

A Lentiviral System for RNAi Transgenesis and the Ena/VASP Triple-Knockout Defines  
Neuronal and Non-Neuronal Functions in Mouse Development

by

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B.S. and M.S. Molecular Biophysics and Biochemistry  
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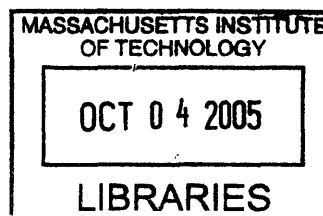
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Submitted to the Department of Biology on September 6, 2005 in Partial Fulfillment of the  
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ABSTRACT

Mammalian development extends and exploits signaling pathways that function exclusively in axon guidance in lower organisms. This emerging paradigm employs complex expression patterns of expanded protein families to achieve the complexity and specificity required in mammalian development. For example, the *Drosophila* axon guidance ligands, Netrin and Slit, have recently been implicated in the development of several mammalian organ systems. While the characterization of extra-neuronal functions of ligands and receptors has emerged, the conservation of intracellular signaling pathways remains unclear. The Ena/VASP protein family is a common downstream effector of multiple axon guidance signaling cascades. The analysis of the Ena/VASP triple-null mouse allows us to determine the extent to which these intracellular cascades have been conserved in the development of the mammalian nervous system as well as other organs. Within the nervous system, we have uncovered novel roles for Ena/VASP in the initiation of axon extension, guidance of non-commissural axons, and neuronal migration. Outside the nervous system, we have observed a novel role for Ena/VASP in blood vessel physiology. Interestingly, several developmental pathways for which axon guidance receptors have been implicated appear to develop normally in Ena/VASP triple-null embryos. Future work in Ena/VASP developmental biology will analyze the specific roles of Ena/VASP splice isoforms and unique functions of individual Ena/VASP family members. I have developed a lentiviral system for the creation of mouse transgenics including RNAi knockdowns that can be applied to address these questions.

Thesis Supervisor: Frank Gertler

Title: Associate Professor of Biology

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## BIOGRAPHICAL NOTE

Douglas Adam Rubinson was born at 11:55pm on Thursday the 12<sup>th</sup> of August, 1976. With his emergence a week past his due date, and a meager five minutes before Friday the 13<sup>th</sup>, Douglas initiated a lifetime of procrastination that has been punctuated by fortuitous luck.

Douglas's earliest forays into science included the near ubiquitous childhood chemistry project in which a variety of foodstuffs and household cleaning products are combined in ridiculous quantities and arbitrary proportions. The results were often colorful, frequently noxious, and invariably useless- thus presaging his experience in graduate school decades later. In 5<sup>th</sup> grade, a salt water tank housing an octopus was placed in Douglas's care. Despite fastidious attention to the salinity, pH and temperature of the tank, the octopus died within weeks of captivity. Such was Douglas introduced to the fickle nature of biological investigation.

As a high school student, Douglas pursued all manner of activities, many of which were in clear opposition to his natural skill set. These included serving on all manners of honor societies, committees, wrestling (126-142lbs. weight classes), soccer and business administration. The mixed successes Douglas experienced in his extracurricular pursuits were exceeded by the decidedly dreadful results in the realm of teenage romance. On Saturday mornings, Douglas's father drove him into Manhattan to attend science classes at Columbia University as part of the Science Honors Program. He graduated in June of 1994 as the Valedictorian, a National Merit Scholar, AP Scholar, and a USA Today Academic All-American Honorable Mention.

In the fall of 1994 Doug enrolled at Yale University with the intention of majoring in Molecular Biophysics and Biochemistry- having little conception what that was. Despite his initial ignorance, Douglas proved quite adept and became fascinated by his program of study. After his sophomore year he began a research project in the laboratory of Sandra Wolin studying the biogenesis of small RNAs. Sandra Wolin proved an extraordinary scientific mentor, and encouraged him to complete a Master's Thesis and pursue a future career as a physician scientist. Outside of the classroom Douglas was involved in political activism. Although his activism was primarily focused on environmental issues, his participation in a protest against Playboy Magazine® resulted in his naked appearance in the October 1995 issue of Playboy as part of their "Women of the Ivy League" issue. In his personal life, Douglas proved more successful in hedonistic pursuits than genuine romance. In May of 1998 Douglas graduated with both his B.S. and M.S. degrees in Molecular Biophysics and Biochemistry, Magna Cum Laude, with distinction in his major.

Douglas entered Harvard Medical School in the fall of 1998 at the age of 22 as an MD/PhD student. Prior to the first day of classes Douglas bet a fellow student, Rahul Kohli, that neither would have completed the program by their 30<sup>th</sup> birthday. Douglas lost this bet, but unfortunately not due to his own rapid progress through the program. The initial two years of medical school were rather unremarkable academically. However, in the course of learning the pathophysiology of human disease he was introduced to a wonderfully idiosyncratic set of colleagues from which rapidly emerged a tight-knit group of friends. He is forever indebted to one of these friends, Robert Hagan, who unknowingly and unwittingly delivered his future wife, Tala Klinck, into his living room one winter afternoon.

In September of 2000, Douglas formally began his Ph.D. training in the Biology Department at MIT. His rationale for enrolling in MIT Biology as opposed to Harvard was convoluted at the time, and in retrospect, is, at best rubbish. After several fits and starts in a variety of labs, Douglas arrived in the lab of Frank Gertler in January of 2001. During the course of his thesis research he initiated a collaboration with Christopher Dillon in the Center for Cancer Research. The eight months that they spent developing, validating, and employing a lentiviral system for RNAi transgenesis were in its best moments, exhilarating, and in its worst moments, crushing. Their publication became one of the most cited papers of 2003, and the technology has been used by hundreds of labs and licensed by several biotech companies. After completing this work, Douglas initiated a collaboration with a fellow student in Frank Gertler's lab, Adam Kwiatkowski. Their work in describing the phenotype of the Ena/VASP triple-null mouse forms the basis for the bulk of this thesis. During his graduate work Douglas received a Ludwig Cancer Fellowship.

After completing his thesis Douglas's plans include completing his medical training at Harvard, and marrying the love of his life, Tala Klinck.

Publications to date:

Chakshumathi, G., Kim, S. D., Rubinson, D. A., and Wolin, S. L. (2003). A La protein requirement for efficient pre-tRNA folding. *Embo J* 22, 6562-6572.

Dillon, C. P., Sandy, P., Nencioni, A., Kissler, S., Rubinson, D. A., and Van Parijs, L. (2005). Rnai as an experimental and therapeutic tool to study and regulate physiological and disease processes. *Annu Rev Physiol* 67, 147-173.

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## ACKNOWLEDGMENTS

Each piece of data that found its way into this thesis sits upon a pile of false starts, faulty hypotheses, failed experiments and scooped ideas. In addition to the fits and starts of scientific discovery, were the unexpected moments of ethical and interpersonal difficulty. A graduate education is a minefield hidden in a labyrinth, and I was fortunate to have had invaluable guidance from colleagues, mentors, family and friends as I negotiated my path.

I am extraordinarily indebted to my thesis advisor, Frank Gertler, who, after some hesitation, agreed to let me join his lab. As a mentor, Frank provided me with the freedom and support to pursue my scientific whims, the guidance to reel me in when my pursuits became too far-flung, and a consistent engagement in encouraging my scientific endeavors and mentoring my career development. But in addition to his role as a mentor, Frank has been a friend, confidant, partner in inebriation, and occasional travel companion- roles that I look forward to continuing in the future.

I have valued all of my interactions with my co-workers in the Gertler lab (even the negative ones). I have had the pleasure of collaborating closely and successfully with Adam Kwiatkowski, Radhika Jagannathan and Chris Dillon. All of whom have displayed uncommon patience in accommodating my logorrhea, hair-brained hypotheses, and nocturnal work habits. Jim Bear and Joe Loureiro were essential in guiding me through the first years of my PhD with priceless bovine analogies. I also need to thank Gerry Strasser for easing the burden of cake baking as the lab grew, Gretchen Baltus for promoting sanity maintenance, Erik Dent for sharing his expertise in embryonic brain microsurgery, Matthias Krause for his brightly colored pants and consistent scientific focus, Angelique Dousis for her unbridled enthusiasm during the cold

dark Boston winters, and Leslie Mebane whose friendship and spontaneity sustained me during the grueling process of writing this thesis.

My parents have been unwaveringly supportive of all of my scientific (and non-scientific) pursuits. As a child they ferried me to all manner of science and nature classes and events. They have made sacrifices, financial and otherwise, to assure that I could take advantage of the opportunities that were presented to me. Together with my brother, David, and extended family, they have provided me with a secure foundation from which I could take risks, pursue my dreams, explore, and forsake monetary needs to enroll in an interminable MD/PhD program.

Finally, I would like to acknowledge my fiancée, Tala Klinck. She is my strongest advocate, my most honest critic, the love of my life, and my partner in exploring the world. She has introduced me to formerly alien concepts of style and fashion. She has inspired me with her passion for architecture and life. She has dragged me away from lab and taken me to Turkey, China, Nova Scotia, the Canadian Rockies and Japan. And our adventure in life is only just beginning.



## PREFACE

The work described within this thesis consists of two fairly distinct projects: the development of a lentiviral system for the induction of RNAi and an exploration into the biology of Ena/VASP proteins. I intend to treat these topics independently as separate chapters within this thesis. In terms of organization, I will present an introductory chapter followed by two chapters discussing my work on Ena/VASP biology. I will follow with an introductory chapter on RNAi and a chapter describing my work on lentiviral RNAi transgenesis. I will conclude with a single chapter detailing conclusions from both fields of work and proposing a possible future set of experiments that would employ the lentiviral system that I have developed (or a derivative thereof) to further the analysis of Ena/VASP biology.

*Chapter 1*

**Ena/VASP Biology: Roles Within and Outside the Nervous System**

## Introduction

Genetic approaches in the lower metazoans *D. melanogaster* and *C. elegans* have uncovered a wealth of ligands, receptors, and downstream signaling molecules that function in axon guidance. Biochemical, cell biological and additional genetic approaches have described how these molecules exert their effects by regulating the assembly, disassembly and architecture of the actin cytoskeleton. By regulating the actin cytoskeleton, these molecules exert control on the processes of membrane protrusion and withdrawal, the fundamental events that govern cell morphology, polarity, and directed motility. The events downstream of ligand/receptor interaction that result in alterations of the actin cytoskeleton are extraordinarily complex. A single receptor may independently activate or repress several different pathways leading to actin rearrangement, and multiple receptors may make use of overlapping subsets of these pathways. Furthermore, dependent upon intracellular conditions, a ligand binding to its receptor can result in opposing responses. In mammals, numerous examples have emerged identifying roles for putative axon guidance ligands and receptors in diverse developmental processes including renal, lung, heart, and blood vessel development. Mammals appear to have exploited these putative axon guidance pathways to accomplish a wide array of complex developmental processes. However, it is unclear to what extent the intracellular signaling pathways downstream of the ligand/receptor interactions are conserved in these non-axon guidance functions.

Growth cone migration, like changes in cell morphology, polarity and directed motility, relies upon the dynamic regulation of the underlying actin cytoskeleton. The regulated extension and retraction of cell protrusions such as a broad lamellipodial protrusions or narrow filopodial projections provides the basis for cell motility. The neuronal growth cone provides one model for how actin rearrangement can mediate changes in cellular morphology and movement. The

growth cone provides an extending axon a broad “antenna” across which it can sense gradients in surrounding ligands. It enhances this spatial resolution by extending long filopodial protrusions along the periphery of the growth cone. In response to an extracellular cue, the growth cone can respond in several ways: filopodial protrusion, growth cone collapse or growth cone protrusion. Furthermore, by limiting these responses to a subset of the growth cone area sidedness can be generated necessary to achieve a turning response.

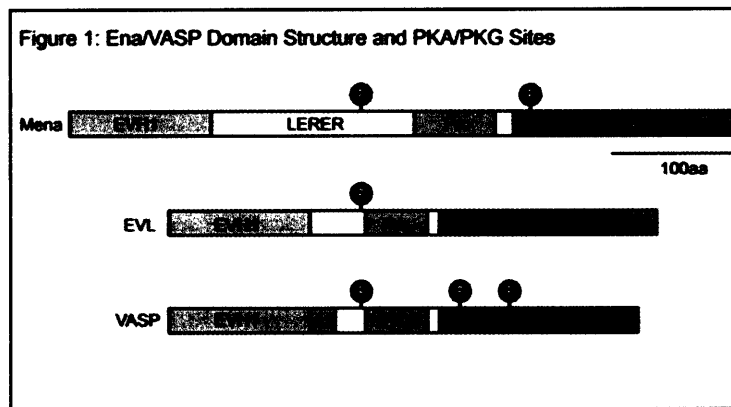
What are the mechanisms of protrusion, and filopodia generation, and collapse? Cell shape is generated by the functional antagonism between membrane tension and cytoskeletal support. An actin meshwork underlies the membrane surface throughout the cell. The importance of the actin network to cell shape is demonstrated by the consequences of pharmacologic disruption of the actin network by the depolymerizing drug Latrunculin B (LatB). Exposure of a cell to LatB causes a dramatic alteration in morphology as the cell “rounds up” under the force of membrane tension. Changes in cell shape are likewise mediated by alterations in this underlying cytoskeleton. Actin forms a polar polymer with barbed and pointed ends. This nomenclature reflects the appearance of myosin decoration along actin filaments by electron microscopy. This structural polarity reflects a functional polarity. Within a cell, ATP-Actin monomers are continually added to an extending barbed end, hydrolyze ATP within the filament, and are lost as ADP-Actin from the pointed end. When these processes are in dynamic equilibrium there is no net change in filament length. Actin filaments can be regulated by disrupting this equilibrium, which can be achieved by modifying any of the following processes: rate of barbed-end polymerization, rate of pointed-end depolymerization, severing of filaments, de novo nucleation of new filaments, nucleation of new filaments from the sides of existing filaments and bundling of actin filaments(Pollard and Borisy, 2003). For example, filopodia may be generated by

enhancing filament bundling in conjunction with increased barbed-end polymerization. In comparison, growth cone collapse may be effected through the inhibition of barbed-end polymerization and/or severing of filaments. The cell contains a wealth of proteins with activities affecting one or more of these properties of actin. The scope of these proteins and activities is beyond the scope of this thesis, and I intend to focus this introduction on the activity of one actin regulatory protein family, Ena/VASP.

Ena/VASP proteins directly interact with actin and regulate its assembly and geometry. Ena/VASP proteins protect actin barbed ends from capping protein, and thus support the generation of long actin filaments. Ena/VASP activity also inhibits the nucleation of new filaments from the sides of existing filaments. Ena/VASP activity thus promotes the formation of long, unbranched filaments. Depending upon the intracellular milieu, these filaments may interact with the membrane individually where they provide a biomechanically less effective structure to resist membrane tension(Bear et al., 2002), or they may be assembled by bundling proteins to produce filopodial protrusions(Mejillano et al., 2004).

The Ena/VASP family is well situated to act as a convergence point in several signaling pathways that function in axon guidance. Within these signaling cascades, Ena/VASP activity is regulated by both its phosphorylation state as well as the repertoire of proteins to which it is bound. Previous work in mice using single or double-knockouts of Ena/VASP family members had identified defects in the guidance of commissural axons as well as mild defects in several other developmental and physiological pathways. *In vitro* experiments and construction of several putative Ena/VASP dominant-negative transgenics have greatly extended the list of developmental and physiological functions assigned to Ena/VASP to include heart, skin, and

immune system development and function. As discussed in Chapters 3 and 4 of this thesis, several of these proposed functions are not recapitulated in the Ena/VASP triple-null embryo.



In this introduction, I will discuss the molecular characteristics and function of Ena/VASP proteins, and examine how their functions are exploited by axon guidance pathways in worms, flies, and vertebrates. I will also review the proposed functions of Ena/VASP outside of axon guidance in a variety of developmental and physiological contexts.

### **Ena/VASP Protein Family**

The Ena/VASP protein family consists of *Drosophila* Enabled (Gertler et al., 1990), *C. elegans* Unc-34 (Colavita and Culotti, 1998), *Dictyostellium* dVASP (Han et al., 2002), and three orthologs in vertebrates mammalian enabled (Mena), vasodilator stimulated phosphoprotein (VASP) and Ena/VASP-like (EVL) (Gertler et al., 1996; Reinhard et al., 1992). This protein family shares a common domain structure consisting of an NH<sub>3</sub>-terminal Ena/VASP homology 1 (EVH1) domain and a COOH-terminal EVH2 domain that flank a central proline-rich region (Figure 1). Mena contains an additional protein region not found in EVL or VASP. This region contains several repeats of the highly charged sequence LERER. The function of this region is not known. Mammalian Ena/VASP mRNAs are also subject to alternative splicing. Several isoforms of Mena have been identified including the widely expressed 80kDa and 88kDa isoforms, an immune specific 75kDa isoform, and a 140kDa isoform that is only expressed in neurons and contains targets for tyrosine phosphorylation (Gertler et al., 1996; Tani et al., 2003).

A second isoform of EVL exists, EVL-I, that has an additional 21 amino acids within the EVH2 domain(Lambrechts et al., 2000). Very little is known about the specific biology of these isoforms, but they likely mediate the formation of distinct protein complexes by displaying unique sites for protein-protein interaction(Klostermann et al., 2000; Krause et al., 2003; Toyofuku et al., 2004). All three vertebrate proteins function equivalently in fibroblast random motility assays suggesting that if a family member possesses a unique function it is only relevant in specific physiologic situations(Loureiro et al., 2002).

## **Ena/VASP Structure/Function**

### **EVH1 Domain**

The NH<sub>3</sub>-terminal EVH1 domain bears structural but not sequence homology to the pleckstrin homology (PH) domain(Fedorov et al., 1999; Prehoda et al., 1999). This PH structural scaffold is not used to bind phosphoinositides, but instead mediates high-affinity interactions with proteins containing the peptide motif (D/E)(F/W/Y/L)PPPPX(D/E)(D/E) (abbreviated FPPPP)(Ball et al., 2002; Carl et al., 1999; Niebuhr et al., 1997; Smith et al., 1996). These motifs are found in a diverse array of proteins implicated in signaling to the cytoskeleton (Table 1), including the axon guidance receptor Sax-3/Robo(Bashaw et al., 2000; Yu et al., 2002), the focal adhesion proteins zyxin and vinculin(Brindle et al., 1996; Drees et al., 2000; Niebuhr et al., 1997; Reinhard et al., 1995b), the T cell adaptor protein Fyb/SLAP/ADAP(Coppolino et al., 2001; Krause et al., 2000), the guanine nucleotide exchange factors (GEFs) RIAM and Lamellipodin(Krause et al., 2004; Lafuente et al., 2004), and several other proteins(Boukhelifa et al., 2004; Moeller et al., 2004). This high-affinity interaction is also exploited by the intracellular pathogen *Listeria monocytogenes* to sequester Ena/VASP proteins to the surface of the bacterium(Chakraborty et al., 1995; Gerstel et al., 1996; Pistor et al., 1995). Accumulation of

Ena/VASP on the surface of *Listeria* is required for the actin polymerization based rocketing of the bacteria within the cytoplasm(Ireton and Cossart, 1997).

<b>Table1: Ena/VASP Ligands</b>			
<b>Ligand</b>	<b>Binding Domain</b>	<b>Function</b>	<b>Reference</b>
Zyxin	EVH1	Recruitment to focal adhesions	(Drees et al., 1999)
Vinculin	EVH1	Recruitment to focal adhesions	(Brindle et al., 1996)
Lamellipodin	EVH1	Lamellipodial dynamics	(Krause et al., 2004)
RIAM	EVH1	Adhesion, Rap1 signaling	(Lafuente et al., 2004)
Fyb/SLAP/ADAP	EVH1	Immune responses	(Krause et al., 2000)
Robo/Sax-3	EVH1	Axon guidance	(Bashaw et al., 2000)
Robo4	EVH1	Angiogenesis	(Park et al., 2003)
Palladin	EVH1	Stress fiber, lamellipodial dynamics	(Boukhelifa et al., 2004)
Fat1	EVH1	Cell polarization and motility	(Moeller et al., 2004)
ActA	EVH1	Recruitment to <i>Listeria</i>	(Pistor et al., 1995)
Sema6A	EVH1	Axon guidance	(Klostermann et al., 2000)
Sema6D	EVH1	Heart development	(Toyofuku et al., 2004)
Profilin	Pro	Actin dynamics	(Reinhard et al., 1995a)
Fe65	Pro		(Ernekova et al., 1997)
Abl	Pro	Tyrosine kinase, axon guidance	(Gertler et al., 1995)
Src	Pro	Tyrosine kinase	(Gertler et al., 1995)
Tuba	Pro	Exocytosis, vesicle trafficking	(Salazar et al., 2003)
Abi/NESH	Pro	Interaction with Abl	(Loureiro, unpublished)
IRSp53	Pro	Actin dynamics	(Krugmann et al., 2001)
G-actin	EVH2	Actin dynamics	(Van Troys et al., 1996)
F-actin	EVH2	Actin dynamics	(Bachmann et al., 1999)
Ena/VASP	EVH2	Tetramerization	(Zimmermann et al., 2002)
Trim9/Bad-1	?	Link to DCC/Unc-40/Unc-5	(Hao et al., submitted)
Gephyrin	?	Postsynaptic actin dynamics	(Giesemann et al., 2003)

### **Proline-Rich Central Region**

The central proline-rich contains mediates protein-protein interactions and is a target for protein phosphorylation. This region binds profilin and contains numerous binding sites for SH3 and WW domain containing proteins including the tyrosine kinases Abl and Src(Ahern-Djamali et al., 1998; Ernekova et al., 1997; Gertler et al., 1996; Reinhard et al., 1995a). *Drosophila* Enabled is tyrosine phosphorylated at multiple residues, and the vertebrate Ena/VASP proteins share a conserved PKA/PKG phosphorylation site within the proline-rich region (discussed below).



