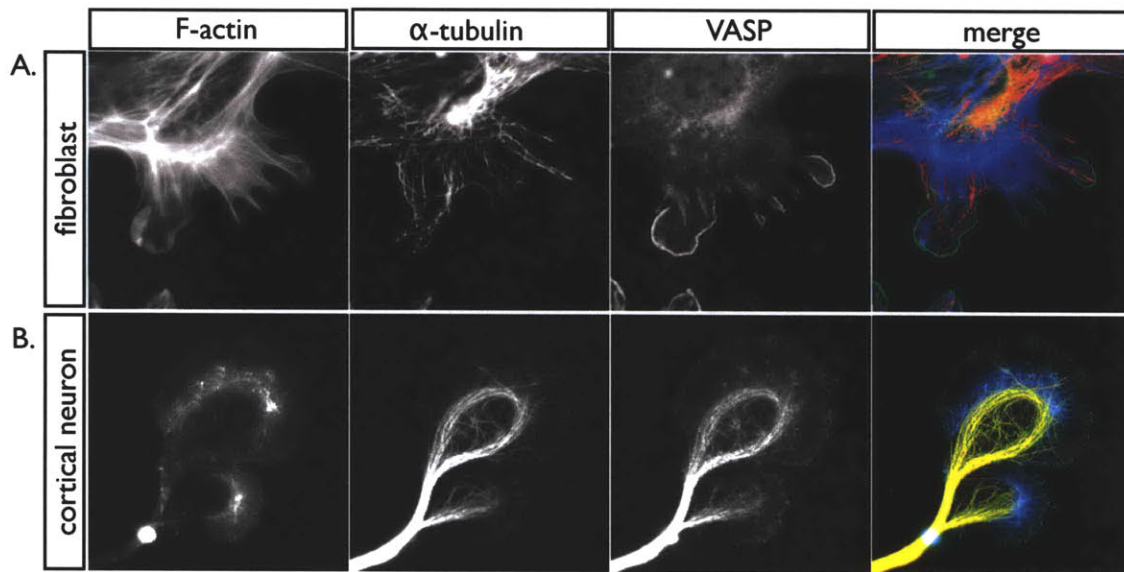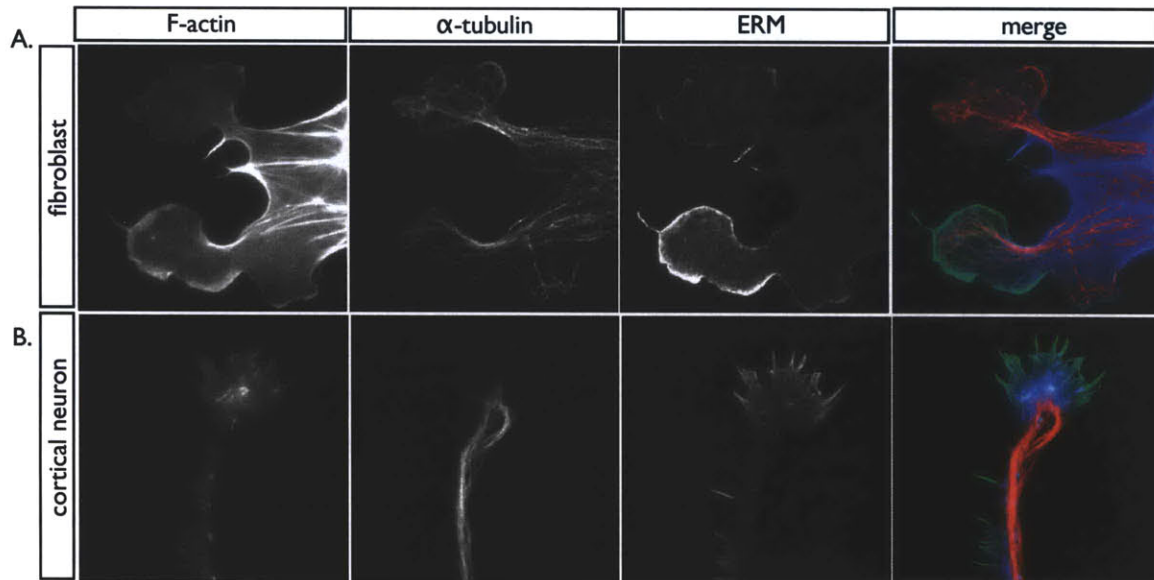

Figure A-1: Ena/VASP proteins localize to microtubule in neurons
A: Menegial fibroblast cultured with cortical neurons shows expected VASP staining at the leading edge of lamellipodia. B: In a population of cortical neurons VASP co-localizes with microtubules and the tips of filopodia.