It has been proposed that the interaction between Ena/VASP proteins and profilin is essential for actin regulation(Reinhard et al., 1995a). Profilins bind G-actin, promote the exchange of ADP to generate ATP-actin, and enhance the barbed-end polymerization of actin(Blanchoin et al., 2000; Paavilainen et al., 2004). *Listeria* motility requires the presence of the proline-rich region(Geese et al., 2002), and is enhanced by the inclusion of profilin both *in vivo* as well as in reconstitution experiments *in vitro*(Geese et al., 2000; Loisel et al., 1999). Knockout of *profilin-1* enhances the phenotype of the *mena* knockout mouse, uncovering a neurulation phenotype (Lanier et al., 1999). Surprisingly, in a fibroblast assay for Ena/VASP activity, the central proline-rich region was dispensable in inhibiting whole cell motility(Loureiro et al., 2002).

#### **EVH2 Domain: Multimerization and Actin Binding**

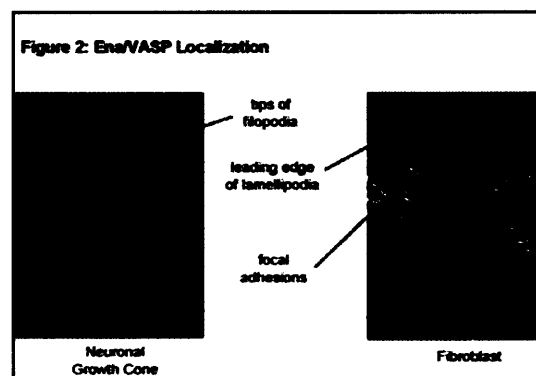
The 160-190aa COOH-terminal domain of Ena/VASP proteins contains a highly conserved F-actin binding motif, G-actin binding motif, and coiled-coil region(Bachmann et al., 1999). The F-actin binding motif can bind actin directly *in vitro* consistent with the proposed molecular function of Ena/VASP as direct effectors of the actin cytoskeleton (described below)(Bachmann et al., 1999; Bear et al., 2002; Lambrechts et al., 2000). The G-actin binding motif bears sequence homology to the actin binding site of Thymosin- $\beta$ 4(Van Troys et al., 1996). The coiled-coil domain forms a right-handed coiled-coil and mediates both hetero- and homo-oligomerization of Ena/VASP proteins(Ahern-Djamali et al., 1998; Kuhnel et al., 2004; Zimmermann et al., 2002). All three of these regions are essential for Ena/VASP function in fibroblast motility(Loureiro et al., 2002). Surprisingly, the EVH2 domain alone was sufficient to rescue the whole-cell motility phenotype of Ena/VASP-deficient cells(Loureiro et al., 2002). This suggests that one basic molecular function of Ena/VASP is mediated by the EVH2 domain,

and that the NH<sub>3</sub>-terminal two-thirds of the molecule may function in the regulation of the protein required for directed motility. Interestingly, the domain requirements for Ena/VASP function in cell motility differ greatly from those required for *Listeria* motility, suggesting that Ena/VASP proteins may be used in distinct ways by different actin-driven processes.

### Localization

The function of Ena/VASP proteins is intimately associated with their localization. Ena/VASP proteins localize within the cell to sites consistent with their roles in regulating the actin cytoskeleton: the leading edge of protruding lamellipodia, the tips of filopodia, focal adhesions, and stress fibers (Figure 2)(Gertler et al., 1996). Localization to focal adhesions

requires the interaction of the NH<sub>3</sub>-terminal EVH1 domain with the FPPPP motif found in the focal adhesion associated proteins vinculin and zyxin(Niebuhr et al., 1997; Reinhard et al., 1995b). Competition for EVH1 binding by exogenous expression of an FPPPP peptide causes



delocalization from focal adhesions(Bear et al., 2000). The intracellular pathogen *Listeria monocytogenes* co-opts Ena/VASP activity by displaying several FPPP repeats within its ActA protein to sequester Ena/VASP on the bacterial surface to facilitate its actin-based intracellular motility(Pistor et al., 1995; Pollard, 1995).

The mechanism of leading edge localization has additional complexity. Structural mutants of Ena/VASP proteins have been used to dissect the mechanism of localization. Because Ena/VASP proteins can hetero- and homo-oligomerize through a coiled-coil within the COOH-terminal EVH2 domain(Bachmann et al., 1999; Zimmermann et al., 2002), localization studies

required the use of an Ena/VASP-deficient cell line(Bear et al., 2000). EGFP-tagged EVH2 domain localized to a broad swath behind the leading edge of the cell, but was not sufficient for restriction to the tip(Loureiro et al., 2002). This broad band of EVH2 localization corresponds with a high concentration of free actin barbed ends. The EVH2 domain contains both F- and G-actin binding motifs and deletion of either motif causes delocalization from the leading edge(Loureiro et al., 2002). Furthermore, Cytochalasin D, a drug that binds actin barbed ends, also eliminates Ena/VASP localization from the leading edge(Bear et al., 2002). Restriction of Ena/VASP localization to the tip of the leading edge requires the EVH1 domain, but it alone is not sufficient to recapitulate normal Ena/VASP expression. EGFP-tagged EVH1 domain localizes to focal adhesions, and weakly to the leading edge(Bear et al., 2000). Recently, two proteins containing EVH1-binding motifs, RIAM and Lamellipodin, have been identified that may function to bring Ena/VASP proteins to the tip of the leading edge(Krause et al., 2004; Lafuente et al., 2004). These proteins each contain PH domains and GEF domains and thus may provide a general link between Ena/VASP localization and phosphoinositide and small GTPase signaling. Ena/VASP proteins have also been shown to interact directly with axon guidance receptors that contain a consensus binding site for EVH1 domains. These include Robo, Sema6A and Sema6D(Klostermann et al., 2000; Toyofuku et al., 2004; Yu et al., 2002).

## **Molecular Function**

### **Lamellipodial Dynamics**

In the classic model of cell locomotion, cells extend an initial actin based lamellipodium which adheres to the substratum. The cell then either actively or passively draws its cell body towards this extension while withdrawing at the trailing edge(Pollard and Borisy, 2003). The delineation of Ena/VASP function within this paradigm was initially complicated by seemingly

contradictory observations. Ena/VASP proteins drive the polymerization of actin by *Listeria*, and preferentially associate with the leading edge of protruding lamellipodia (Bear et al., 2000; Laurent et al., 1999; Rottner et al., 1999). In addition, the initial *in vitro* characterization of VASP led several groups to report an actin nucleation activity (Bachmann et al., 1999; Huttelmaier et al., 1999). Surprisingly, overexpression of Ena/VASP inhibited cell motility, and inhibition of Ena/VASP increased cell speeds (Bear et al., 2000). This apparent paradox was resolved by detailed observation of a protruding lamellipodium and electron microscopic analysis of its underlying actin ultrastructure. Consistent with Ena/VASP's association with protruding lamellipodia, the speed of membrane extension was directly proportional to the concentration of Ena/VASP. However, the protrusions from Ena/VASP overexpressing cells were not productive, instead withdrawing as membrane ruffles. Underlying this membrane behavior, the actin architecture in Ena/VASP overexpressing cells consisted of long unbranched filaments running parallel to the membrane. In contrast, Ena/VASP-deficient cells possessed shorter, highly branched filaments oriented perpendicular to the membrane (Bear et al., 2002). The latter structure has been suggested to more effectively counter membrane tension to drive stable membrane protrusion (Pollard and Borisy, 2003).

### **Biochemical Basis of Ena/VASP Function**

What is the biochemical basis for the alteration to the actin ultrastructure? Several lines of evidence suggest that the primary activity of Ena/VASP is as a functional antagonist to heterodimeric capping protein (CP). Actin filaments are polar structures with a barbed end and a pointed end (Pollard and Borisy, 2003). Within a cell, actin assembly occurs through the addition of actin monomer to free actin barbed ends found near the leading edge. This process is terminated through the irreversible binding of heterodimeric capping protein (Pollard and Borisy,

2003). Ena/VASP proteins bind to actin filaments through an F-actin binding motif within the EVH2 domain leading to colocalization with the regions of the cell containing free barbed ends, the filopodia and lamellipodia. This localization can be disrupted by low doses of the barbed-end binding toxin Cytochalasin D. *In vitro*, Ena/VASP proteins can compete with capping protein to permit actin polymerization (Bear et al., 2002). Finally, inhibition of capping protein, similar to Ena/VASP overexpression, causes filopodial protrusion in fibroblasts (Mejillano et al., 2004). Thus, within a fibroblast, overexpression of Ena/VASP inhibits the ability of capping protein to terminate filament elongation resulting in aberrantly long actin filaments. Filopodia formation in neurons requires Ena/VASP activity, suggesting that the observations in fibroblasts are generally applicable to other cell types (Lebrand et al., 2004).

The paucity of filament branching is inadequately explained solely by Ena/VASP anti-capping activity. The seven member protein complex Arp2/3 binds to the sides of actin filaments and overcomes the rate-limiting step in actin nucleation by mimicking an actin dimer. The new filament is generated at a 70° angle from the existing filament, and thus the iterative action of Arp2/3 proteins can generate the dendritic actin architecture observed in normal fibroblasts and enhanced in Ena/VASP deficient cells. Interestingly, Arp2/3 together with Ena/VASP are the two proteins that are recruited by and required for *Listeria* actin assembly (Cossart, 2000; Geese et al., 2002; Loisel et al., 1999). The absence of branches suggests that Ena/VASP proteins directly affect Arp2/3 function. Several possible mechanisms exist for this activity: 1. Ena/VASP and Arp2/3 may compete for overlapping binding sites at or near the actin filament barbed end. 2. Ena/VASP proteins may have a debranching activity. 3. Arp2/3 activity may require barbed end capping to nucleate a new filament.

Recently, several other protein families have been described as regulating barbed end capping. The formins function as processive cappers, allowing for the addition of new actin monomer without dissociating from the extending barbed end(Harris et al., 2004; Kozlov and Bershadsky, 2004; Otomo et al., 2005; Romero et al., 2004; Zigmond et al., 2003). Conversely, the Eps8 protein has been shown to have capping activity(Croce et al., 2004; Disanza et al., 2004). Interestingly, Ena/VASP proteins share common interacting partners with both of these protein families. A yeast two hybrid screen identified Formin Binding Protein 3 (Fnbp3) as a potential interactor of Evl (Jagganathan, Rubinson, and Gertler, unpublished observations). Eps8 binds Abi1(Disanza et al., 2004), an Ena/VASP interactor, as well as RN-Tre which was also identified in the Evl yeast two hybrid screen(Lanzetti et al., 2000)( Jagganathan, Rubinson, and Gertler, unpublished observations). This suggests that macromolecular complexes exist containing one or more proteins acting at the barbed end. The significance, function and regulation of these complexes remain unclear.

### **Filopodia Formation**

Ena/VASP proteins are critical in the formation of filopodia(Mejillano et al., 2004) (Lebrand et al., 2004). Filopodia emerge from the dendritic actin array from the polymerization of unbranched, bundled actin filaments(Svitkina et al., 2003; Vignjevic et al., 2003). Ena/VASP proteins form tetramers *in vivo*(Zimmermann et al., 2002), with each member of the tetramer capable of binding to and promoting elongation of an actin barbed end. As described above, Ena/VASP activity also inhibits actin filament branching. Inhibition of Ena/VASP activity blocks the generation of filopodia, and inhibition of capping protein by RNAi results in enhanced filopodia formation, suggesting that the levels of capping protein and Ena/VASP activity regulates the formation of lamellipodial versus filopodial protrusions(Mejillano et al., 2004).

## **Focal Adhesions and Stress Fibers**

The function of Ena/VASP proteins at focal adhesions remains uncertain. The specific elimination of Ena/VASP from focal adhesions has no effect on fibroblast motility (Bear et al., 2000). Recently, a role for Ena/VASP proteins in cell adhesion and spreading has been described downstream of the Rap1 GEF RIAM (Jenzora et al., 2005; Lafuente et al., 2004). More substantive roles for Ena/VASP at focal adhesions have been suggested for cell:cell and cell:matrix contacts in epithelium and endothelium (discussed below).

## **Ena/VASP Regulation**

### **Serine/Threonine Phosphoregulation**

Upstream signaling pathways converge and regulate Ena/VASP proteins through one or more phosphorylation sites. In contrast with *Drosophila* Enabled, which is phosphorylated by the Abelson tyrosine kinase (Gertler et al., 1995), the phosphorylation of Ena/VASP proteins in mammals is mediated primarily by cyclic-nucleotide dependent kinases. The initial identification of VASP was based upon it acting as a substrate for phosphorylation by Protein Kinase A (PKA) and Protein Kinase G (PKG) within platelets (Eigentlicher et al., 1992; Reinhard et al., 1992; Waldmann et al., 1987). Mammalian Ena/VASP proteins can also be phosphorylated by the Ca<sup>2+</sup>-regulated Protein Kinase C (PKC) (Chitaley et al., 2004). The three mouse Ena/VASP homologs each possess one or more phosphorylation sites. Mena, VASP, and Evl share a phosphorylation site within the central proline-rich region. However, Mena contains an additional site within the EVH2 domain, whereas VASP contains this site and an additional site within the EVH2 domain (Figure 1) (Butt et al., 1994; Gertler et al., 1996; Lambrechts et al., 2000).

Significant evidence suggests that phosphorylation is essential to Ena/VASP biology. Ena/VASP-deficient cell lines show increased rates of cell motility. Expression of wildtype Ena/VASP proteins, but not those containing phospho-site Serine→Alanine mutations could restore wildtype motility(Loureiro et al., 2002). Elimination of VASP from platelets destroys the normal inhibition of aggregation mediated by cAMP or cGMP(Aszodi et al., 1999), suggesting that VASP is the critical PKA/PKG target in this process. In addition, the phosphorylation state affects both the spectrum and avidity of Ena/VASP binding proteins(Harbeck et al., 2000; Lambrechts et al., 2000; Laurent et al., 1999). Several groups have demonstrated changes in VASP phosphorylation state associated with cell spreading (Howe et al., 2002; Lawrence and Pryzwansky, 2001) or the formation of cell:cell contacts(Comerford et al., 2002). Within the neuronal growth cone, Ena/VASP proteins are phosphorylated downstream of the axon guidance receptor DCC, and this phosphorylation is associated with protrusion of growth cone filopodia(Lebrand et al., 2004).

The phosphorylation of all three vertebrate Ena/VASP proteins results in a sizable band shift in SDS-PAGE(Gertler et al., 1996; Halbrugge et al., 1992; Lambrechts et al., 2000). Tagging the NH<sub>3</sub>- and COOH- terminus of Evi with YFP and CFP respectively resulted in a change in Fluorescence Resonance Energy Transfer (FRET) when treated with the catalytic subunit of PKA *in vitro* (Rubinson and Gertler, unpublished observation). These data suggested a simple model whereby Ena/VASP phosphorylation caused a major structural shift in the protein generating an active conformation. However, the phosphorylation state of purified Ena/VASP proteins did not alter the anti-capping activity in an *in vitro* assay (Barzik and Gertler, submitted). This suggests that while phosphorylation of Ena/VASP may mediate a structural



change, the phosphoactivation of Ena/VASP likely requires the dissolution of a 3<sup>rd</sup>-party inhibitory molecule within the cell.

### **Tyrosine Phosphoregulation**

In *Drosophila*, Enabled is regulated by tyrosine phosphorylation. *Drosophila enabled* was initially identified as a genetic suppressor and a substrate for the non-receptor tyrosine kinase Abl(Gertler et al., 1995; Gertler et al., 1990). Ena also interacts both biochemically as well as genetically with the tyrosine phosphatase Dlar(Wills et al., 1999). This antagonism between tyrosine kinases and phosphatases functions to regulate Ena activity downstream of axon guidance receptors(Bashaw et al., 2000; Gertler et al., 1995; Wills et al., 1999). Ena is not a substrate of PKA/PKG phosphorylation, making tyrosine phosphorylation the sole mechanism of its phosphoregulation.

The role of tyrosine phosphorylation in the biology of vertebrate Ena/VASP proteins is more complicated. In contrast with *Drosophila* Ena, tyrosine phosphorylation does not appear to be conserved as the basic mechanism of vertebrate Ena/VASP regulation. The Ena tyrosine phosphorylation sites are not conserved in the vertebrate proteins, and Ena does not rescue the cell motility phenotype in Ena/VASP-deficient mouse fibroblasts(Loureiro et al., 2002). None of the potential tyrosine phosphorylation sites are conserved between vertebrate Ena/VASP proteins. However, several recent studies provide evidence that Ena/VASP tyrosine phosphorylation does occur. Vertebrate Ena/VASP proteins coimmunoprecipitate in a complex with Abl(Howe et al., 2002) and directly interact with the Abelson interacting (Abi) family of proteins at the cell leading edge through SH3 binding to the Ena/VASP proline-rich region (Loureiro and Gertler, unpublished observations)(Tani et al., 2003). Abi proteins direct the formation of a protein complex containing Ena/VASP proteins and Abl, and promote

phosphorylation of Mena at Y296(Tani et al., 2003). More recently, Abl phosphorylation of Mena at Y296 has been implicated in regulating the interaction of Mena and Sema6D during heart development(Toyofuku et al., 2004).

### **Phosphoregulation During Axon Guidance**

Genetic evidence in *Drosophila* and *C. elegans* places Ena/VASP proteins in pathways regulated by the antagonistic function of tyrosine kinases including Abl and tyrosine phosphatases such as Dlar and clr-1(Bashaw et al., 2000; Chang et al., 2004; Lanier and Gertler, 2000; Wills et al., 1999). However, in mammals, only Mena appears to be a target of tyrosine phosphorylation(Gertler et al., 1996; Tani et al., 2003; Toyofuku et al., 2004). The conservation of tyrosine phosphorylation as the mechanism of Ena/VASP regulation downstream of axon guidance receptors is unclear. As described above, the PKA-dependent protein phosphorylation of Ena/VASP has been observed downstream of Netrin/DCC signaling in vertebrates(Dent and Gertler, 2003). An intriguing possibility is that PKA/PKG phosphorylation has supplanted tyrosine phosphorylation as the primary means of modulating Ena/VASP activity.

Several lines of evidence suggest that cAMP regulation of PKA plays an integral role downstream of several axon guidance cues in vertebrates. Establishment of a cAMP gradient across a neuronal growth cone is sufficient to cause a turning response(Lohof et al., 1992) and cAMP is required for turning in response to a Netrin cue(Ming et al., 1997). In a series of seminal papers from the Poo laboratory, attractive or repulsive responses could be mediated from a single ligand/receptor interaction dependent upon extracellular matrix or the intracellular levels of second messengers including cAMP and  $Ca^{2+}$ (Hopker et al., 1999; Nishiyama et al., 2003; Song et al., 1997). This switching behavior has been observed with several ligands including netrin, SDF-1, acetylcholine, SemaIII, and myelin-associated glycoprotein (MAG) suggesting

that modulating intracellular cAMP and  $\text{Ca}^{2+}$  provides a generalized mechanism to regulate guidance responses(Henley et al., 2004; Hong et al., 2000; Nishiyama et al., 2003; Song et al., 1998; Song et al., 1997; Xiang et al., 2002). Presumably, within the developing nervous system this provides a fine level of control on axon guidance. Ena/VASP proteins, as substrates for PKA/PKG and PKC, provide an intriguing potential target to participate in mediating this switching.

## **Axon Guidance**

During development, neurons direct an axon towards a distant target. Guidance decisions are generated within the neuronal growth cone, a specialized structure at the end of a growing axon. Within the developing brain, complex patterns and gradients of soluble and fixed guidance cues are generated(Tessier-Lavigne, 1994). These cues include soluble axon guidance ligands such as Netrin and Slit, bifunctional transmembrane proteins such as Ephs and their partner Ephrins, Semaphorins and their partner Plexins, and extracellular matrix molecules such as Laminin(Tessier-Lavigne and Goodman, 1996). These ligands can be either soluble or fixed, and either attractive or repulsive. The growth cone must decipher these cues and respond to them in a specific manner. Two axons with distinct targets must be able to pass through the same microenvironment and respond independently(Song and Poo, 2001). It is believed that the guidance of an individual axon is accomplished through a series of milestones. Axons destined for a distant target pass through a series of intermediate targets along their journey. These intermediate targets serve as choice points so that axons destined for different final targets make divergent decisions upon reaching the same intermediate target. Intracellular conditions such as the concentration of cAMP and  $\text{Ca}^{2+}$  permit growth cones to respond distinctly to identical guidance cues. Guidance receptors activate a number of signaling cascades to mediate changes in

the axon cytoskeleton including Rho GTPases, WASP and Arp2/3, tyrosine kinases, tyrosine phosphatases, serine/threonine kinases, and Ca<sup>2+</sup>-dependent kinases (reviewed in (Korey and Van Vactor, 2000). A detailed review of the signaling cascades is beyond the scope of this work, and I will focus my discussion to the developmental role of Ena/VASP proteins in mediating effects on the actin cytoskeleton.

### **Midline Guidance**

The biological challenge of axon guidance is typified by the challenge of directing axon growth across the midline of an embryo. Midline crossing is an essential event in a nervous system that exerts contralateral motor control and sensation. To cross the midline, axons must first be attracted towards the midline and then upon crossing must lose the attractive signal and be repulsed to prevent recrossing. The molecules controlling this event were elucidated first in *C. elegans* navigation of circumferential axons and then in *Drosophila* by identifying mutants that impact the architecture of the ventral nerve cord. Two signaling pathways function in concert to control midline crossing. In *Drosophila*, axon attraction towards the midline is mediated by the ligands Netrin-1 and Netrin-2 binding their receptor frazzled/DCC, and repulsion after crossing is controlled by the ligand Slit binding its receptors Robo1, Robo2, and Robo3. Mutations in Netrins(Harris et al., 1996; Mitchell et al., 1996) or frazzled/DCC(Keino-Masu et al., 1996; Kolodziej et al., 1996) prevent the formation of commissural axons. The ventral nerve cord in these mutants consists of two parallel tracts without crossing fibers. Mutations in Slit (Kidd et al., 1999; Rothberg et al., 1988; Rothberg et al., 1990) or Robo(Kidd et al., 1998) caused collapse of the ventral nerve cord onto the midline. The axons in these mutants approached the midline through Netrin/DCC but no longer had a repulsive cue to prevent iterative recrossing. In order to approach the midline the repulsive Slit/Robo pathway is inhibited by the cytoplasmic

protein commissureless (Comm) which prevents surface expression of Robo until after crossing has occurred(Keleman et al., 2005; Seeger et al., 1993; Tear et al., 1996).

As mentioned above, the genes involved in *Drosophila* midline crossing were initially identified in *C. elegans* as mutations that regulate the ventral/dorsal guidance decisions in the circumferential axons causing uncoordinated phenotypes. Surprisingly, Unc-6, the *C. elegans* Netrin ortholog, can direct both the ventral and dorsal migration of circumferential axons(Hedgecock et al., 1990; Ishii et al., 1992). *C. elegans* possess two receptors for Unc-6/Netrin, Unc-40/DCC and Unc-5, and the response to Unc-6/Netrin is dependent upon which combination of the two receptors is expressed on the growth cone(Wadsworth, 2002). When expressed by itself, the *C. elegans* DCC homolog Unc-40 mediates ventral attraction towards a gradient of Unc-6/Netrin(Chan et al., 1996; Hedgecock et al., 1990). When Unc-5 is coexpressed with Unc-40, they direct ventral growth away from the Unc-6/Netrin gradient(Colavita and Culotti, 1998; Hamelin et al., 1993). Similarly, Sax-3/Robo acts as a receptor for SLT-1/Slit in directing ventral axon guidance in the worm(Zallen et al., 1999). Surprisingly, *C. elegans* Unc-40 binds to Sax-3/Robo and participates in mediating the repulsive guidance downstream of Slit(Yu et al., 2002).

In order to identify genes that participate in downstream signaling pathways, screens have been conducted to identify genetic interactors of axon guidance receptors(Colavita and Culotti, 1998; Gallo and Letourneau, 1999). *Drosophila enabled* was initially identified by its suppression of pupal lethality caused by mutations in the non-receptor tyrosine kinase *abl*(Gertler et al., 1989; Gertler et al., 1990). Subsequent analysis of the *enabled* mutant phenotype showed defects in axon pathfinding including in motor axons and commissural axons of the ventral nerve cord(Gertler et al., 1995; Wills et al., 1999). Genetic interaction with the

tyrosine phosphatase Dlar demonstrated that Ena activity in axon guidance is regulated by the antagonistic balance of the Abl tyrosine kinases and the tyrosine phosphatase Dlar. The *C. elegans enabled* homolog, *unc-34*, was first identified in a screen for mutations causing a lack of coordinated worm movement, and subsequently identified as a suppressor of phenotypes induced by ectopic expression of the repulsive Unc-6/Netrin receptor Unc-5 (Colavita and Culotti, 1998). In addition to mediating repulsion through Unc-5, Unc-34 also has been placed downstream of Unc-6/Netrin attraction through Unc-40. The attractive response to Unc-6/Netrin mediated by Unc-40 occurs through two downstream pathways; one pathway proceeds through the Rac GTPase Ced-10, and the other pathway proceeds through Unc-34/Ena (Gitai et al., 2003). The signaling pathways linking Unc-40 and Unc-5 to Unc-34 have not been elucidated. A recent collaboration has identified Bad-1 as a genetic interactor within the Unc-40/Unc-5 repulsive guidance pathway. Independently, a yeast two-hybrid screen conducted in our lab has identified Trim9, the mammalian Bad-1 homolog, as a binding partner for Ena/VASP proteins. These data suggest a direct signaling pathway from Unc-40/DCC through Bad-1/Trim9 to Unc-34/Ena that may be conserved in mammals (Hao et al., submitted; Jagganathan, Rubinson, and Gertler, unpublished).

The *unc-34* mutant also confers axon pathfinding defects in several neurons that partly phenocopy mutations in Sax-3/Robo (Yu et al., 2002). Construction of *sax-3;unc-34* double-mutant worms provided additional genetic evidence that Unc-34 acts within the Sax-3/Robo pathway. In contrast with Unc-6/Unc-40/Unc-5 signaling, Sax-3/Robo receptors contain an EVH1 binding motif, and interact with Unc-34/Ena biochemically (Yu et al., 2002), providing a mechanism for Sax-3/Robo regulation of Unc-34/Ena.

Emerging evidence suggest that the basic functions of the Netrin/DCC and Robo/Slit pathways are conserved in vertebrates. *In vitro*, Slit proteins can repel axons from motor, retinal, and forebrain neurons (Brose et al., 1999; Nguyen Ba-Charvet et al., 1999; Ringstedt et al., 2000). Vertebrate Netrin signaling is more complicated. Netrins repel trochlear axons (Colamarino and Tessier-Lavigne, 1995), and can mediate the attraction or repulsion of spinal commissural neurons depending upon whether the neurons co-express the Unc-5 receptor (Hong et al., 1999; Kennedy et al., 1994). Similarly, Netrins can either attract or repel retinal axons depending upon factors such as ECM proteins and intracellular cAMP concentrations. (de la Torre et al., 1997; Hopker et al., 1999; Ming et al., 1997). Deletion of *netrin-1* or *DCC* results in defective commissural axon guidance (Fazeli et al., 1997; Serafini et al., 1996). The *in vivo* analysis of Slit/Robo pathways has been complicated by the expansion of these gene families and overlapping expression of its members. Vertebrates appear to have three Slit and four Robo genes, which display overlapping function and expression patterns (Li et al., 1999; Yuan et al., 1999). Construction of the *slit1/slit2* double-knockout revealed midline crossing defects in the forebrain but failed to reveal defects in spinal cord commissural neurons (Bagri et al., 2002). However, the construction of the *slit1/slit2/slit3* triple-knockout mouse caused a phenotype that mimicked the ventral nerve cord phenotype in the *slit* mutant fly (Long et al., 2004). In contrast, only three of the four Robo genes are involved in commissural axon guidance. Robo1 and Robo2 function analogously to *Drosophila* Robo in mediating the repulsive signals following midline crossing (Long et al., 2004). In another parallel to the fly, the expression of both Robo1 and Robo2 is restricted to the postcrossing portion of the commissural axon (Long et al., 2004). However, vertebrates do not have a *comm* homolog and the mechanism of pre-crossing Robo inhibition had long been unknown. The recent characterization of the third Robo gene, *rig-*

*1/robo3*, has revealed a function in suppressing Robo signaling until after reaching the midline (Marillat et al., 2004; Sabatier et al., 2004). Mutations in the human Rig-1/Robo3 homolog result in analogous axon guidance defects that cause the horizontal gaze palsy with progressive scoliosis syndrome (HGPPS)(Jen et al., 2004). Unlike Comm, Rig-1/Robo3 does not appear to prevent Robo1/Robo2 expression but instead acts to suppress signaling(Sabatier et al., 2004). The mechanism by which vertebrates partition Robo1/Robo2 expression to the post-crossing axon remains unknown.

The conservation of midline guidance function in vertebrates suggests that the downstream pathways are likewise conserved. As discussed earlier, Ena/VASP is required for the burst of filopodial activity in the growth cone induced by Netrin-1 *in vitro*. *In vivo*, the generation of the *mena* and *mena/vasp* knockout mice has uncovered several defects in axon guidance at the midline. The *mena* knockout mouse fails to generate the cortico-cortico axon tract and the corpus callosum(Lanier et al., 1999). The *mena/vasp* double-knockout mouse has defects in the major forebrain commissures that are also affected in the *slit1/slit2* knockout mouse(Bagri et al., 2002; Menzies et al., 2004). The various mutant combinations of *mena* and *vasp* demonstrate an inverse relationship between Ena/VASP dosage and phenotypic penetrance. No defects in spinal commissural axons are observed in the *mena/vasp* double-knockout mice. The three Ena/VASP family members share a broad and overlapping expression pattern(Gambaryan et al., 2001; Lanier et al., 1999; Menzies et al., 2004) as well as overlapping function(Bear et al., 2000; Loureiro et al., 2002). The presence of EVL, the remaining Ena/VASP family member, can likely partly compensate for loss of Mena and VASP in tissues in which it is expressed. The generation of the triple-knockout mouse should allow for the



examination of the conserved roles of Ena/VASP as a downstream target of midline guidance receptors.

### **Additional Roles for Axon Guidance Molecules Outside of the Nervous System**

Numerous processes in development and physiology require the dynamic reorganization of the cytoskeleton to mediate changes in cell morphology or cell movement. This is reflected in part by the expanded use of axon guidance receptors in developmental pathways outside the nervous system. Even within *Drosophila*, mutations within the Robo/Slit pathway have phenotypes in tracheal development(Lundstrom et al., 2004). Reflecting the importance of directed guidance in development, the generation of mouse knockouts of several axon guidance molecules has uncovered unexpected phenotypes outside of the nervous system. A common developmental challenge uses the recursive branching of a primary tube to generate a complex structure. This process occurs in lung development in which the initial formation of a lung bud at the terminus of the tracheal bifurcation produces the highly branched mature lung through a series of branching steps between E10.5 and E16.5 of embryo development (Cardoso, 2001). Similarly, vasculogenesis and angiogenesis, the generation of blood vessels through the formation of a large tube followed by a series of branching and sprouting events to eventually create a capillary bed, occurs through a branching mechanism. During blood vessel branching and extension, the new blood vessel is directed by a leading tip cell whose morphology and function has been compared to the neuronal growth cone(Gerhardt et al., 2003). Reflecting this visual and functional similarity, the Unc5 receptor, Unc5h2 (also referred to as Unc5b), and its binding partner Netrin-1 have recently been implicated in both lung branching and angiogenesis(Liu et al., 2004; Lu et al., 2004). The relationship between lung branching morphogenesis, angiogenesis, and axon guidance has been further extended through the

identification of several proteins integral to all three functions. Angiogenesis is regulated by the action of the soluble ligand VEGF (Vascular Endothelial Growth Factor). The action of VEGF occurs via binding to two classes of receptors- VEGFR (VEGF Receptor) and a group of receptors classically involved in axon guidance, NRPs (Neuropilins)(Whitaker et al., 2001). Interestingly, VEGF is expressed within neurons, and can affect axon outgrowth and neuron survival *in vitro*(Sondell et al., 1999). Other axon guidance molecules that have been implicated in angiogenesis and/or lung branching include Slit-2, Robo4, Sema3A, PlexinD1, and the NRPs Npn-1 and Npn-2(Bachelder et al., 2003; Bates et al., 2003; Ito et al., 2000; Kawasaki et al., 1999; Park et al., 2003; Shoji et al., 2003; Suchting et al., 2005; Torres-Vazquez et al., 2004). This extensive use of axon guidance molecules in the angiogenesis and lung branching morphogenesis reflects both a similarity in the mechanisms of development as well as a physical linkage between the two processes as the lung and its vasculature develop together(reviewed in (Autiero et al., 2005). Interestingly, Ena/VASP activity has been reported downstream of Slit/Robo, Netrin/Unc5, and several Sema/Npn/Plexin combinations(Bashaw et al., 2000; Forsthoefel et al., 2005; Gitai et al., 2003; Klostermann et al., 2000; Lebrand et al., 2004; Toyofuku et al., 2004; Yu et al., 2002), suggesting that Ena/VASP may play a role in angiogenesis and lung branching morphogenesis analogous to its role in axon guidance.

### **Beyond Axon Guidance - Ena/VASP in Physiology and Development**

The broad expression of Ena/VASP proteins and their proposed function as direct effectors of the actin cytoskeleton indicate their potential involvement in numerous processes outside of axon guidance. In addition to the nervous system phenotypes described above, Ena/VASP have been implicated through a variety of *in vitro* and *in vivo* experiments in a spectrum of activities. The following section will delineate some of the reported functions for

Ena/VASP proteins outside of axon guidance, and speculate on the possible pathways that regulate their activity.

### **Platelet Aggregation**

VASP was originally identified as the major substrate for Protein Kinase A and Protein Kinase G within platelets. VASP is the only Ena/VASP family member expressed in platelets so that the construction of the VASP-null mouse provided platelets deficient of all Ena/VASP proteins (Aszodi et al., 1999). Thrombus formation is an exquisitely regulated process that requires a robust, rapid, and specific response to a wound. Inappropriate thrombus results in various pathologies including stroke, heart attack, and thromboembolisms. The inhibition of platelet aggregation and platelet adhesion to vessel walls is in part mediated by the action of cGMP and cAMP dependent kinases. Platelet activation is typified by a dramatic actin-dependent change in morphology and a change in adhesive properties. Incubation of platelets with collagen results in platelet aggregation. Addition of pharmacologic cAMP or cGMP analogs inhibits this aggregation. Elimination of VASP prevented the cAMP/cGMP-dependent inhibition of platelet aggregation (Aszodi et al., 1999; Hauser et al., 1999). In addition to affecting platelet morphology, phosphorylation of VASP also inhibits the activation of the GPIIb-IIIa integrin thereby inhibiting platelet interactions with the blood vessel wall *in vivo* (Massberg et al., 2004).

### **Cortical Neuronal Migration and Architecture**

Roles for Ena/VASP proteins have also been proposed in the migration of neurons in development. During vertebrate cortical development newborn pyramidal neurons must migrate radially from the ventricular zone outward to the cortical plate (Olson and Walsh, 2002). Their movement is directed by the secretion of reelin by Cajal-Retzius cells that are present in the thin

relatively acellular marginal zone that intervenes between the cortical plate and the pial membrane(D'Arcangelo et al., 1995; Hirotsune et al., 1995). Essential for establishing cortical organization, elongated radial glia provide tracts that neurons associate with during their outward migration and their foot processes interact with one another and with the external ECM to form the inner aspect of the pial membrane(Halter et al., 2002). Within the cortical plate, neurons are arranged in an outside-in orientation with later born neurons migrating beyond earlier born neurons to form the outer layers of the cortex(Olson and Walsh, 2002).

Ena/VASP mutation in lower organisms causes neuronal migration phenotypes. In *C. elegans*, *unc-34* mutation results in inappropriate migration of neurons(Withee et al., 2004; Yu et al., 2002). In mice, individual pyramidal neurons in which Ena/VASP proteins were inactivated by a dominant negative construct migrated to inappropriately superficial positions within the cortex(Goh et al., 2002). The *mena/vasp* double-knockout mouse does not have a neuronal migration phenotype likely due to the continued expression of EVL in neurons.

### **At the Synapse**

Several studies have suggested roles for Ena/VASP proteins at both the pre- and post-synaptic cleft. Ena/VASP proteins are part of a large protein complex that includes Dynamin and the “Wave inhibitory complex” that localizes to synapses and functions in endocytosis and vesicle trafficking(Salazar et al., 2003). Ena/VASP proteins may also have functions in synaptic exocytosis through a potential interaction with Trim9 (Jagganathan, unpublished), which has previously been shown to regulate SNAP-25(Li et al., 2001). Recently, actin dynamics within dendritic spines have been implicated in both Long Term Potentiation (LTP) and Long Term Depression (LTD). Actin-dependent changes in dendritic spine morphology are necessary for induction of LTP and LTD(Matsuzaki et al., 2004; Zhou et al., 2004). The interaction of

Ena/VASP proteins with scaffolding molecules Gephyrin and IRSp53 within dendritic spines suggests a potential role in mediating actin-based changes in learning and memory(Choi et al., 2005; Giesemann et al., 2003; Krugmann et al., 2001).

### **Epithelial and Endothelial Function**

The localization of Ena/VASP proteins to focal adhesions within both fibroblasts and epithelial cell types prompted several investigations into potential roles in epithelial formation and function. The expression of a COOH-terminal Mena fragment that includes the coiled-coil domain (termed TD for Tetramerization Domain) was used as a putative dominant-negative construct within skin epithelia *in vivo*(Vasioukhin et al., 2000). Expression of this construct caused a skin blistering phenotype. Furthermore, microscopic analysis of keratinocyte epithelial sheet formation *in vitro* showed adherens junction formation via a zippering of filopodial-like extensions from adjacent cells. Inhibition of Ena/VASP activity prevented adherens junction formation, leading to a proposed function for Ena/VASP proteins in the integrity of epithelial monolayers(Vasioukhin et al., 2000). Neither the specificity of the TD construct nor the ability of the TD to inhibit Ena/VASP function (as opposed to simply altering function) have ever been established.

In a related process, *Drosophila* dorsal closure requires directed movement and fusion of epithelial sheets. *Abl* loss-of-function mutations delay dorsal closure and can be partly suppressed by loss of *ena*. *Ena* loss-of-function mutations cause mild dorsal closure phenotypes. The severity of this defect is enhanced when combined with loss-of-function mutations in *armadillo*, the *Drosophila* homolog to the adherens junction protein beta-catenin(Grevengoed et al., 2001). *Ena* mutations disrupt the fusion of epithelial sheets but do not affect their integrity. The fusion of epithelia requires the apposition of intact epithelia, which then fuse via the knitting

of filopodial processes and adherens junction creation. If Ena had a general role in adherens junction biology, mutations would be expected to cause global disruption of *Drosophila* epithelia.

In mice, an equivalent process controls neurulation. The fusion of the neuroepithelium requires a series of morphological changes that together with cell movements bring the neural folds into apposition. This complicated process can be affected by a wide range of mutations including cytoskeletal proteins and adhesion molecules. Combinations of both *mena/profilin-1* and *mena/vasp* knockouts cause defects in neurulation that result in exencephaly (Lanier et al., 1999; Menzies et al., 2004). The existence of exencephaly is consistent with a role for Ena/VASP proteins in dorsal closure; however, the complexity of neurulation and the lack of an available *in vitro* model for the process can not rule out Ena/VASP functioning in a morphological change rather than in cell:cell adhesion.

### **Immune System**

Immune cell activation and function require actin reorganization. Engagement of the T Cell Receptor (TCR) by MHC/antigen on an Antigen Presenting Cell (APC) induces a well-described cascade of tyrosine and serine/threonine kinases. Essential to the complete activation of a T cell is the establishment of an immunological synapse in which an actin-rich cup forms beneath the engaged TCR and nearby integrins are activated to stabilize T cell:APC interaction (Ryser et al., 1982; Valitutti et al., 1995). The importance of actin rearrangement in T cell function is reflected by mutations in WASP (Wiskott-Aldrich Syndrome Protein), a regulator of Arp2/3 activity, resulting in an X-linked immunodeficiency (Nonoyama and Ochs, 1998). The potential link between Ena/VASP proteins and immune function is suggested by several pieces of evidence. VASP and EVL are strongly expressed in immune cells including T cells, and Mena

has been reported to be expressed in B cells(Lanier et al., 1999; Tani et al., 2003). The T cell adaptor protein Fyb/SLAP/ADAP is phosphorylated downstream of TCR engagement, binds Ena/VASP proteins through an EVH1-binding motif, and binds WASP, suggesting the existence of a regulatable complex controlling actin assembly. Finally, inactivation of Ena/VASP with a dominant negative construct impairs the *in vitro* polarization of actin assembly necessary for T cell immunological synapse formation and macrophage phagocytosis(Coppolino et al., 2001; Krause et al., 2000). Surprisingly, deletion of Fyb/SLAP/ADAP did not affect actin assembly in an *in vitro* T cell activation assay(Peterson et al., 2001). The discrepancy between these results has not been resolved but may reflect the presence of an alternate pathway that may activate Ena/VASP proteins.

### **Heart Development**

Mutations within the actin binding components that compose the myocyte sarcomere result in the human cardiomyopathies including hypertrophic and dilated cardiomyopathy. It is believed that the disruption of the sarcomere causes myocardial disorganization dysfunction that causes these diseases. Within a myocyte Ena/VASP localizes via an EVH1 interaction to the intercalated disks that serve to join adjacent myocytes(Eigenthaler et al., 2003). Disruption of Ena/VASP function by the overexpression of an EVH1 transgene causes a dilated cardiomyopathy(Eigenthaler et al., 2003). In comparison with other dominant negative approaches in which a mitochondrial-targeted FPPPP-containing peptide is used to sequester Ena/VASP on the surface of mitochondria, the overexpression of EVH1 may bind to a variety of FPPP-containing proteins potentially disrupting their function. Recently a role for Semaphorin/Plexin signaling in the development of the heart was reported(Toyofuku et al., 2004). The myocardial architecture of the heart is defined by two parameters: wall thickness and

trabeculation. The Abl-dependent phosphorylation of Mena regulates Mena's interaction with Sema6D and controls the thickness and trabeculation of the myocardium (Toyofuku et al., 2004). The study demonstrates a biochemical interaction of Mena, but not VASP or EVL, with Sema6D, suggesting a unique role for Mena in heart development. However, this potential role is undermined by the lack of a heart phenotype in the *mena* knockout mouse.

### **Summary**

Ena/VASP proteins directly affect the architecture of the actin cytoskeleton, localize to focal adhesions, and are targets for tyrosine and serine/threonine kinases. *In vivo*, Ena/VASP proteins function downstream of axon guidance receptors in *Drosophila* and *C. elegans* to translate signals from extracellular ligands into directed cell motility. In mice, construction of the *mena*, *vasp*, and *mena/vasp* double-knockout mice have uncovered roles for Ena/VASP proteins in forebrain commissure formation, neurulation, and platelet function. Numerous additional roles have been assigned to Ena/VASP proteins based upon *in vivo* experiments using questionable dominant interfering strategies or *in vitro* assays. The generation of the *mena/vasp/evl* triple-null mouse will allow the complete description of Ena/VASP function in nervous system (Chapter 2) and extra-nervous system functions (Chapter 3). Future work to identify family-member and splice-isoform specific functions will require the construction of additional transgenic and knockout mice. This process will be greatly enhanced by a lentiviral system for RNAi (Chapter 5).