The ERM protein family consists of three proteins ezrin, radixin and moesin that are enriched in the nervous system and function to link actin filaments with the membrane (Arpin et al., 2011). These proteins localize to the tips of filopodia in the

growth cone, as seen in Figure A-2, yet do not co-localize with microtubules using the same fixation conditions as in Figure A-1.


Figure A-2: Other actin-associated proteins in the growth cone do not localize to microtubules
A: Menegial fibroblast shows ERM proteins localize to the leading edge of lammelipodia B: ERM proteins localize to the tips of filopodia but do not co-localize with microtubules

Multiple antibodies recognize Ena/VASP proteins at microtubules: VASP polyclonal antibody, Mena polyclonal antibody and Mena monoclonal antibody (Figure A-3). We found that Ena/VASP proteins localize to microtubules in 8% of neurons. All of the neurons observed with Ena/VASP at microtubules had large paused growth cones and Ena/VASP proteins specifically localized to C domain microtubules. This small population of neurons in the cortex may have a specialized role in guidance or represent a signaling state that occurs during guidance.

| F-actin | α-tubulin | Mena (monoclonal) | merge |

A.

B.
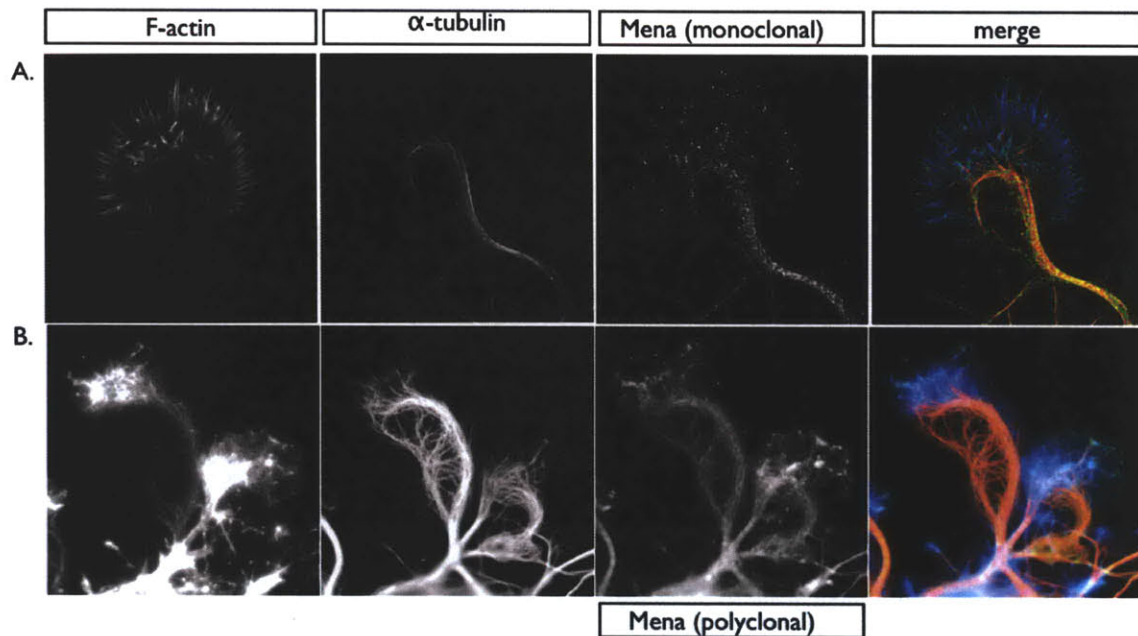
Mena (polyclonal)

Figure A-3: Polyclonal and Monoclonal antibodies to Mena localize to microtubules
A-B: In cortical neurons Mena co-localizes with microtubules A: Immunocytochemistry with
Mena monoclonal antibody. B: Immunocytochemistry with Mena polyclonal antibody.