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*Chapter 2:*

**Requirements for Ena/VASP proteins in cortical organization, neuritogenesis and axon outgrowth suggest analogous functions with integrins in the developing nervous system**

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Adam Kwiatkowski and this author contributed equally to this work. The data in Figure 1 was generated by Adam V. Kwiatkowski and Jon Leslie. Jiangyang Zhang and Susumu Mori contributed Figure 4 in work performed at Johns Hopkins University. Reinhard Fässler provided the VASP knockout mouse. Erik W. Dent supplied the data in Figures 3 and 6A-E,G. Roderick T. Bronson assisted in analyzing the mouse pathology. All mouse work and other data in this chapter was generated by Adam V. Kwiatkowski and this author. The text was generated by Adam V. Kwiatkowski, Frank Gertler and this author.

## Abstract

A newly born neuron must migrate to its correct location within the developing nervous system and elaborate axons and dendrites that connect with their synaptic targets. Previously, analysis of mice lacking Mena and VASP uncovered roles for Ena/VASP in the midline guidance of forebrain axons (Lanier et al., 1999; Menzies et al., 2004). We report here the generation of an Ena/VASP-deficient nervous system through the disruption of the remaining Ena/VASP family member, EVL. Neurons lacking all three Ena/VASP family members fail to elaborate axons both *in vivo* and *in vitro* in the absence of the extracellular matrix (ECM) component laminin. In the presence of laminin, axons are initiated but are defective in outgrowth. Finally, brains from these mice possess numerous ectopic neuronal outgrowths resembling those observed in mice with disruptions in laminin (Halfter et al., 2002) or its integrin receptors (De Arcangelis et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001).



## Introduction

Nervous system development requires the coordination of neuronal differentiation, neuronal migration, axogenesis, axon extension, axon guidance and synapse formation. As described in the previous chapter, Ena/VASP proteins regulate the actin cytoskeleton and function in signaling cascades directing axon guidance. This chapter will focus on Ena/VASP function in the nervous system including neuronal migration, neuritogenesis and axon extension. To avoid redundancy in text, the reader is referred to chapter 1 for introduction to axon guidance and general background on Ena/VASP proteins.

### Cortical Neuronal Migration

Newborn neuroblasts migrate outward from the proliferative ventricular zone along radial glia to establish the mature 6-layered cortex. During cortical plate development, newer born neuroblasts migrate beyond their predecessors to establish the outer cortical layers. Two major classes of human congenital disorders affect cortical organization, lissencephaly and cobblestone cortex. In addition, a spontaneous mouse mutation *reeler* causes disruption of motor coordination and inversion of cortical layering. The identification of the genes causing these disorders and their analysis in mice has elucidated the basic pathways underlying cortical development.

Human lissencephalies are defined by the gross absence of cerebral gyri in humans (mice do not possess cortical folds) and the histologic disruption of cortical layering (Olson and Walsh, 2002). Superficial to the cortical plate is a relatively neuron-free marginal zone. The secretion of Reelin (product of the *Reln* gene that is mutated in the *reeler* mouse) by the Cajal-Retzius (C-R) cells within the marginal zone directs the outward migration of neuroblasts through its action at the LDL-receptors VLDLR and ApoER2 (D'Arcangelo et al., 1999; Hiesberger et al., 1999).

Downstream signaling through Dab1 affects cytoskeletal dynamics through unknown mechanisms(Howell et al., 1999).

Mutations in several cytoskeletal proteins, such as filamin, result in cortical migration defects in mice and humans(Couillard-Despres et al., 2001; Fox et al., 1998). Interestingly, inactivation of Ena/VASP proteins in individual neuroblasts by retroviral infection *in utero* resulted in the inappropriate migration of infected cells beyond the appropriate layer(Goh et al., 2002).

Mutation of *C. elegans* Unc-34 also causes misguided neuronal migration(Forrester and Garriga, 1997; Withee et al., 2004). These results suggest a role for Ena/VASP proteins in coordinating neuronal migration.

Neuroblast migration beyond the pia mater generates ectopias causing the bumpy appearance of cobblestone cortex. The etiology of cobblestone cortex involves disruptions of pial membrane integrity(Yamamoto et al., 1997). The pia mater is generated by adherens junctions between radial glia endfeet and adhesion between glial endfeet and the extracellular matrix (ECM).

Mouse knockouts of the ECM component laminin or its neuronal integrin receptors ( $\alpha_6\beta_1$  and  $\alpha_3\beta_1$ )(Delwel et al., 1996; Mercurio and Shaw, 1991) cause cobblestone cortex and show immunohistochemical evidence of radial glia endfoot and ECM disorganization(De Arcangelis et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Halfter et al., 2002).

Furthermore, disruption of the intracellular signaling molecule *focal adhesion kinase (FAK)* in either radial glia or meningeal fibroblasts also generates a cobblestone cortex(Beggs et al., 2003).

The pial gaps that result allow C-R cell placement outside of the pia which directs the invasion of neuroblasts to form ectopias(Halfter et al., 2002).

### **Axogenesis and Axon Outgrowth**

The process by which neuroblasts establish cell polarity to generate an axon has been extensively modeled *in vitro* (Dotti et al., 1988). Neurons progress from the elaboration of a lamellar and filopodial veil (Stage 1) to the outgrowth of minor processes (Stage 2) with the eventual selection and extension of a single process to generate an axon (Stage 3) (Dotti et al., 1988). The initial establishment of neuronal processes (Stage 1 to Stage 2) appears to involve reorganization of the cytoskeleton from the actin-based protrusion of lamellapodia and filopodia to the microtubule-based protrusion of cell processes (Yu et al., 2001). The preponderance of research on neuronal polarity has focused on the selection of an axon during Stage 2 to Stage 3 transition (Andersen and Bi, 2000; Bradke and Dotti, 2000; Inoue et al., 2005; Nishimura et al., 2004; Shi et al., 2003). The physical extension of an axon, axon outgrowth, is essential to both the formation and eventual guidance of an axon to its target. *In vitro*, outgrowth can be enhanced by the ECM component laminin (Lein et al., 1992) as well as signaling by neurotrophins and netrins (Gates et al., 2000; Loeb et al., 1991; Serafini et al., 1994). A recent report suggests that Netrin/DCC and neurotrophin/Trk stimulated outgrowth depends upon the NFAT family of transcription factors (Graef et al., 2003). Disruption of the NFAT c2/c3/c4 genes prevented axon outgrowth *in vivo* and *in vitro* but did not disrupt neuronal differentiation (Graef et al., 2003). The transcriptional program turned on by NFAT signaling necessary for outgrowth remains to be determined.

### **Role of Integrins and ECM in axon guidance**

Significant crosstalk exists between axon guidance factors and integrin/ECM signaling. Netrins contain structural homology to the ECM component laminin (Ishii et al., 1992; Serafini et al.), and can bind integrins to mediate development outside of the nervous system (Yebra et al., 2003). Conversely, laminin can influence axon guidance (McKerracher et al., 1996) such as by

converting Netrin-mediated axon attraction to repulsion *in vitro* (Hopker et al., 1999). Finally, integrins can modulate the signaling by slits (Stevens and Jacobs, 2002) and function as a receptor for semaphorins (Pasterkamp et al., 2003) in axon guidance.

### **Summary**

Ena/VASP proteins have been implicated as sites of convergence in axon guidance signaling cascades (Lebrand et al., 2004) and are associated with sites of cell:cell and cell:ECM junctions (Bachmann et al., 1999; Reinhard et al., 1995; Vasioukhin et al., 2000). Disruption of Mena, alone or in combination with VASP, causes defects in axon guidance at the midline (Lanier et al., 1999; Menzies et al., 2004) but does not affect neuronal migration, axogenesis or axon outgrowth. To determine the complete spectrum of Ena/VASP activities in the nervous system, we have knocked out the remaining Ena/VASP family member, EVL, and generated embryos lacking all three Ena/VASP proteins. The subsequent chapter will discuss Ena/VASP loss of function phenotypes observed elsewhere in the mouse.

## Results

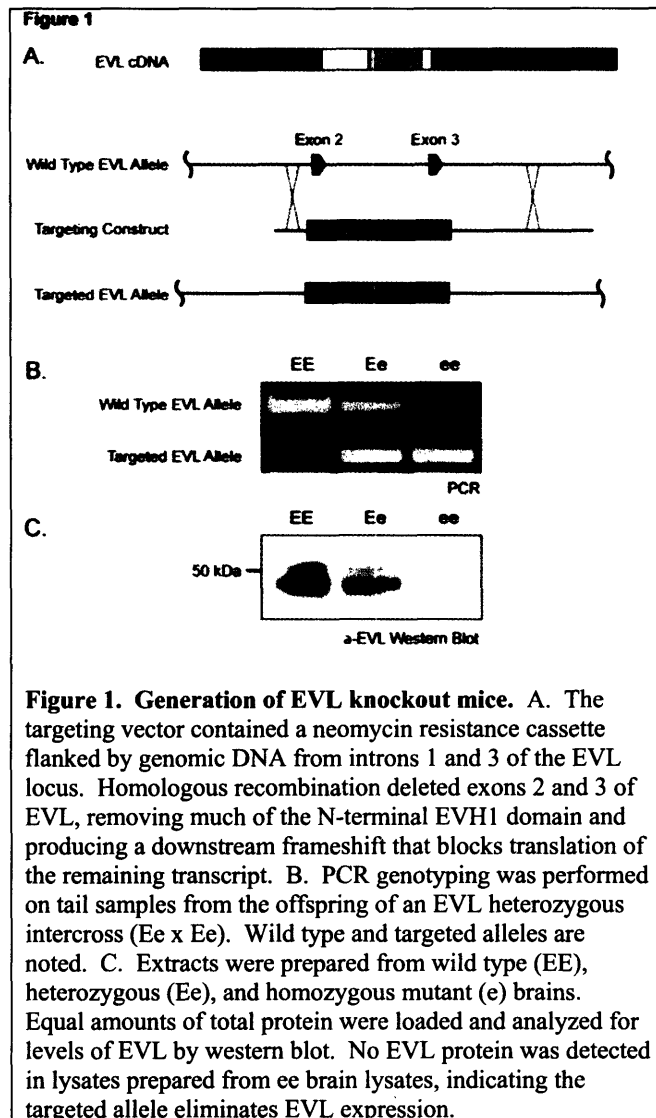
### Generation of EVL-deficient Mice

To investigate the requirement of Ena/VASP proteins in nervous system development, we generated a targeted disruption of the *EVL* locus. Exons 2 and 3 of *EVL* were replaced with a cassette encoding neomycin resistance

(Fig. 1A), deleting most of the NH<sub>3</sub>-terminal coding region of EVL and producing a frameshift downstream. The *EVL* locus was targeted in embryonic stem cells, and recombination determined by PCR and Southern blot (data not shown).

Five independent targeted ES clones were isolated and used to generate chimeric mice. Chimeric mice experiencing germline transmission were used to generate heterozygous mice that were then intercrossed to produce mice homozygous for the mutant EVL allele. Mice homozygous for the EVL disruption are fully viable and observed at the expected Mendelian ratios (data not shown). Gross

morphological analysis of mice lacking EVL did not reveal any obvious phenotypes. Western blot analysis of brain lysates from wild type (EE), heterozygous (Ee), and homozygous mutant



(ee) mice, as determined by PCR genotyping (Fig. 1B), was performed to determine if the targeted locus eliminated EVL protein expression. As expected, expression of EVL was reduced in the heterozygous mutant animal and eliminated in the homozygous mutant animal (Fig. 1C).

### **Ena/VASP proteins are required for proper DRG and trigeminal nerve formation**

EVL knockout mice were crossed to existing Mena and VASP mutant lines. Viable Ena/VASP triple mutant combinations were then mated to each other to generate mice null for all Ena/VASP proteins (mmvvee). Ena/VASP-deficient mice were never observed as adults; however, the expected number of mmvvee embryos were recovered at E10.5. The majority (83%) of mmvvee embryos displayed defects in neural tube closure, a defect noted previously in embryos lacking both Mena and VASP {Menzies, 2004; discussed in Chapter 3}. Occasionally, mmvvee embryos were isolated that appeared smaller and possibly developmentally delayed when compared to littermate controls. Although we can not formally exclude the possibility, the phenotypes reported below for mmvvee embryos are likely not the simple consequence of developmental delay. The *in vivo* phenotypes discussed in this study were observed consistently throughout development and were present in both normal-sized and the smaller embryos. The *in vitro* analyses discussed below were conducted at both E13.5 and E14.5 and equivalent results were observed in both sets of experiments.

Whole-mount neurofilament staining was performed on E10.5 mmvvee embryos to visualize the developing nervous system. Significant defects in dorsal root ganglia (DRG) development and spinal nerve outgrowth were observed in mmvvee embryos (N=4) (Fig. 2, A-D). Though present, the DRGs in mmvvee embryos appeared poorly formed based on neurofilament staining at E10.5 (Fig. 2D), and were significantly smaller and less well developed at E14.5 (Fig 2J, white arrows) compared to control littermates (Fig. 2I, white arrows).

**Figure 2**



**Figure 2. Loss of Ena/VASP causes severe defects in spinal nerve formation.** (A-F) Whole-mount immunostaining with anti-neurofilament antibody was performed to visualize the developing nervous system in E10.5 embryos. Compared to control littermates (A, C, E), mmvvee embryos (B, D, E) showed severe defects in spinal nerve (white arrows in C and D) and trigeminal nerve outgrowth (white arrows in E and F). The three branches of the trigeminal ganglia are labeled in the control (e): ophthalmic (op), maxillary (mx), and mandibular (md). Note the lack of outgrowth of all three nerves, particularly the ophthalmic, in the triple null embryo (F). (G, H) Transverse sections through E10.5 control (G) and mmvvee (H) embryos stained with anti-βIII-tubulin and counterstained with hematoxylin. Dorsal is up. Spinal nerve outgrowth and development was severely limited in mmvvee embryos (black arrows, H) compared to littermate controls (black arrows, G). In addition, defects in commissural axons reaching the floor plate (fp) were observed frequently in mmvvee embryos (black asterisk, G and H). (I, J) Spinal cords and attached dorsal root ganglia (DRG) were dissected from control (I) and mmvvee (J) E14.5 embryos. Though present, DRGs (white arrows) were smaller and poorly developed in mmvvee embryos compared to littermate controls.

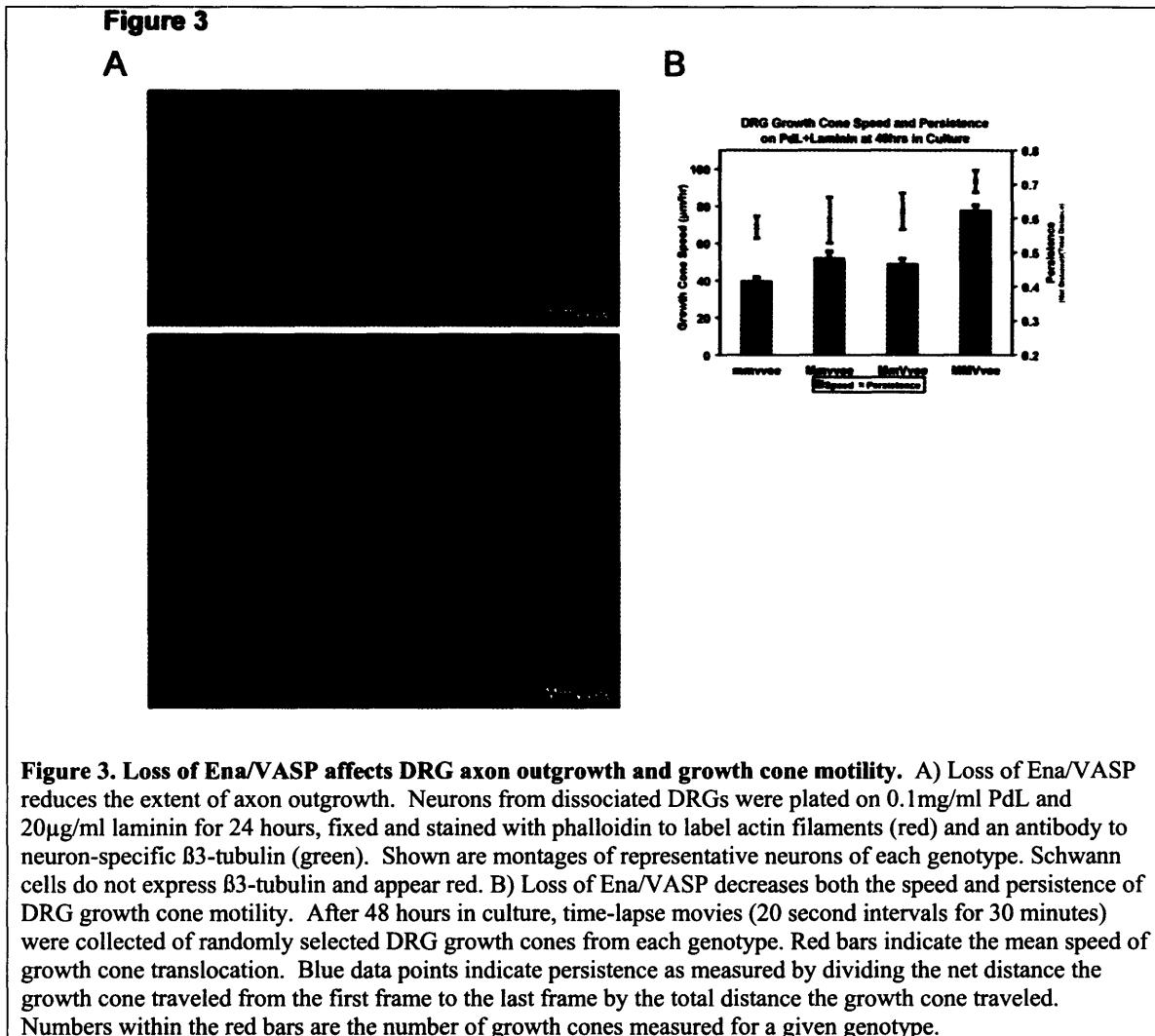
Peripheral axonal projections from the DRGs were severely shortened in mmvvee embryos, and in some instances, appeared misrouted (Fig. 2D, white arrows).

Profound defects were also noted in the axonal projections of the trigeminal ganglia in mmv<sup>vee</sup> embryos (Fig. 2, E and F). Some neurite outgrowth from the three branches - the ophthalmic, maxillary and mandibular - was observed, but the axonal projections were severely stunted, particularly the projections forming the ophthalmic branch (Fig. 2F). Defects in trigeminal ganglia and spinal nerve development were far more severe in mmv<sup>vee</sup> embryos compared to littermate embryos possessing one Ena/VASP allele (e.g. mmV<sup>vee</sup> and mmv<sup>Ee</sup>), indicating that all three vertebrate Ena/VASP proteins participate in nervous system development (see below).

Outgrowth defects observed in mmv<sup>vee</sup> embryos did not appear to arise from neurons failing to differentiate, as similar levels of B3-tubulin staining were observed in transverse sections through the DRGs of mmv<sup>vee</sup> and control embryos (Fig 2, G and H). As neurons were able to differentiate, we cannot account for the smaller size of DRGs in mmv<sup>vee</sup> embryos, but it may reflect a defect in sensory neuron migration to the developing DRG. B3-tubulin staining did provide further evidence for the lack of DRG axonal outgrowth of mmv<sup>vee</sup> mutants compared to control littermates (Fig 2, G and H, black arrows). A potential defect in commissural axon growth was also noted. In control embryos, commissural axons were observed at the floor plate (fp) and crossing the midline (Fig 2G, black asterisk). However, in mmv<sup>vee</sup> embryos commissural axons are rarely observed at the floor plate (Fig 2H, black asterisk). This defect will be explored further using specific markers for commissural axons, such as TAG-1.

To explore the defect in axonal outgrowth further, DRGs were isolated from mutant embryos, dissociated, and neurons cultured *in vitro*. Neurite outgrowth was observed in DRG neurons isolated from mmv<sup>vee</sup> embryos (Fig. 3A, top panel), but axonal length was significantly reduced compared to the littermate MmV<sup>vee</sup> control (Fig. 3A, bottom panel). These





observations are consistent to what was observed *in vivo*, and provide further support for the role of Ena/VASP proteins in axonal extension. These results also strongly suggest the defects observed are cell autonomous, given the low number of non-neuronal cells in dissociated cultures.

Ena/VASP proteins regulate cell motility and axon guidance, and play a critical role in controlling growth cone morphology. Therefore, we questioned whether loss of Ena/VASP affected growth cone motility. Time lapse movies of DRG growth cones revealed that loss of

Ena/VASP decreased both the speed and persistence of growth cone movement (Fig. 3B). A marked reduction in actin bundles and filopodial production in growth cones of *mmvvee* neurons was also observed (data not shown), consistent with a previous report implicating Ena/VASP function in filopodial formation (Lebrand et al., 2004).

### **Loss of Ena/VASP causes defects in neuronal migration and fiber tract formation in the developing brain**

Defects observed in E10.5 *mmvvee* embryos suggested a role for Ena/VASP proteins in axonal outgrowth in the peripheral nervous system. Past work has clearly defined a role for Ena/VASP proteins in CNS development. To determine how loss of Ena/VASP affects CNS development, we analyzed triple null embryos later in development. Approximately 75% of *mmvvee* embryos survive to E16.5, but the majority of these embryos are exencephalic, precluding analysis of brain development. *mmvvee* embryos typically died between E16.5 and E18.5 from cardiovascular defects (discussed in Chapter 3). We were, however, able to collect a few non-exencephalic *mmvvee* embryos at E16.5 and older. Since mutant embryos were limiting, we applied a combination of MRI and classic histological techniques to probe for defects in brain development.

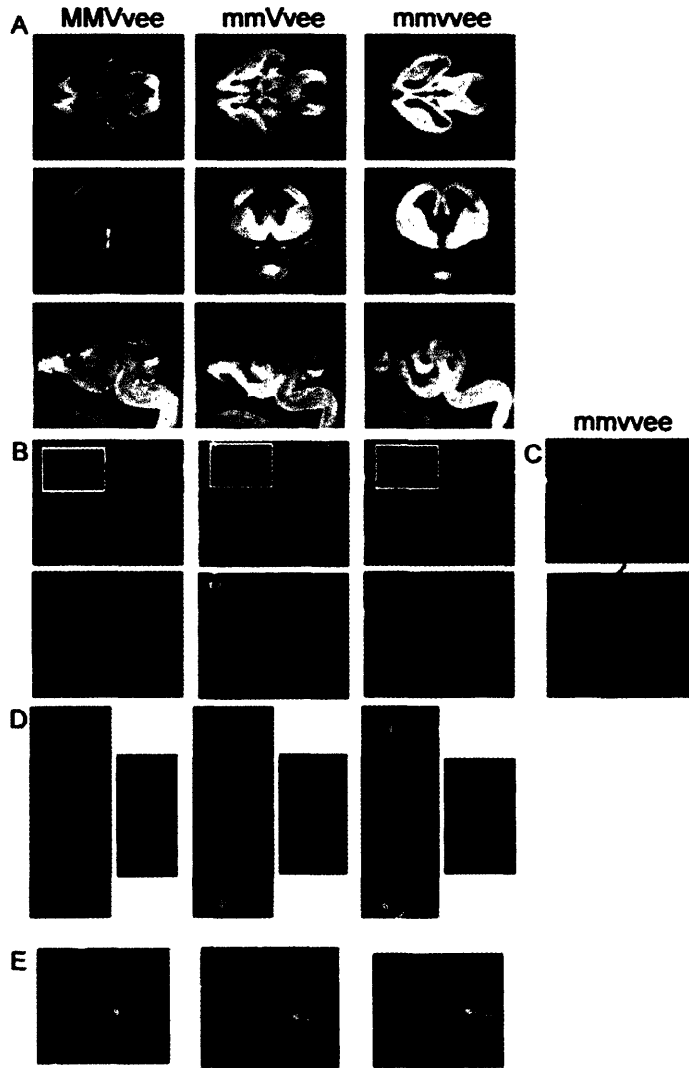
MRI provides a non-invasive method to examine brain morphology in three dimensions. New MRI techniques such as diffusion tensor microimaging ( $\mu$ DTI) permit increased resolution of morphological structures, and have been used successfully to visualize cortical layering and fiber tract formation in the mouse brain (Mori et al., 2001; Zhang et al., 2002). Three E16.5 embryo littermates – *MMVvee*, *mmVvee*, and *mmvvee* - were analyzed using a combination of standard MRI (Fig 4A) and  $\mu$ DTI (Fig 4, B-E) techniques.

A number of striking defects were observed in both mmvvee and mmVvee brains, though all defects observed were more severe in the mmmvee brain. Both brains were hydrocephalic, possessing enlarged ventricles (Fig. 4A, black regions in center of brain). Hydrocephaly was accompanied by a significant reduction in brain matter, particularly in the mmvvee brain. Histological examination of additional mmvvee brains revealed similar defects in ventricle size and total brain matter with no evidence of widespread apoptosis in the developing brain (data not shown).

Major defects in cortical development were observed in the Ena/VASP-deficient brain. Tensor imaging revealed that the intermediate zone was poorly developed and disorganized in the mmvvee brain, and thin in the mmVvee brain compared to the MMVvee littermate (Fig. 4B, orange arrow in bottom panels points to intermediate zone). In addition, the ventricular zone appeared thin in some regions in the mmvvee brain (data not shown). H/E staining of mmvvee brains revealed that the intermediate zone was present, but lacked cellularity and showed signs of disorganization compared to control, and that the ventricular zone was thin in areas (Fig. 5, G and H). Cortical layering appeared largely intact in mmvvee brains, with the exception of the ectopias discussed below (Fig. 4C, blue arrows). The intermediate zone is comprised largely of axons extending from the cortical plate, and the defects observed could be indicative of a defect in axonal extension.

Strikingly,  $\mu$ DTI analysis revealed the presence of extra-cortical layers (ectopias) throughout the cortex in the mmvvee brain. Ectopias are produced by the aberrant migration of neurons across the basal lamina, and are hallmarks of the human congenital disorder cobblestone cortex. Ectopias were identified clearly by H/E staining (Fig. 4, B and D), and observed in all late stage non-exencephalic brains studied (n=4), but never in brains from embryos expressing

Figure 4



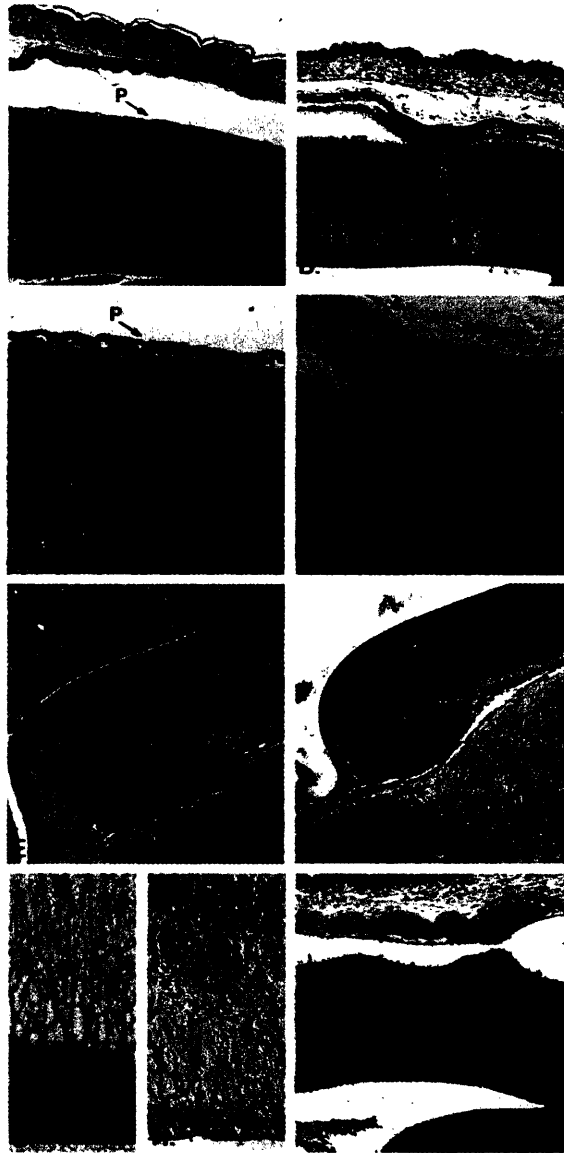
**Figure 4. MRI reveals structural defects Ena/VASP-deficient brains.** A) Horizontal (top row), coronal (middle row) and sagittal (bottom row) T<sub>2</sub>-weighted MRI images of E16.5 brains. Hydrocephalus is evident in mmVvee and mmvvee brains in comparison to control (MMVvee) with enlargement of all ventricles (black regions within brain). Increase in ventricle size correlates with a reduction of brain matter particularly in the midbrain. B)  $\mu$ DTI imaging of a coronal sections reveals disorganization within the intermediate zone. Magnification of the area enclosed in the white box reveals a well-defined cortical plate (green arrow), intermediate zone (orange arrow) and ventricular zone (purple arrow) in the MMVvee brain. The thickness (distance between cortical plate and ventricular zone) and organization (green signal in control intermediate zone indicates alignment of tissue) of the intermediate zone is decreased in the mmVvee and mostly absent in the mmvvee brain. C) Triple-null brains exhibit Type-II lissencephaly. In addition to the three cortical layers, extracortical layers (blue arrows) can be seen in multiple planes from an mmvvee brain. D)  $\mu$ DTI reveals a defect in optic chiasm formation in embryos lacking Ena/VASP. The optic nerves from an mmvvee brain are poorly organized and fail to generate an optic chiasm. The optic nerves from an mmVvee brain generate a poorly formed chiasm in comparison to the MMVvee brain. E) Hippocampal fiber tracts (yellow arrow) are observed in both mmvvee and mmVvee brains, but are not as well defined as in the MMVvee brain.

one or more *Ena/VASP* allele (data not shown). B3-tubulin staining demonstrated that these ectopias were, as expected, comprised of differentiated cortical neurons that migrated past the Pial membrane and accumulated in the arachnoid space. (Fig. 5I). In many instances, the ectopic neurons appeared to spread into or along the arachnoid membrane, sometimes migrating well past the point of escape (Fig. 5, B and D). Finally, H/E staining suggested that these ectopic neurons extend axons across the Pial membrane and marginal zone and into the poorly formed intermediate zone (Fig. 5D, black arrows). Additional staining experiments are needed to determine if these ectopic neurons are projecting axons into the cortex.

Additional brain structures were poorly formed in brains from *mmvvee* embryos.  $\mu$ DTI imaging indicated the layered structure of the hippocampus was poorly developed in *mmvvee* brains, and the dentate gyrus was not well defined (data not shown). H/E staining confirmed that the hippocampus was not well developed (Fig. 5F). Interestingly, though poorly organized, numerous fiber tracts were observed emerging from the hippocampus by MRI to form the ventral hippocampal commissure fiber tract (Fig. 4E), although the ventral hippocampal fiber tract failed to reach the midline. In contrast, we were unable to identify fiber tracts for the other major forebrain commissures, including the corpus callosum and the anterior commissure (data not shown). Mice lacking *Mena* and *VASP* also fail to form forebrain commissures, but misguided fiber tracts could be identified for all of the major commissures (Menziés et al., 2004). In addition, the cerebellum was small, lacked some convolution, and was devoid of most white matter (data not shown). Also, the amygdala was reduced in size (data not shown).

Dosage-sensitive defects in optic nerve outgrowth and optic chiasm formation were observed in *mmVvee* and *mmvveee* embryos. The optic chiasm is an X-shaped structure found at the midline that is formed by the posteriorly projecting axons of retinal ganglion cells. In the

**Figure 5**



**Figure 5. Loss of *Ena/VASP* affects brain development** A-D) Ectopias are observed in mmvvee brains. H/E staining of a control E18.5 embryo brain shows normal cortical layering (A, higher magnification in C). In mmvvee brains, neurons born in the ventricular zone (VZ) have migrated beyond the cortical plate (CP), through the marginal zone (MZ) and across the pial (P) membrane where they accumulate in large clusters (D, higher magnification in D). In some instances, these clusters appear to send out processes, presumably axons, back across the marginal zone and cortical plate and into the intermediate zone (IZ) (black arrow in B, higher magnification in D). E,H) mmvvee brains show defects in hippocampus formation. Compared to control (E), the hippocampus in mmvvee brains appears disorganized and poorly formed. G, H) Cortical layering defects in mmvvee brains. The VZ is extremely thin and the intermediate zone shows signs of disorganization and a lack of cellularity in mmvvee mutants (H, control in G). I) B3-tubulin staining confirms that the ectopias are composed of differentiated neurons in mmvvee brains.

mmV<sup>vee</sup> mutant, the optic nerves were formed and projected into the brain, but inappropriately stopped at the midline and failed to generate the optic chiasm (Fig. 4D, middle panels). This phenotype is more severe in the mmv<sup>vee</sup> mutant, where the optic nerves appear thinner and never reach the midline. A more subtle defect in optic chiasm formation was previously reported in mice lacking Mena (Menzies et al., 2004), and these results provide further evidence that Ena/VASP proteins are required for optic nerve and optic chiasm formation.

### **Ena/VASP proteins are required for cortical neuron development**

The misorganization of the intermediate zone observed in the mmv<sup>vee</sup> brain suggested potential defects in cortical neuron axon outgrowth. To explore this possibility further, we cultured cortical neurons from mmv<sup>vee</sup> brains *in vitro*. Due to the lack of non-exencephalic mmv<sup>vee</sup> brains, neurons were isolated and cultured from exencephalic mutants. Cortical neurons isolated from exencephalic mutants behaved similar to neurons isolated from non-exencephalic mutants of the same genotype, and when differentiated, assumed the same morphology as cortical projection neurons (data not shown).

Cortical neuritogenesis in culture follows a well defined set of stages. Stage 1 cortical neurons display extensive lamellipodia and filopodia along their periphery. Stage 2 neurons possess multiple minor processes, but have not established an axon. Stage 3 neurons have extended a single axon (Fig. 6A). Intriguingly, EGFP-Mena expressed in cortical neurons localizes to the tips of actin-rich lamellipodia and filopodia in Stage 1 and Stage 2 of development (Fig. 6A). The majority of wild type neurons typically reach stage 3 of development after 48 hours in culture. Cortical neurons were isolated from either E13.5 or E14.5 mutant embryos, plated onto poly-D-lysine coated coverslips, and scored for developmental stage after 48 hours in culture. Strikingly, the majority (74%) of cortical neurons from mmv<sup>vee</sup> mutants

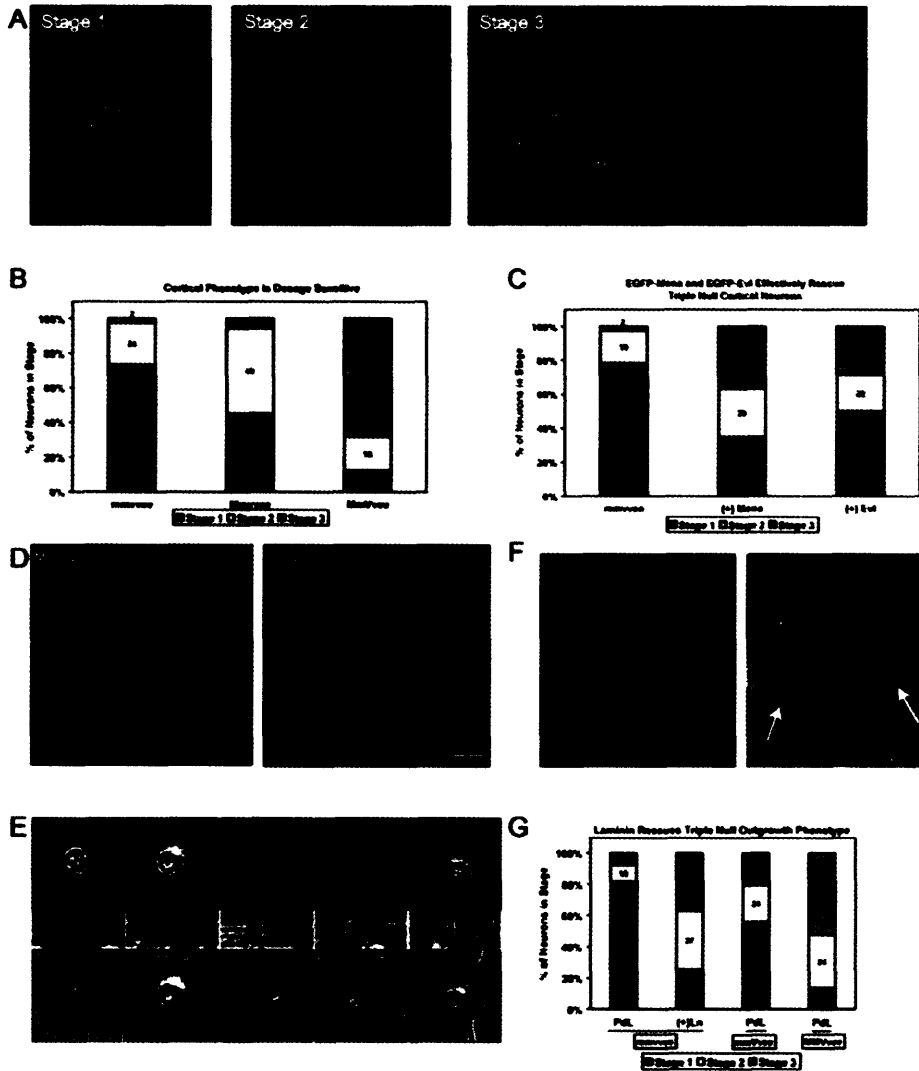
were observed in stage 1, with only 2% of neurons reaching stage 3 (Fig 6B). This was in sharp contrast to neurons from an MmV<sup>vee</sup> littermate, where 69% of neurons developed to stage 3 (Fig. 6B). The defect in mmv<sup>vee</sup> cortical neurons was largely a failure to develop from stage 1 to stage 2, defined as neurite initiation or neuritogenesis, as similar levels of stage 2 neurons were observed in all three genotypic classes (Fig. 6B). Neurons from an Mmv<sup>vee</sup> mutant displayed an intermediate phenotype, suggesting the defect was sensitive to Ena/VASP dosage (Fig. 6B). Furthermore, Mena was the most critical allele for neuritogenesis, as one allele of Mena promoted maturation more effectively than one allele of either VASP or EVL (see below and data not shown).

We attempted to rescue the defect in mmv<sup>vee</sup> neuritogenesis by restoring Ena/VASP function. EGFP-Mena was transfected into mmv<sup>vee</sup> cortical neurons immediately after dissection, and stage development of EGFP-expressing neurons scored and compared to untransfected neurons. Expression of EGFP-Mena was sufficient to restore cortical neuritogenesis to levels observed in neurons expressing two or more alleles of Ena/VASP (Fig. 6C). Consistent with Mena being the most critical allele for development, expression of EGFP-EVL rescued neuritogenesis, but was less effective than EGFP-Mena (Fig. 6C).

To determine the nature of the developmental defect in Stage 1 mmv<sup>vee</sup> neurons, we analyzed the cellular morphology and membrane dynamics of mmv<sup>vee</sup> cortical neurons. Usually few and occasionally no filopodia were observed in fixed stage 1 neurons, and there was a marked reduction in actin bundles along the periphery of the cell (Fig. 6D). The architecture of both the peripheral actin and the central microtubule cytoskeletons appeared disorganized (Fig. 6D). Lamellipodial and filopodial dynamics of stage 1 neurons were measured by time-lapse video microscopy. Kymographic analysis revealed a marked decrease in protrusive activity in



**Figure 6**



**Figure 6. Loss of Ena/VASP from cortical neurons inhibits axon formation.** A) Stages of cortical neuron development *in vitro* plated on poly-d-lysine (PdL). Cortical neurons expressing EGFP-Mena (green), stained with phalloidin to label actin filaments (red) and labeled with an antibody to tyrosinated tubulin (blue) to label microtubules. Stage 1 neurons exhibit extensive lamellipodia and filopodial-rich periphery with no processes. Stage 2 neurons possess one or more minor processes but have not yet extended an axon, while stage 3 neurons have extended a single axon. B) Cortical axon formation is sensitive to Ena/VASP dosage. Cortical neurons were scored for developmental stage after 48 hours in culture. Progression from Stage 1 to Stage 3 is enhanced with additional alleles of Mena or VASP. C) Transfection with Mena or rescues cortical neuron development. mmvvee neurons were transfected with a plasmid expressing EGFP-Mena [(+)Mena]. Untransfected neurons in the same dish were used as controls. Transfection of EGFP-Mena rescues cortical development similar to neurons containing one allele of Mena (Mmvvee). D) Stage 1 neurons from mmvvee cortices have a marked decrease in filopodial protrusions. Cortical neurons were stained with phalloidin to label actin filaments (red) and neuron specific  $\beta$ 3-tubulin (green), indicating that these cells are indeed differentiated neurons. E) Dynamics of Stage 1 neurons are sensitive to Ena/VASP dosage. Time-lapse movies of individual Stage 1 neurons were collected at 5 sec intervals for 10 minutes. Representative frames are shown for each genotype (top row). Single pixel wide kymographs (middle row) generated along the lines indicated in the top left panel show protrusions and retractions along those lines during the movie. Arithmetic minima from each frame of the time-lapse were

overlaid (bottom row) showing filopodial and lamellipodial activity over the ten minute time course. F) Expression of laminin-1 in the E13.5 spinal column and brain. Laminin-1 immunoreactivity is limited to the pial membrane surrounding the forebrain and midbrain (left panel) and is present in the DRGs (right panel, white arrows). G) Laminin-1 rescues the Ena/VASP-deficient axon outgrowth phenotype *in vitro*. mmvvee cortical neurons were plated on PdL or PdL supplemented with 20 $\mu$ g/ml laminin-1 [(+)Ln] and scored for developmental stage after 48 hours in culture. Development of mmvvee neurons grown on laminin is similar to MMVvee neurons grown on PdL alone.

mmvvee neurons (Fig. 6E). Intriguingly, the few filopodia that were elaborated in mmvvee Stage 1 neurons were far less motile than those in neurons possessing one or two alleles of Ena/VASP (data not shown). Similar to the developmental defects, lamellipodial and filopodial dynamics were sensitive to the number of Ena/VASP alleles present as increasing the level of Ena/VASP increased protrusive activity (Fig. 6E).

The defect in cortical neuron neuritogenesis *in vitro* might explain the disorganized intermediate zone and lack of axonal outgrowth *in vivo*. However, we observed axonal outgrowth and fiber tract formation in specific regions of the brain, most notably from cortical ectopias, retinal ganglia, and the hippocampus. Axons, although stunted, also formed from DRGs *in vivo* and *in vitro*. Interestingly, it appeared that cortical neurons generated axons from within ectopias but failed to do so from within the cortical plate. We hypothesized that ectopic neurons may be exposed to a different ECM microenvironment. The pial membrane is generated by the fusion of radial glial endfeet and adhesion between the endfeet and laminin in the surrounding ECM. Staining of wildtype brains for laminin demonstrated that laminin is found solely in the surrounding pia but not within the cortical plate (Fig. 6F). In the PNS, laminin expression was also observed within DRGs (Fig. 6F).. We hypothesized that neuritogenesis may occur via two pathways: one requiring Ena/VASP function and the other requiring laminin. To test this hypothesis, we attempted to rescue neuritogenesis with laminin *in vitro*. Cortical neurons from an mmvvee mutant were plated onto poly-D-lysine coated coverslips with or

without laminin. Strikingly, the presence of laminin rescues the Ena/VASP-deficient neuritogenesis defect almost completely (Fig. 6G).