A mass spectrometry screen for novel Ena/VASP binding partners uncovered a

possible interaction with the microtubules associated protein Map1b, which can localize

to both actin and microtubules structures. In addition, Ena/VASP proteins and Map1b co-

localize at stable C domain microtubules (Figure A-4). It is possible that an interaction

between these two proteins could recruit a population of Ena/VASP to microtubules.

| F-actin | Mena | Map1b | merge |
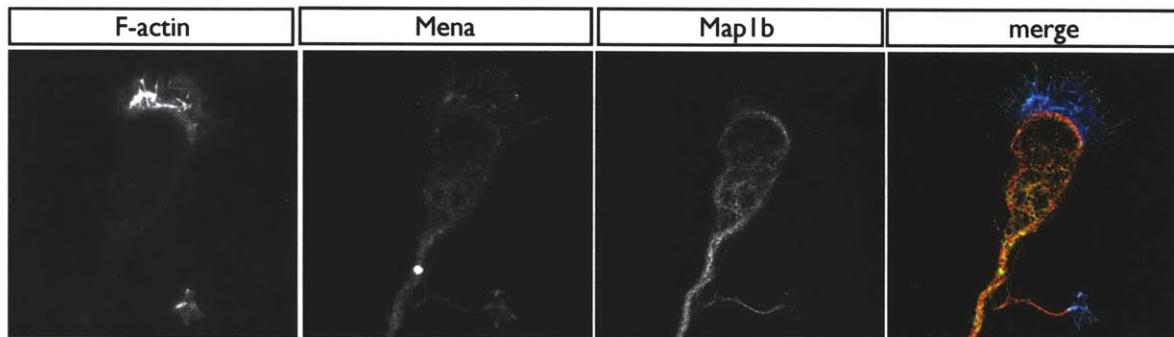
Figure A-4: Mena and Map1b co-localize at microtubules
Mena and Map1b co-localize at microtubules in cortical neurons.

A.4 Conclusion

Studies in brain slices and organotypic cultures have shown that over many hours of development, growth cones in different regions of the corto-callosal pathway have remarkably different behaviors (Halloran and Kalil, 1994). Growth cones advancing toward the corpus collosum rapidly undergo continual shape changes, do not make turns, and often extend past cortical targets. In contrast, once the axon has crossed the corpus collosum, growth cones just beneath and approaching cortical targets have elaborate morphologies characterized by large paused growth cones which undergo repeated cycles of collapse, withdrawal, and resurgence. It is thought that these behaviors reflect recognition of cortical target signals and the dramatic changes in the cytoskeleton result in the formation of precise synaptic connections (Kalil et al., 2000).

Ena/VASP co-localizes with C domain microtubules only in large paused growth cones. Future work will determine if this localization is due to an interaction with Map1b downstream of a particular guidance cue or an extracellular matrix cue. The coordination of microtubule and actin dynamics is critical to neuronal development, and there are a growing number of proteins that regulate both of these cytoskeletal components. Identification of more proteins that coordinate cytoskeletal crosstalk may identify master regulators in growth cone pathfinding.

A.5 Materials and Methods

Antibodies and Reagents

The following antibodies were used in this study: anti-Mena Monoclonal (generated in Gertler lab), anti-VASP rabbit polyclonal (generated in Gertler lab), anti-Mena rabbit polyclonal (generated in Gertler lab), anti-ERM (clone 13H9 gift from F. Solomon, MIT) anti-map1b (clone13725 from Lifespan Biosviences), anti- α-tubulin monoclonal (clone DM1A Sigma), and anti-tyrosinated tubulin rat monoclonal (Millipore). Alexa Fluor Phalloidin 350, 488 or 647 (Invitrogen).

Cortical neuron culture

Cortical neuron cultures were prepared from embryonic E14.5-E16.5 mice as previously described (Kwiatkowski et al., 2007). Cortices were dissected, trypsinized and dissociated. Neurons were initially cultured in 5% FBS (Hyclone) in Neurobasal Medium (GIBCO) and switched to serum free media supplemented with B27 (GIBCO) after 2 hrs in culture. Neurons plated on glass and plastic were coated with 0.2-1mg/ml poly-D-lysine (Sigma) at 5000 cells/cm^2.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde-PHEM with 0.1% Trition-X100 (Strasser et al., 2004), rinsed with PBS, permeabilized with 0.2% Triton-X100, and blocked with 10%BSA/PBS. The samples were then incubated with primary antibody for one hour, washed 3 times with PBS and incubated with fluorescently labeled secondary antibody at 1:500 (Millipore) and fluorescently coupled phalloidin for one hour. Images were collected on a Deltavision microscope and deconvolved using Softworxs software (Applied Precision).

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