## Discussion

### **Ena/VASP Proteins are Required for Neurite Initiation, Axonal Outgrowth, and Neuronal Migration**

Ena/VASP function is required for multiple processes during nervous system development. Loss of Ena/VASP resulted in poorly formed spinal nerves that exhibited reduced outgrowth both *in vivo* and *in vitro*. Reduced outgrowth was also noted in hippocampal and retinal fiber tracts, and the majority of forebrain commissures failed to form in Ena/VASP-deficient brains. Analysis of the cortex revealed a thin ventricular zone and disorganized intermediate zone where cortical fiber tracts were either poorly formed or absent. However, no obvious defects were noted in cell differentiation or cell death. Loss of Ena/VASP function caused a severe form of cobblestone cortex, the possible result of a poorly formed pial membrane and increased cortical neuron migration. Analysis of cortical neuron development *in vitro* revealed Ena/VASP proteins are required for neuritogenesis. This defect could be rescued with laminin, indicating that Ena/VASP protein and laminin function in independent pathways to promote neurite initiation.

This study clearly demonstrates, for the first time, that Ena/VASP proteins are required for neuritogenesis and axon outgrowth. It is interesting to note that the observed defects in the vertebrate nervous system are more severe than might be expected from studies of Ena/VASP function in worm and flies. Multiple genetic studies in *C.elegans* and *D. melanogaster* have shown that loss of Ena/VASP causes cell autonomous defects in axon guidance (Gertler et al., 1995; Gitai et al., 2003; Yu et al., 2002); no defects in neurite initiation or axon extension have ever been reported (discussed in more detail below). In fact, loss of Enabled cause a bypass defect in the ISNb motor axon guidance in *Drosophila* - the motor axon extends too far as it fails

to recognize and respond to a choice point (Wills et al., 1999). One possibility is the activation of an Ena/VASP-independent pathway for neuritegenesis and outgrowth in the fly and worm, similar or identical to the laminin-dependent pathway described below. Also, the most severe defects were observed in the cortex, a mammalian-specific brain structure, suggesting that Ena/VASP proteins have evolved to meet a unique and specific requirement for neural development.

The Ena/VASP-dependent outgrowth defects share some striking similarities to outgrowth defects observed in mice lacking calcineurin-NFAT signaling. The NFAT transcription complex is regulated by calcineurin, and calcineurin-NFAT signaling controls aspects of neuronal morphogenesis (Crabtree and Olson, 2002). Ablation of the NFAT transcription complex proteins NFATc2, NFATc3 and NFATc4 (c2/c3/c4) causes striking defects in DRG and trigeminal ganglion outgrowth as well as defects in commissural axon projection *in vivo*, all similar to loss of Ena/VASP function. Furthermore, cell autonomous defects in sensory axon outgrowth *in vitro* were observed, similar to the reduction in outgrowth observed in mmvvee DRG neurons *in vitro*. Phenotypes observed in NFAT (c2/c3/c4) knockout mice were not due to an increase in cell death or neuron differentiation. Interestingly, netrin was found to stimulate NFAT-dependent transcription through calcineurin and the netrin receptor DCC, and inhibiting calcineurin signaling blocked netrin-dependent outgrowth from DRG explants (Graef et al., 2003). Ena/VASP functions downstream of Netrin signaling during axon guidance (Colavita and Culotti, 1998; Gitai et al., 2003; Yu et al., 2002), and Netrin stimulates Ena/VASP-dependent filopodia formation (Lebrand et al., 2004). Given the evidence presented in this report that Ena/VASP regulated changes in the actin cytoskeleton are required for

neuritegenesis, it is intriguing to speculate that Ena/VASP proteins cooperate with calcineurin/NFAT signaling to promote outgrowth downstream of netrin.

### **Axon Outgrowth and Guidance**

Ena/VASP mutations in the fly and worm primarily cause defects in axon guidance (Colavita and Culotti, 1998; Gertler et al., 1995; Gitai et al., 2003; Wills et al., 1999; Yu et al., 2002). The axons in these mutants form, extend, but take an inappropriate path due to a failure to respond to guidance cues, including the midline guidance cues Netrin and Slit (Bashaw et al.; Colavita and Culotti, 1998; Forsthoefel et al., 2005; Gitai et al., 2003; Yu et al., 2002). In addition to the genetic links with midline guidance pathways, Ena/VASP proteins physically interact with the axon guidance receptors Robo, and Semaphorin 6A (Bashaw et al., 2000; Klostermann et al., 2000). Consistent with this, mice lacking both Mena and VASP fail to generate the major forebrain commissures. The axon tracts from these mice extend towards but fail to reach the midline (Menziés et al., 2004). Filopodia formation in response to Netrin requires Ena/VASP proteins, suggesting a molecular mechanism for Ena/VASP involvement in guidance decisions (Lebrand et al., 2004).

The analysis of axon guidance in mice completely deficient for Ena/VASP proteins is complicated by several factors. First, cortical neurons failed to complete axogenesis and thus did not form fiber tracts (discussed below). Second, the axons that were formed, including spinal nerves and hippocampal fibers, appeared severely stunted, indicative of a defect in axon outgrowth. In support of this, dissociated DRG neurons form shorter neurites when cultured *in vitro*. Microscopic analysis of growth cone dynamics revealed Ena/VASP-dependent defects in the rate of growth cone extension and persistence. These defects exist in the absence of guidance or neurotrophic factors and represent a fundamental defect in growth cone mechanics. Consistent

with this, we observed a decrease in actin bundling and filopodia extension in DRG neurons lacking Ena/VASP proteins. These results suggest that Ena/VASP proteins play an essential role in neurite initiation and extension through their regulation of actin dynamics. Therefore, analysis of specific guidance decisions during development was precluded by Ena/VASP-dependent neurite development.

### **Neuritogenesis in Ena/VASP-deficient mice requires laminin**

Based upon both in vivo and vitro observations, we propose that there are two pathways for neurite initiation, one requiring Ena/VASP proteins and a second that is ECM-dependent. In vivo, we observed a nearly complete agenesis of the major cortical fiber tracts including those forming the corpus callosum in mice lacking all Ena/VASP proteins. This is in contrast with mice lacking only Mena and VASP. While major forebrain commissures were absent in these mice, the axon tracts were present but failed to reach the midline (Menziés et al., 2004). Similarly, mice expressing only a single allele of VASP also formed cortical fiber tracts, suggesting that a complete loss of Ena/VASP expression is required to block axogenesis. Consistent with a failure to observe cortical fiber tracts by MRI, the intermediate zone underlying the cortical plate was disorganized and contained few axons. Interestingly, several non-cortical axon tracts appeared to form but had obvious defects in outgrowth and guidance. Notably, fiber tracts were observed initiating from retinal ganglia, hippocampi, and DRGs. Surprisingly, axonal outgrowths were also noted emerging from cortical neurons present in ectopias. The ability of cortical neurons to complete axogenesis from within an ectopia but not from within the cortical plate strongly suggested an underlying difference in the microenvironment of an ectopic cortical neuron. Ectopic neurons sit on top of the pial basal lamina whose primary ECM component is laminin-1 (Georges-Labouesse et al., 1998). Laminin-

1 has previously been implicated in the extension and guidance of axons (Anton et al., 1999; Lein et al., 1992). Similarly, expression of laminins has been reported in the developing retina, hippocampus and DRGs, but is notably absent from the cortical plate {Georges-Labouesse, 1998; Libby, 2000; Sharif, 2004; De Arcangelis, 1999; and this study}. Laminins are bound by several integrin receptors including  $\alpha_6\beta_1$ ,  $\alpha_6\beta_4$  and  $\alpha_3\beta_1$  (Hynes). Both  $\alpha_6\beta_1$  and  $\alpha_3\beta_1$  are expressed throughout the brain including cortical neurons (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001). The ability of laminin-1 to rescue Ena/VASP-deficient cortical neuron axogenesis *in vitro* further supports the model that either Ena/VASP or integrin signaling support axogenesis. Mice deficient in both  $\alpha_6$  and  $\alpha_3$ , or  $\beta_1$  have been generated and do not possess any defects in fiber tract formation (De Arcangelis et al., 1999; Graus-Porta et al., 2001). This likely reflects the ability of broadly expressed Ena/VASP proteins to compensate for loss of integrin signaling (Lanier et al., 1999; Menzies et al., 2004). We propose that disrupting Ena/VASP activity in  $\beta_1$ -deficient neurons would block axogenesis throughout the nervous system.

### **Molecular insight into neurite initiation**

Ena/VASP proteins regulate the geometry of the actin cytoskeleton in both fibroblasts and neurons. In fibroblasts, Ena/VASP overexpression promotes the formation of long, unbranched actin filaments in extending lamellipodia, whereas loss of Ena/VASP creates short, highly branched networks of actin filaments (Bear et al., 2002). In neurons, overexpression of Ena/VASP increases the number of filopodia and actin bundles in the growth cone, while loss of Ena/VASP decreases filopodia and actin bundles and creates a dense, slightly disorganized actin network (Lebrand et al., 2004). Ena/VASP-dependent alterations in the lamellipodial actin network create profound changes in lamellipodial dynamics and affect cell movement in fibroblasts (Bear et al., 2000; Bear et al., 2002). Therefore, we hypothesize that Ena/VASP-



regulated actin assembly in cortical neurons is required for neurite initiation in the absence of laminin.

The cytoskeletal architecture of stage 1 Ena/VASP-deficient cortical neurons plated on poly-D-lysine was markedly different from control neurons. Wild type Stage 1 neurons display lamellipodia rich with actin bundles and filopodial projections that are unique from the classic fibroblast lamellipodia. In contrast, few if any filopodial protrusions or actin bundles were noted in the lamellipodia of Ena/VASP-deficient neurons. Furthermore, the microtubules appeared fragmented and disorganized. Inhibiting Ena/VASP function in primary hippocampal neurons causes similar defects in actin organization of the growth cone (Lebrand et al., 2004). Thus, the observed defects are consistent with the current model for Ena/VASP function in regulating actin geometry.

How might Ena/VASP-regulated actin bundles and filopodia promote neurite initiation? One possibility is that actin bundles play an important role in supporting microtubule elongation and stabilization. The interplay between actin filaments and microtubules is thought to be critical for axon outgrowth, turning, and guidance (Dent and Gertler, 2003), and has been suggested to play an important role in neurite initiation (Dehmelt and Halpain, 2004). In this model, Ena/VASP proteins promote the formation F-actin bundles along the stage 1 neuronal lamellipodial veil. These actin bundles serve as guides for probing microtubules, and stabilization of microtubules at points along the cell periphery promotes outgrowth and neurite formation. In the absence of bundled actin, microtubule stability would be compromised and outgrowth ablated.

Our results indicate that neurite initiation can occur independent of Ena/VASP proteins through laminin. If the primary function of Ena/VASP in neurite initiation is regulating actin

assembly, specifically the formation of actin bundles, it seems likely that another protein or set of proteins are functioning in place of Ena/VASP to promote the assembly of bundled actin. The formin family of proteins is an attractive candidate for this role. Formins promote actin assembly at the barbed-end of actin filaments and promote filament elongation (Zigmond, 2004) and function downstream of Rho GTPase signaling (Peng et al., 2003; Watanabe et al., 1999). Furthermore, the formin mDia2 has been found to function downstream of Cdc42 and Rif in the formation of filopodia (Pellegrin and Mellor, 2005). As noted above, laminin is bound by multiple integrins, and integrin engagement is known to activate multiple signaling pathways, including Rho GTPase signaling (Hynes, 2002). Rho GTPase activation could in turn stimulate mDia2 or another formin protein and promote actin filament and bundle formation, and thus provide the structural requirements for neurite initiation.

### **Neuronal Migration and Pial Integrity**

Mutation of either ECM components (laminins or perlecan) or their integrin receptors generate cortical abnormalities resembling those observed in the Ena/VASP-deficient brain (De Arcangelis et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Halfter et al., 2002). Cobblestone cortex is also a feature of several human congenital disorders including Fukuyama Muscular Dystrophy (FCMD), Walker-Warburg Syndrome (WWS), and Muscle-Eye-Brain Disease (MEB) (Olson and Walsh, 2002). The genes responsible for these human syndromes encode  $\alpha$ -dystroglycan or proteins involved in its glycosylation (Brockington et al., 2001; Hayashi et al., 2001; Yoshida et al., 2001). Glycosylation of  $\alpha$ -dystroglycan is essential for its binding to laminin and is required for basal lamina integrity (Brockington et al., 2001; Chiba et al., 1997). Mutation of Ena/VASP proteins generates focal ectopias, but through the majority of the cortical plate neurons respect the marginal zone barrier. Histologically, the brain has a

laminin-1 positive pial membrane. Together these results suggest that the basal lamina can form in Ena/VASP-deficient brains, but that it likely has defects in its structural integrity.

How might Ena/VASP-deficiency disrupt basal lamina integrity? Histologic analyses of mouse models of cobblestone cortex have identified defects in both radial glial endfoot organization and disruption of the overlying ECM. Recently, *focal adhesion kinase (FAK)*, has been identified as the first intracellular signaling molecule generating cobblestone cortex (Beggs et al., 2003). Surprisingly, deletion of FAK from either meningeal fibroblasts or radial glia but not neurons was sufficient to generate cortical ectopias (Beggs et al., 2003). This further suggests that the inappropriate neuronal migration in cobblestone cortex is a secondary effect of basal lamina disruption, and that basal lamina disruption can be mediated by cell autonomous defects in either radial glia or fibroblasts (Beggs et al., 2003; Shearer and Fawcett, 2001; Sievers et al., 1994). FAK is a nonreceptor tyrosine kinase that, like Ena/VASP proteins, is associated with integrin-mediated focal adhesions. Through both its tyrosine kinase and protein scaffold activities, FAK is required for focal adhesion integrity and signaling through actin in fibroblasts (Ilic et al., 1995; Sieg et al., 1998). In contrast, fibroblasts lacking Ena/VASP proteins still form focal adhesions and have no obvious adhesive or motility defect (Bear et al., 2000). The generation of a cobblestone cortex may indicate a link between FAK and Ena/VASP signaling downstream of integrin engagement to laminin. An alternative hypothesis for cobblestone cortex is that Ena/VASP proteins function at adherens junctions may disrupt interactions between glial endfeet. Ena/VASP proteins localize to adherens junctions, and have been implicated in their generation and integrity (Egenthaler et al., 2003; Vasioukhin et al., 2000). Further work will be needed to discern between these two possibilities.

Intriguingly, the ectopias observed in both the *Ena/VASP*-deficient and *FAK*-deficient brains are distinct from those seen in integrins or laminin mutants. In the latter brains, neuronal invasion does not extend significantly beyond the marginal zone into the pial membrane. In contrast, disruption of either *Ena/VASP* or *FAK* causes the invasion of neuroblasts beyond the pial membrane into the subarachnoid space, with the inward extension of axons back through the cortical plate into the intermediate zone. However, the ectopias present in *Ena/VASP*-deficient mice showed far greater invasion beyond the pia into the arachnoid space. While the formation of the ectopias reflect defects in pial integrity, the subsequent extensive invasion observed in *Ena/VASP*-deficient mice may reflect a cell autonomous increase in neuronal motility. Such an increase would be consistent with the previously observed enhanced motility of individual neurons lacking *Ena/VASP* proteins in vivo (discussed below)(Goh et al., 2002).

Disruption of *Unc-34/Ena* in *C. elegans* affected the migration of many neurons and disruption of *Ena/VASP* function in individual mouse neuroblasts caused their inappropriate positioning within the cortical plate(Forrester and Garriga, 1997; Goh et al., 2002; Withee et al., 2004). Surprisingly, the cortical plate in *Ena/VASP* deficient brains appears grossly normal; however, we can not rule out subtle neuron autonomous defects in cortical positioning or layering. Our inability to generate sufficient numbers of nonencephalic *Ena/VASP*-deficient brains has thus far precluded neuronal birthdate analysis. Further work will be necessary using both birthdate analysis and employing layer-specific antibodies to determine whether neuronal migration is affected in these brains.

## **Experimental Procedures**

**Generation of EVL knockout mouse.** A BAC mouse genomic DNA clone was obtained from Genome Systems that contained the EVL locus. The targeting vector was constructed by subcloning regions surrounding exons two and three of the EVL locus into the vector pPGKneobpAlox2PGKDTA (a gift from Philip Soriano). The final targeting vector contained a neomycin resistance cassette under the control of the PGK promoter flanked by a 1.0 kb genomic DNA fragment upstream of exon 2 and 5.0 kb fragment downstream of exon 3. The targeting vector was electroporated into early passage R1 embryonic stem (ES) cells. Over 1000 G418 resistant ES colonies were picked and screened for homologous recombination by PCR. 5 clones were identified, and homologous recombination reconfirmed in these clones by Southern blot. All 5 clones were injected into blastocysts to generate chimeric mice. 4 of the 5 clones produced high percentage chimeras, but subsequent breeding revealed that only one clone went germline. This clone was used to establish the EVL knockout line used in this study. To confirm loss of EVL protein, adult mouse brain lysates were probed with anti-EVL polyclonal antibody 1404 by Western blot.

**Embryonic stem cell culture.** R1 ES cells were cultured in DME (Specialized Media) plus 15% ES cell tested fetal calf serum (HyClone) and supplemented with L-Glutamine (Specialized Media), Penicillin/Streptomycin (Specialized Media),  $\beta$ -mercaptoethanol (Specialized Media) and LIF-conditioned media. When necessary, G418 was added to a final concentration of 300  $\mu$ g/ml. R1 cells were cultured on a monolayer of irradiated primary mouse embryonic fibroblasts (MEFs).

**Mouse Colony.** Chimeric mice were initially crossed to C57/B6 mice to determine germline transmission of the mutated EVL allele. Progeny from these crosses were then backcrossed into the inbred lines 129/Sv, C57/B6, and Balb/c. All inbred mouse lines were obtained from Taconic. All experiments described were conducted with mutant mice on a mixed background, primarily a mix of Balb/c and 129/Sv.

Small (less than 1 cm) pieces of tail were cut from P10 mice for genotyping purposes. Genomic DNA was prepared from these tail samples and used for PCR. To genotype embryos, DNA was isolated from either yolk sacs (E10.5) or tails (E12.5-E18.5).

For timed pregnancies, mating pairs were set up in the evening and checked for vaginal plugs the following morning. Plugged females were removed from the mating pair and sacrificed at the appropriate time. The day of the plug was deemed embryonic stage E0.5.

**Whole-mount Neurofilament Staining.** Embryos were collected at E10.5 and fixed in 80% MeOH, 20% DMSO at 4°C overnight. The following day, embryos were bleached in 60% MeOH, 20% DMSO, and 20% H<sub>2</sub>O<sub>2</sub> at room temperature for 6 hours, washed in MeOH and then stored in MeOH at -20°C until staining. For staining, embryos were rehydrated by washing in a series of MeOH in PBS dilutions. After rehydration, embryos were blocked overnight at 4°C in PBS + 0.5% Triton X-100 + 2% nonfat dry milk + 3% normal donkey serum. The following day, anti-neurofilament monoclonal antibody H3 (Developmental Studies Hybridoma Bank) was added at 1:200 and incubated overnight at 4°C. Post-primary antibody washes were performed using PBS + 0.5% Triton X-100 + 2% nonfat dry milk. Embryos were washed at least 6 times for one hour. Embryos were then incubated with preabsorbed donkey anti-mouse antibody conjugated to horseradish peroxidase (HRP) overnight at 4°C. Embryos were washed extensively before labeling with diaminobenzidine (DAB) plus NiCl<sub>2</sub>. After staining, embryos

were dehydrated in EtOH and either stored at -20°C or imaged after being cleared in methyl salicylate.

**Histology and Immunohistochemistry.** Tissues for histology were fixed in either 10% formalin or Bouin's fixative (Electron Microscopy Sciences). Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (using standard techniques). For immunohistochemistry, sections were dewaxed in xylene, rehydrated through ethanol and when appropriate, incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes to extinguish endogenous peroxidase activity. Samples were treated with Retrieval A (pH 6.0) (BD Pharmingen) to improve antigen exposure. Staining was performed using the TSA Biotin System (PerkinElmer Life Sciences) for signal amplification. Sections were blocked in TNB buffer and incubated overnight in antibodies to  $\beta$ III-tubulin (anti- $\beta$ III-tubulin, Promega) and laminin (anti-laminin, Sigma). Following the overnight incubation, samples were washed and incubated for 1 hour with HRP-conjugated anti-mouse or anti-rabbit antibodies. Biotin-labeled tyramide was then added to amplify the signal for 10 minutes. Finally, sections were incubated in streptavidin-HRP (1:1,000) or streptavidin-fluorescein (1:500) (PerkinElmer Life Sciences). Samples labeled with streptavidin-HRP were developed with diaminobenzidine (Vector Labs), counterstained with hematoxylin and mounted. Samples labeled with streptavidin-fluorescein were mounted in Fluoromount-G (Electron Microscopy Sciences). Bright field images were taken on an inverted Nikon microscope using Nomarski optics and recorded with a CCD camera. Fluorescent images were captured on confocal microscope (described in more detail below).

**MRI.** Before imaging, we placed specimens in PBS for more than 24 h to wash out the fixation solution and transferred them into home-built MR-compatible tubes. The tubes were then filled

with fomblin (Fomblin Profluidropolyether, Ausimont, Thorofare, New Jersey, USA) to prevent dehydration.

Imaging was performed using an 11.7 Tesla spectrometer with microimaging gradient unit (300 Gauss/cm maximum). A saddle coil (10mm diameter Bruker Biospin, Billerica, MA, USA) was used as both the radio frequency signal transmitter and receiver. The NMR sequence was based on a 3D fast echo sequence with navigator-echo phase correction scheme and segmented k-space acquisition (Mori et al.) with an echo train length of 4. 3D diffusion-weighted images were acquired with a repetition time of 0.9 s, an echo time (TE) of 25 ms, and four signal averages. The field of view 16 mm × 9 mm × 9 mm and the native imaging resolution was approximately 0.09 mm x 0.09 mm x 0.09 mm. At least six diffusion weighted images with b values of 1000–1200 s/mm<sup>2</sup>. Diffusion sensitizing gradients were applied along six different orientations: [0.707, 0.707, 0], [0.707, 0, 0.707], [0, 0.707, 0.707], [-0.707, 0.707, 0], [0.707, 0, -0.707], [0, -0.707, 0.707]. We also acquired at least one image with a b value of 150 s/mm<sup>2</sup>. The imaging time for DTI was approximate 20 h.

The diffusion tensor was calculated using a multivariate linear fitting method, and three pairs of eigenvalues and eigenvectors were calculated for each pixel (1,2). The eigenvector associated with the largest eigenvalue was referred to as the primary eigenvector. For the quantification of anisotropy, fractional anisotropy (FA) was used. Color map images were generated by combining the images of primary eigenvector and FA into RGB images. In the color map images, the ratio among R(ed), G(reen), and B(lue) components of each pixel was defined by of the ratio of the absolute values of x, y, and z components of the primary eigenvector, and the intensity was proportional to the FA. Red was assigned to the anterior–posterior axis, green to the medial-to-lateral axis, and blue to the superior–inferior axis.



**Plasmids.** For all neuronal transfections, Ena/VASP constructs were cloned into a pCAX vector containing a beta-actin promoter and a CMV enhancer (REF). These vectors contained an EGFP or mCherry (Shaner et al., 2004) sequence N-terminal to Ena/VASP for visualization.

**Cortical and DRG cell culture, Transfection and Immunocytochemistry.** Primary dissociated cortical neurons were prepared from E14.5 mice and cultured in serum free medium essentially as described (Lebrand et al., 2004). For exencephalic embryos, the most anterior regions of cortex were removed. Neurons were initially cultured in 5% fetal bovine serum (FBS) (Hyclone) in Neurobasal Medium (Gibco) with B27 supplements and glutamine, and later switched to serum free medium after one hour in culture. Neurons were plated on coverslips coated with 1.0 mg/ml poly-d-lysine (PdL) (Sigma) at a concentration of 5000 cells/cm<sup>2</sup>. Laminin-1 (BD Biosciences) was added to the cultures at 20µg/ml. Primary dissociated dorsal root ganglion neurons (DRGs) were dissected from E13.5-E14.5 mice and cultured on 0.1mg/ml PdL and 20µg/ml laminin-1 at a concentration of 1000 cells/cm<sup>2</sup> in 5% FBS in Neurobasal medium with B27 supplements and glutamine.

Neurons were transfected in suspension, before plating, with the aid of a Nucleopator (Amaxa) according to manufacturers instructions with the exception that 10-15µg of DNA was used in each transfection.

Neurons were fixed in warmed 4% paraformaldehyde/0.25% gluteraldehyde in Krebs' solution with 0.4M sucrose (Lebrand et al., 2004) to preserve the cytoskeleton and fine morphological features such as filopodia. Cells were blocked in 10% BSA/PBS and extracted with 0.2% Triton X-100 in block. Cultures were stained with Alexa-phalloidin (Molecular Probes) at 1:100 dilution, neuron specific β3-tubulin at 1:1000 (Promega) and tyrosinated tubulin

(clone YL1/2) at 1:1000 (Chemicon). Alexa-conjugated secondary antibodies were used at 1:1000 (Molecular Probes). Neurons were imaged with the aid of TE2000 microscope (Nikon) equipped with a spinning disk confocal head (Yokagawa), a Lambda 10-2 multiposition filter wheel (Sutter) and Orca-ER cooled CCD camera (Hamamatsu). This system was coupled to a Coherent 70C 2.5W multi-band laser. Wavelengths were selected with separate excitation and emission filters (Chroma) in the Sutter filter wheels. Live-cell phase contrast images for morphological quantification and growth cone translocation were collected with an Orca-ER coupled directly to the Nikon TE2000 microscope so as to avoid image dimming and degradation through the spinning disk head. All hardware was controlled by Metamorph software (Molecular Devices).

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## *Chapter Three*

### **Ena/VASP proteins: Roles in Epitheliogenesis and Development**

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The author and Adam V. Kwiatkowski contributed equally to this work. The text was generated by this author. All figures and tables were generated by this author and Adam V. Kwiatkowski except for Figure 4A and 4B which were generated by Craig Furman. All work was conducted in the lab of Frank Gertler.

## **Abstract**

Ena/VASP proteins have conserved roles in regulating the actin cytoskeleton downstream of signaling cascades and are required for a variety of developmental processes. Throughout development, most cells express one or more Ena/VASP proteins. Previous studies *in vitro* and *in vivo* using transgenic mice have led to proposed roles for Ena/VASP in a range of processes including heart development, skin formation, immune cell function and endothelial cell function in addition to their known roles in nervous system development. To investigate the extent of Ena/VASP involvement in these processes we generated embryos lacking all three Ena/VASP proteins. Ena/VASP-deficient embryos suffered from severe subdermal edema and survived until late in embryogenesis before succumbing to intraamniotic hemorrhage. Histological and molecular analysis of the embryos showed defects in endothelial cell function, and epithelial sheet fusion. In contrast to several published reports, we did not observe defects in skin formation or heart development.

## Introduction

Regulation of actin dynamics is essential for cell morphology, polarity and motility. As reviewed extensively in Chapter 1, the Ena/VASP protein family, consisting of *C. elegans* Unc-34, *Drosophila* Ena and the vertebrate Mena, VASP and EVL, has emerged as direct modulators of actin architecture acting downstream of a number of signaling pathways. The protein family shares a common domain structure, overlapping expression patterns, and has interchangeable functions in many *in vitro* cell assays (Bear et al., 2000; Loureiro et al., 2002).

As discussed in previous chapters, Ena/VASP proteins have roles in neurite formation and axon guidance. This chapter will focus on the roles for Ena/VASP proteins at sites of adhesion as it applies to the formation, fusion, and structural integrity of epithelia. Other roles for Ena/VASP in immune cell function and branching morphogenesis will be discussed.

### **Focal Adhesions: Sites of cell:matrix interaction**

The preponderance of Ena/VASP research has focused upon its function regulating actin dynamics in filopodia and lamellipodia. However, the majority of Ena/VASP within the cell is associated with cellular adhesion structures such as focal adhesions and adherens junctions (Gertler et al., 1996). Integrin mediated focal adhesions provide cells with a tether to the underlying extracellular matrix (ECM). Regulated assembly of focal adhesions beneath protruding lamellipodia coordinated with disassembly of adhesions at the cell's trailing edge is essential for productive motility (Mitra et al., 2005). Integrin contacts with the ECM are mediated intracellularly by linkage with the actin cytoskeleton. Within the cell, actomyosin stress fibers emerging from focal adhesions provide a tensile apparatus with which the cell can engage ECM.

A wealth of proteins associated either directly or in complex with the cytoplasmic domains of integrin molecules have roles in regulating actin dynamics. The focal adhesion proteins Zyxin and Vinculin recruit Ena/VASP proteins to focal adhesions through EVH1 interactions (Brindle et al., 1996; Reinhard et al., 1995). However, the function of Ena/VASP at focal adhesions has remained unclear. The specific delocalization of Ena/VASP proteins from focal adhesions does not affect the speeds of migrating fibroblasts *in vitro* (Bear et al., 2000). However, interaction with the Rap1-binding protein RIAM suggests a role for Ena/VASP activity in cell spreading and focal adhesion formation downstream of Rap1 (Lafuente et al., 2004). Other groups have identified changes in Ena/VASP phosphorylation that correlate with cell spreading and trypsinization (Garcia Arguinzonis et al., 2002; Howe et al., 2002; Lawrence and Pryzwansky, 2001).

*In vivo*, the *VASP* knockout has a defect in the inhibition of agonist induced aggregation of platelets (Aszodi et al., 1999; Massberg et al., 2004). Platelet aggregation employs adhesion and signaling through  $\alpha$ IIb $\beta$ 3 integrin binding to fibrinogen (Andrews and Berndt, 2004). Platelet aggregation is inhibited by prostacyclin- or nitric oxide (NO) - induced activation of PKA and PKG respectively. VASP is the only Ena/VASP family member expressed in adult platelets, and is the critical substrate of PKA/PKG in mediating inhibition of aggregation (Aszodi et al., 1999). The mechanism by which Ena/VASP affects  $\alpha$ IIb $\beta$ 3 function has not been determined, but may involve its interactions with RIAM and Fyb/SLAP/ADAP (Danielewski, 2005; Oberfell, 2001}. Compensation by other Ena/VASP proteins has complicated attempts to describe a role for Ena/VASP in cell:ECM adhesion in other tissues.

### **Ena/VASP in cell:cell interactions**

Adherens junctions are distinct adhesive structures that mediate cell:cell interactions, but like focal adhesions are sites of Ena/VASP localization(Bachmann et al., 1999). Adherens junctions form through the Ca<sup>2+</sup>-dependent homophilic interaction of cadherins (Drubin and Nelson, 1996; Geiger and Ayalon, 1992). Although some adherens junctions are used in transient cell:cell contacts, epithelial sheets require robust and stable adhesion between neighboring cells. To achieve this stability epithelia employ both E-Cadherin containing adherens junctions as well as specialized cadherin structures that link to intermediate filaments called desmosomes(Kowalczyk et al., 1999). The molecular basis for adherens junction formation during development has been postulated to require the initial interdigitation of filopodia (Vasioukhin et al., 2000). Ena/VASP localizes to both the tips of filopodia as well as to adherens junctions and has been implicated in the integrity of cell:cell contacts *in vivo*, presenting the attractive hypothesis that Ena/VASP may be central to adherens junction formation. Disruption of Ena/VASP function in keratinocytes with the TD construct did not affect cell:ECM adhesion but caused blister formation due to a failure in cell:cell adhesions between basal keratinocytes(Vasioukhin et al., 2000).

Further evidence of Ena/VASP function at cell:cell adhesions comes from cardiac myocytes. Similar to the skin epithelium, cardiac myocytes also form desmosomes but in the context of a specialized cell:cell contact called an intercalated disk(Severs, 1985). Intercalated disks allow for the coordinated muscular contraction across sheets of myocytes necessary for proper heart function. Ena/VASP proteins localize to intercalated disks(Markert et al., 1996), and the myocyte expression of the VASP EVH1 domain disrupts this localization *in vivo* (Egenthaler et al., 2003). The dislocalization of VASP from intercalated disks correlated with changes in intercalated disk integrity and the development of dilated cardiomyopathy(Egenthaler et al., 2003).

## **Epithelial Fusion**

The generation of cell:cell contacts is recapitulated in the fusion of epithelial sheets. Fusion events occur at specific sites during mouse development including neurulation, craniofacial fusion, eyelid fusion, periumbilical and perigenital midline fusion, and digit sculpting(Martin and Parkhurst). Disruption of Mena in combination with either VASP or the actin monomer binding protein Profilin-1 causes craniofacial defects(Lanier et al., 1999). Failure of cephalic neural tube closure in these mice results in exencephaly. Neural tube closure requires the formation of a hinge in the neuroepithelium placing the neural folds in apposition; these folds can then fuse to close the neural tube(Copp and Greene, 2000; Copp et al., 2003). The mechanism that causes the failure in neurulation in *Ena/VASP* mutants has not been elucidated. The roles of *Ena/VASP* in regulating cell shape and motility suggest that any or multiple steps in closure may be affected.

Intriguingly, mutations in *Drosophila ena* cause defects in the related process of dorsal closure(Grevengoed et al., 2001). *Drosophila* dorsal closure has provided a model for epithelial fusion(Martin and Parkhurst, 2004). Epithelial sheets extend toward one another through the coordinated action of a leader edge directing the movement of the trailing sheet and leader edge compression by a contractile actin ring(Redd et al., 2004). The knitting of filopodial extensions from the apposed sheets initiates the fusion of epithelia, which then matures through the generation of adherens junctions(Martin and Wood, 2002). The role of *ena* in dorsal closure may reflect defects in leader edge motility, filopodial formation and knitting, or adherens junction assembly. Regarding the latter possibility, while the fusion of apposed epithelia is defective, the integrity of the cell:cell contacts within the epithelial sheets is normal. This is a clear discrepancy from the epithelial failure causing mouse skin blistering. Thus, the role of *Ena/VASP* in the formation and integrity of cell:cell contacts remains elusive.

## **Epithelial and Endothelial Function**

Ena/VASP has also been implicated in the physiology of mature epithelium and endothelium. The generation of a barrier is an essential function of the endothelial lining of the vasculature. With the exception of the capillaries of the renal glomerulus and liver, the endothelial monolayer reliably excludes the transudation of large macromolecules across the vessel wall. Failure in the barrier results in the loss of albumin to the interstitium generating an osmotic pressure that supports the loss of water resulting in edema, or hydrocephalus if it occurs at the blood brain barrier (Rubin and Staddon, 1999). Furthermore, the regulated transmigration of immune cells across the endothelial monolayer results in the temporary formation of gaps that must be repaired to preserve barrier function. The association of Polymorphonuclear cells (PMNs) promotes the regeneration of the barrier following their transmigration, and this promotion is in part mediated by PKA (Comerford et al., 2002). Both endothelial and epithelial barriers are generated by the formation of occludens junctions between cells mediated by homophilic interactions between ZO-1 proteins (Harhaj and Antonetti, 2004). It has recently been found that phosphorylated VASP associates with ZO-1 at cell:cell contacts *in vitro*, and that VASP phosphorylation by PKA correlates with increases in endothelial cell barrier function (Comerford et al., 2002). The integrity of endothelial barrier function varies inversely with cell:cell tension (Carbajal and Schaeffer, 1999). This tension may be provided by the action of myosins at actin stress fibers. By their localization to focal adhesions and occludens junctions Ena/VASP proteins provide a link between adhesion molecules and actin stress fibers. Potentially, Ena/VASP could inhibit the transmission of tensile forces from stress fibers between adjacent cells thereby enhancing barrier function.

## **Axon guidance molecules in development**



The mature endothelial monolayer lines the inner aspect of blood vessels. During development the migration of endothelial cells in response to extracellular cues generates the highly branched vascular system in a process called angiogenesis (Risau, 1997). This iterative vascular branching and extension is driven by the migration of an endothelial tip cell that has been proposed to function similarly to a neuronal growth cone (Gerhardt et al., 2003). As in axon guidance, an extending blood vessel must migrate towards a distant target, and in the case of organ vascularization must enter its target at a precise location (Geiger et al., 2005). Recently, further similarities to the growth cone have been elucidated. The guidance of tip cells requires the expression and activity of axon guidance molecules. Similar roles for axon guidance molecules have been postulated in the related developmental process of lung branching morphogenesis. The Slit receptor Robo4 binds Mena and is specifically expressed in endothelial cells and knockdown of Robo4 causes defects in zebrafish angiogenesis (Bedell et al., 2005; Park et al., 2003; Suchting et al., 2005). Netrin interaction with the Unc5b receptor affects the behavior of both endothelial tip cells and respiratory epithelium (Liu et al., 2004; Lu et al., 2004). In flies and worms, Ena/VASP proteins function downstream of both Robo/Slit and DCC/Unc5/Netrin pathways during axon guidance. The extent to which the pathways downstream of axon guidance molecules have been conserved in their extraneuronal functions is not clear.

Ena/VASP activity has also been implicated in cell movements that define the architecture of the heart during development. The heart wall has two basic characteristics- thickness and trabeculation. The generation of a heart wall of proper thickness and trabeculation is mediated by signaling by Plexin-A1 through the Semaphorin 6D receptor (Toyofuku et al., 2004). Semaphorin 6D signaling guides myocyte migration within the heart wall. Interestingly, Semaphorin 6D was found to associate

with both Abl and Mena (but not EVL or VASP), and the tyrosine phosphorylation of Mena by Abl inhibited Mena:Sema6D interactions(Toyofuku et al., 2004).

### **Immune system development and function**

Activation of T cells through interaction with antigen presenting cells (APCs) causes T cell polarization and formation of an actin dependent structure referred to as the immunological synapse(Bromley et al., 2001). Ena/VASP interacts with the immune cell adaptor protein Fyb/SLAP/ADAP, and disruption of Ena/VASP localization in cultured T cells by expressing an EVH1 ligand blocks immune synapse formation(Krause et al., 2000). Similarly, disruption of Ena/VASP in macrophages prevents the formation of the phagocytic cup necessary to internalize opsonized substrates through the Fcγ1 receptor(Coppolino et al., 2001).

## Results

### Characterization of genotypic combinations of Ena/VASP

To address the reported roles for Ena/VASP in physiology and development, and to uncover additional Ena/VASP functions, we established crosses between triple-heterozygotic (MmVvEe) animals to determine the viability of various genotypic combinations (Table 1). As previously reported the mmvvEE mouse is not viable, and as expected we did not recover genotypic combinations lacking both Mena and VASP (Menziez et al., 2004).

Interestingly, we were able to recover MmVvEe mice, albeit at lower than expected frequencies, suggesting that loss of both VASP and EVL can be tolerated and that expression from a single Mena allele is sufficient for mouse development. In contrast, we were unable to recover mmVVee mice indicating that EVL expression is required in the absence of Mena. Together, these results indicate that EVL or VASP function is required for postnatal viability only in the absence of Mena.

<b>Table 1: Recovered Genotypes from Triple-heterozygotic crosses</b>			
<b>Genotype</b>	<b>Odds</b>	<b># observed</b>	<b># expected</b>
MMVVEE	1/64	12	8
MMVVee	1/32	23	16
MMVvEE	1/32	23	16
MmVVEE	1/32	24	16
MMVvEe	1/16	57	31
MmVVee	1/16	58	31
MmVvEE	1/16	33	31
MmVvEe	1/8	77	63
MMVvEe	1/64	12	8
MMVvee	1/32	21	16
MmVVee	1/32	21	16
MmVvee	1/16	44	31
MMvvEE	1/64	13	8
MMvvEe	1/32	19	16
MmVVee	1/32	9	16
MmVvEe	1/16	21	31
mmVVEE	1/64	3	8
mmVVee	1/32	9	16
mmVvEE	1/32	10	16
mmVvEe	1/16	2	31
MMVvee	1/64	5	8
MmVvee	1/32	5	16
mmVVee	1/64	0	8
mmVvee	1/32	0	16
mmvvEE	1/64	1	8
mmvvEe	1/32	0	16
mmvvee	1/64	0	8
<b>Total</b>		<b>502</b>	

**Table 1. MmVvEe animals were crossed and the progeny were genotyped at P10. Several genotypic classes are greatly reduced from expected frequencies**

**mmVVee mice die at birth due to respiratory failure**

To address the specific decrease in mmVVee and mmVVEe mice (Table 1 and Fig. 1A) we established crosses to generate these mice at high frequencies. Embryos were surgically collected and recovered viable at expected Mendelian frequencies throughout development (data not shown). Genetically engineered mice with perinatal lethality are typically lost due to either a failure in respiration or feeding. To determine the mode of lethality we surgically collected embryos just prior to birth at E18.5 and observed them for the

ability to breathe. Despite opening their mouths in an attempt at gasping, all mmVVee and most mmVVEe mice were unable to breathe at birth, and within five minutes of dissection had become strikingly cyanotic and ceased movement (Fig. 1B). When immersed in liquid fixative the mmVVee mice sunk to the bottom in contrast with anesthetized littermates (not shown). Histologically *Ena/VASP*-deficient lungs had normal lobation and size but the failure of mmVVee to inflate their lungs prevented a reasonable comparison with their littermates.

**Lung branching morphogenesis occurs normally in mmVVee mice**

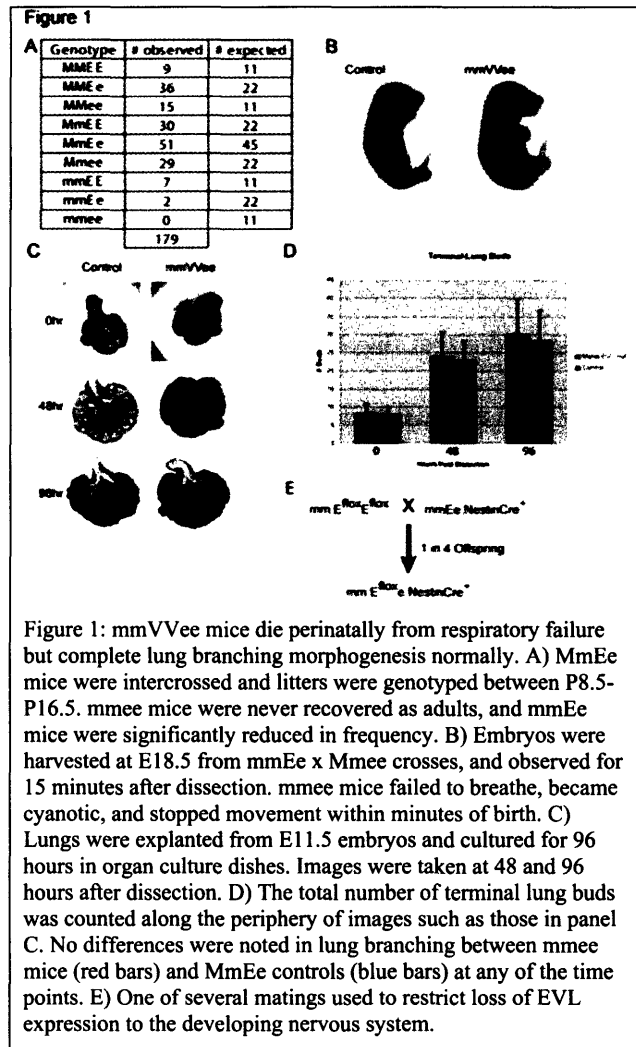


Figure 1: mmVVee mice die perinatally from respiratory failure but complete lung branching morphogenesis normally. A) MmEe mice were intercrossed and litters were genotyped between P8.5-P16.5. mmEe mice were never recovered as adults, and mmEe mice were significantly reduced in frequency. B) Embryos were harvested at E18.5 from mmEe x MmEe crosses, and observed for 15 minutes after dissection. mmEe mice failed to breathe, became cyanotic, and stopped movement within minutes of birth. C) Lungs were explanted from E11.5 embryos and cultured for 96 hours in organ culture dishes. Images were taken at 48 and 96 hours after dissection. D) The total number of terminal lung buds was counted along the periphery of images such as those in panel C. No differences were noted in lung branching between mmEe mice (red bars) and MmEe controls (blue bars) at any of the time points. E) One of several matings used to restrict loss of EVL expression to the developing nervous system.

Respiratory failure is an extraordinarily common cause of death in genetically engineered mice, with a startlingly varied list of etiologies including failures in lung development, formation and innervation of the diaphragm, and central defects in the CNS respiratory center. Recently, several groups have identified roles for axon guidance receptors in lung branching morphogenesis (Anselmo et al., 2003; Ito et al., 2000; Liu et al., 2004). To determine whether Ena/VASP functions in lung branching morphogenesis we dissected the trachea and its associated immature lung buds at E11.5 (Fig. 1C, top row). Dissected lungs were grown *in vitro* in organ culture dishes for 96 hours and the extent of branching was documented at 48 hours and 96 hours post-dissection (Fig. 1C). To determine the extent of lung branching morphogenesis in mmVV<sup>ee</sup> compared to littermate MmVV<sup>Ee</sup> controls, the number of terminal lung branches was counted around the periphery of both lungs. At all stages of lung development analyzed, no differences in the morphology or extent of branching were noted in mmVV<sup>ee</sup> mice when compared to MmVV<sup>Ee</sup> littermate controls (Fig. 1C and 1D).

### **Respiratory failure in mmVV<sup>ee</sup> mice is secondary to a nervous system defect**

To further delineate the etiology of respiratory failure in the mm<sup>ee</sup> mice, we employed a conditional EVL allele (E<sup>flox</sup>) in which exons 2 and 3 are flanked by LoxP sites. The elimination of EVL expression was then restricted to the nervous system by introducing Cre recombinase under the control of the neuron-specific Nestin promoter (Fig. 1D). We were unable to recover mmVV<sup>E<sup>flox</sup>e</sup>;Cre<sup>+</sup> mice suggesting that EVL expression is required within the nervous system for perinatal survival. Surprisingly, similar matings failed to produce MmVV<sup>E<sup>flox</sup>e</sup>;Cre<sup>+</sup> mice. Crosses with the traditional EVL-knockout recovered MmVV<sup>ee</sup> mice at expected ratios (Table 1 and Fig. 1A). The inability to obtain these mice from the conditional disruption of EVL in the

nervous system suggests that the loss of EVL can be compensated for during development but the sudden loss of EVL expression during neuronal differentiation is not tolerated.

### **Ena/VASP-deficient mice have no major defects in early development**

As expected we were unable to generate Ena/VASP-deficient mice of genotyping age (Table 1). To determine the requirement for Ena/VASP during mouse development we established timed pregnancies, denoted the morning the plug was found as E0, and collected embryos throughout embryogenesis. Crosses were established to produce mmvvee mice at 1-in-4 or 1-in-8. Embryos were collected at various times after fertilization and genotyped to determine whether Ena/VASP-deficient mice were being recovered.

To determine whether Ena/VASP plays a role in the patterning of early embryos we collected embryos at E8.5 at which point gastrulation has been completed and basic embryo topology has been established. At E8.5-E10.5, triple-nulls

were generated at the expected frequency without any obvious gross phenotypes (Table 2). This indicates that Ena/VASP is not required for the cell migrations in early mouse development.

### **EVL has limited involvement in neurulation or craniofacial defects**

The mmvvee mouse has previously been reported to have defects in neurulation and craniofacial fusion (Menzies et al., 2004). The defect in neurulation is limited to the cephalic

<b>Genotype</b>	<b>E8.5-E10.5 Observed</b>	<b>E8.5-E10.5 Expected</b>
MMVvEe	15	11.5
MmVvEe	23	23.0
MMVvvee	2	1.5
MMVvee	23	25.1
MmVvvee	3	3.0
MmVvee	60	50.3
MMvvEe	17	21.6
MmvvEe	50	43.3
mmVvEe	9	11.5
MMvvee	34	33.8
Mmvvee	59	67.5
mmVvvee	1	1.5
mmVvee	19	25.1
mmvvEe	24	21.6
mmvvee	35	33.8
<b>Total</b>		<b>374.0</b>

**Table 2. Embryos from various matings were collected at E8.5, E9.5, and E10.5 and genotyped. The expected and observed number of mice at E8.5, E9.5 and E10.5 for each genotypic class were pooled. All genotypic combinations were recovered at expected frequencies.**

neural tube and along with craniofacial defects generates exencephaly and cleft palate and lip (CPL). The fusion of the neural tube occurs at E9.5. To determine whether the loss of EVL contributed to the neurulation defect we examined embryos at E10.5-E18.5. *mmvvee* embryos demonstrated exencephaly and CPL with similar penetrance to that reported for the *mmvEE* mice (82% and 64% respectively)(Table 3). With the exception of a very low penetrance of non-cephalic defects, the triple-null phenotype was limited to the head and palate. *mmVvee* mice completed neurulation normally (Fig. 1B), and *mmVvee* mice had a similar incidence of neural tube defects as the previously reported *mmVvEE* mice (Table 3).

<b>Table 3: Recovery and Phenotypes of Embryos at E18.5</b>				
<b>Genotype</b>	<b>#Observed</b>	<b># Expected</b>	<b>% Exencephalic</b>	<b>% Hemorrhage</b>
<b>MMVvee</b>	17	22	0%	0%
<b>MmVvee</b>	40	43	0%	0%
<b>MMvvEe</b>	20	15	0%	0%
<b>MmvvEe</b>	27	31	4%	4%
<b>MMvvee</b>	83	56	1%	0%
<b>Mmvvee</b>	106	112	6%	5%
<b>mmVvee</b>	29	22	10%	7%
<b>mmvvEe</b>	16	15	63%	56%
<b>mmvvee</b>	34	56	82%	76%
<b>Total</b>	372			
<b>mmvEE (Menzies et al., 2004)</b>			64%	0%
<b>mmVvEE (Menzies et al., 2004)</b>			7%	0%
<b>Table 3. Embryos from various crosses were harvested at E18.5. At dissection they were scored for the presence of exencephaly and intraamniotic hemorrhage. Collected embryos were genotyped and the recovery of genotypic classes was compared with that expected by Mendelian ratios. The recovery of <i>mmvvee</i> was 60% of the expected value.</b>				

The similar penetrance and expressivity of the neurulation defect suggests that EVL does not play an important role in neural tube fusion, and that a critical role for *Ena/VASP* in neurulation is restricted to the head and face. Interestingly, approximately 25% of E10.5 *mmvvee* embryos appeared to be dead or dying by histology. Analysis of the dying E10.5 embryos demonstrated intraembryonic hemorrhage, apoptosis, and tissue necrosis (data not shown). We failed to

observe hemorrhage in the surviving E10.5 embryos and it is unclear if the hemorrhage is endothelial cell autonomous or the proximal cause of embryonic death. The loss of embryos at E10.5 can partly explain the reduction in triple-nulls recovered at E18.5 (Table 3- 40% reduction in viable triple-nulls).

### **Neurulation defects are insensitive to nutrient supplementation**

Women of reproductive age are routinely advised to supplement their diet with folic acid. The administration of folic acid greatly reduces the incidence of the caudal neurulation defect that causes spina bifida (Copp and Greene, 2000). Consistently, administration of folate in mice can rescue some mouse models of neurulation defects (Carter et al., 1999; Gefrides et al., 2002). More recently, a second class of folate-insensitive mutations have been found to respond to administration of inositol (Greene and Copp, 1997). To determine whether the neurulation defects in *Ena/VASP*-deficient mice were sensitive to either inositol or folate, we administered intraperitoneal injections of inositol and folic acid and harvested litters between E14.5 and E18.5. The incidence of exencephaly was similar between treated and untreated females indicating that *Ena/VASP* mutations are neither folate nor inositol responsive (data not shown).

### ***Ena/VASP*-deficient embryos die between E16.5 and E18.5 due to intraamniotic hemorrhage**

E18.5 *mmvvee* embryos could be easily identified by their swollen red appearance within the uterine horn (Fig. 2A). Dissection of the maternal and extraembryonic tissue revealed pallid exencephalic embryos within an amniotic sac swollen with blood (Fig. 2A). At E18.5, 76% of *mmvvee* embryos, and 56% of *mmvvee* embryos suffered from intraamniotic hemorrhage (Table 3). A significant percentage of the *mmvvee* mice had died in the day or two preceding dissection as determined by lack of fetal heartbeat. This defect was not reported in the *mmvvee*



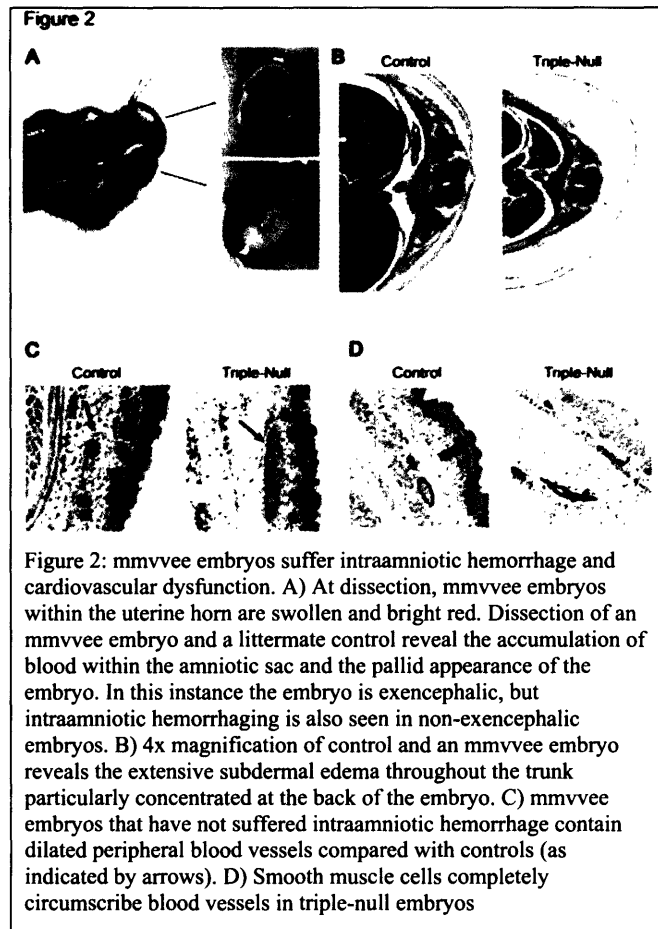
exencephalic embryos indicating that the hemorrhage requires the loss of at least one EVL allele.

The identification of hemorrhagic nonexencephalic mmvvee embryos demonstrates that these two phenotypes occur independently. Hemorrhagic embryos that were still alive as determined by the presence of a heartbeat were

dissected from the amnion and observed to determine the origin of the hemorrhage. Surprisingly, an origin of the hemorrhage could not be identified, suggesting that the accumulation of blood may result from a slow leak and not a catastrophic event.

Consistent with the slow loss of blood, intraamniotic hemorrhage was also

observed at E17.5 and E16.5 albeit with lesser severity and lower penetrance. Thus it is likely that the intraamniotic hemorrhage occurs as a stochastic event late in development that gradually drains the embryo of blood.



Histologic analysis of hemorrhagic E16.5 and E18.5 embryos was conducted to further describe the mechanism and extent of the hemorrhage. To determine whether the intraamniotic hemorrhage was indicative of a general defect in blood vessel integrity, we surveyed the embryo for any evidence of intraembryonic hemorrhage. Consistent with our failure to observe gross evidence of intraembryonic hemorrhage, such as blood pooling or red splotching of the skin, we

found no evidence of major hemorrhage within the embryo. Some Ena/VASP-deficient embryos appeared to contain red blood cells that were immediately outside of the vascular lumen. The observation of scattered extravascular red blood cells but not frank intraembryonic hemorrhage is consistent with small fenestrations within the endothelial lining permitting blood cell extravasation (discussed below). It is formally possible that the intraamniotic blood accumulation could represent the slow extravasation of red blood cells from umbilical or superficial vessels, but the extent of hemorrhage makes this scenario unlikely. However, the pallid appearance of the hemorrhagic embryos strongly suggested that the blood was of embryonic and not maternal origin, and no architectural defects were observed in the placenta (data not shown). The precise origin of the intraamniotic hemorrhage remains under investigation.

### **E18.5 triple-null embryos have phenotypes indicating cardiovascular dysfunction**

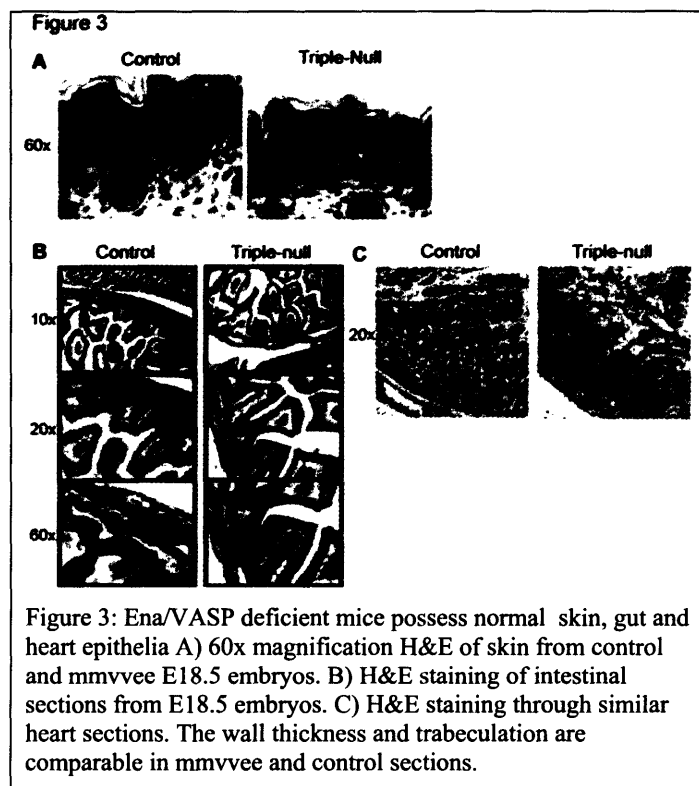
In our histologic analysis of E18.5 embryos we noted the appearance of several phenotypes that were similar to patterns associated with congestive heart failure (CHF). Congestive heart failure typically results in an adult as a sequelae to heart dysfunction after myocardial infarction. Failure to pump blood adequately results in accumulation of fluid causing vascular distention, peripheral edema, and hepatic congestion. Embryos at E18.5 experience profound subdermal edema throughout the trunk (Fig. 2B). There was also evidence of mild hepatic congestion and pericardial edema (data not shown). Within embryos that had not suffered intraamniotic hemorrhage, the peripheral vasculature within the skin was greatly distended (Fig. 2C). The majority of the skin's vasculature should normally be fine-caliber capillaries. Vessels with calibers similar to the aorta could easily be identified passing within the dermis of triple-null embryos (Fig. 2C). Smooth muscle cell (SMC) association with the vasculature is essential for

maintenance of vessel caliber, regulation of blood pressure, and vessel structural integrity. To determine whether there was a defect in SMC migration or organization with the peripheral vasculature we stained sections with an antibody against smooth muscle actin (SMA). SMA immunoreactivity completely circumscribed both central and peripheral vessels and there was no evidence of SMC disorganization (Fig. 2D)

### Heart development does not require Ena/VASP proteins

Several papers have suggested a role for Ena/VASP proteins in the development and function of the heart (Eigenthaler et al., 2003; Toyofuku et al., 2004). The phenotypes observed at E18.5 could be caused by heart muscle dysfunction or a structural defect in the heart. To determine whether an underlying heart abnormality was associated with the cardiovascular phenotypes observed at E18.5 we analyzed the hearts from mmvvee embryos both with and without subdermal edema. No difference was

noted in wall thickness between Ena/VASP-deficient embryos and controls (Fig. 3C). There existed some variability in the degree of trabeculation among mmvvee embryos. All mmvvee embryos exhibited trabeculation, but in some cases the trabeculation was similar to controls (Fig. 3C) whereas in others the trabeculae were thicker and more poorly organized in comparison with



controls. To determine whether there were any underlying anatomic defects in heart and outflow tract development we serially sectioned *Ena/VASP*-deficient hearts at E14.5 and E18.5 for histological analysis. The ventricular septum, atrioventricular septa and valves, aortic valve and root, and pulmonary valve and root all appeared normal (data not shown). We did not directly assess cardiac output or rhythm. However, heart dysfunction typically results in secondary changes in heart morphology including hypertrophic or dilated cardiomyopathies. Both the thickness of the heart wall and the overall size of the heart appeared normal (Fig. 3C and data not shown).

#### **Subdermal edema precedes other phenotypes and can be observed as early as E14.5**

The complicated spectrum of cardiovascular phenotypes at E18.5 could represent one of several etiologies. The observed phenotypes are interrelated and any individual phenotype may both be a sequelae and enhancer of any another phenotype. To determine the primary defect in the triple-null mouse we analyzed embryos at earlier time points to identify the onset and progression of cardiovascular failure. Subdermal edema, but not intraamniotic hemorrhage, vascular distension or hepatic congestion, was observed as early as E14.5 (data not shown). *mmvvee* embryos at E14.5 could be identified by gross examination based upon the balloon-like expansion of skin along their dorsum. Histology confirmed that this expansion represented subdermal edema throughout the trunk particularly concentrated dorsally (data not shown). The earlier appearance of the subdermal edema suggests that phenotype may be the primary event in the cardiovascular failure.

#### ***Ena/VASP* proteins participate in endothelial barrier function**

The etiologies of subdermal edema include any combination of events that change the balance of hydrodynamic forces within the small blood vessels. Increases in intravascular hydrostatic

pressure (such as those secondary to CHF) or decreases in oncotic pressure result in transudation of fluid. Although the presence of distended vessels suggests an increase in hydrostatic pressure, the onset of edema precedes vessel distention thus an increase in hydrostatic pressure is likely not the primary cause of edema. Changes in ionic and protein content of blood plasma are typically caused by dysfunction of the kidney, and recently Slit/Robo signaling has been implicated in kidney development (Grieshammer et al., 2004). However, *mmvvee* mice show normal kidney histology, and Periodic Acid Schiff (PAS) staining failed to show evidence for pathological protein reabsorption in the proximal tubules that typically accompanies inappropriate albumin secretion from the capillaries (data not shown). Edema can also be caused by inadequate drainage by the lymphatic system, leading to the accumulation of fluid in peripheral tissues (Karkkainen et al., 2004). Although a microscopic analysis of lymphatics was not conducted, our histologic analysis of the heart and great vessels showed the presence of a normal thoracic duct which drains collected lymphatic fluid into the superior vena cava (SVC) (data not shown).

Endothelial barrier dysfunction allowing protein loss from the blood plasma can cause edema. Throughout the vasculature, endothelial cells form a tight barrier through tight junctions. The passage of proteins, cells and solutes across this barrier is regulated. Failure of this barrier allows the passage of albumin into the interstitium altering the hydrodynamic forces within the vessel resulting in the transudation of water. Endothelial cells express Ena/VASP proteins and the phosphorylation of Ena/VASP has been reported to correlate with barrier function (Comerford et al., 2002). To determine the localization of Ena/VASP proteins within endothelial cells we expressed a GFP-tagged VASP construct within cultured human umbilical venous endothelial cells (HUVECs). VASP localized to regions of cell:cell contacts and colocalized with actin at

stress fibers (Fig. 4A). To determine whether Ena/VASP proteins play a role in barrier function we employed an Ena/VASP dominant negative construct that sequesters Ena/VASP to the surface of mitochondria by fusing a mitochondrial targeting cassette to the EVH1 ligand FPPPP (the construct is abbreviated as mito). This construct has been well characterized in previous studies as a highly effective and specific inhibitor of Ena/VASP function (Bear et al., 2000). HUVECs expressing either mito, GFP-VASP, or GFP alone were allowed to form a monolayer. We assayed the ability of a fluorescently tagged dextran, of similar size to albumin, to cross the monolayer. When compared to the GFP expressing cells, expression of mito allowed approximately 5x greater permeability to the dextran molecule. Conversely, overexpression of GFP-VASP significantly increased the barrier function of the monolayer (Fig. 4B).

To determine whether loss of Ena/VASP proteins affects barrier function in vivo we analyzed the endothelial monolayer of blood vessels by thin section electron microscopy. In comparison to control venules (Fig. 4, C and D), the endothelial lining of Ena/VASP-deficient venules (Fig. 4, E-H) contains defects in cell:cell junction integrity. The cell:cell junctions in the control cells (Fig. 4, C and D, black arrows) extends across the width of the cell:cell interface. However, the junctions between Ena/VASP-deficient endothelial cells often form gaps (Fig 4, F and H, yellow arrows) between areas of apparently normal junction formation (Fig. 4, F and H, black arrows). We believe that the gaps represent areas of defective occludens or adherens junctions between normal desmosomes. Immuno-EM with junction-specific antibodies will be necessary to clarify the defect. Consistent with inappropriate leakage through endothelial barrier gaps we observed extravasated red blood cells surrounded by rings of deposited fibrin (Fig. 4, E and F, pink asterisks). Elsewhere large deposits of fibrin were observed within the interstitium (Fig. 4G).

**Figure 4**

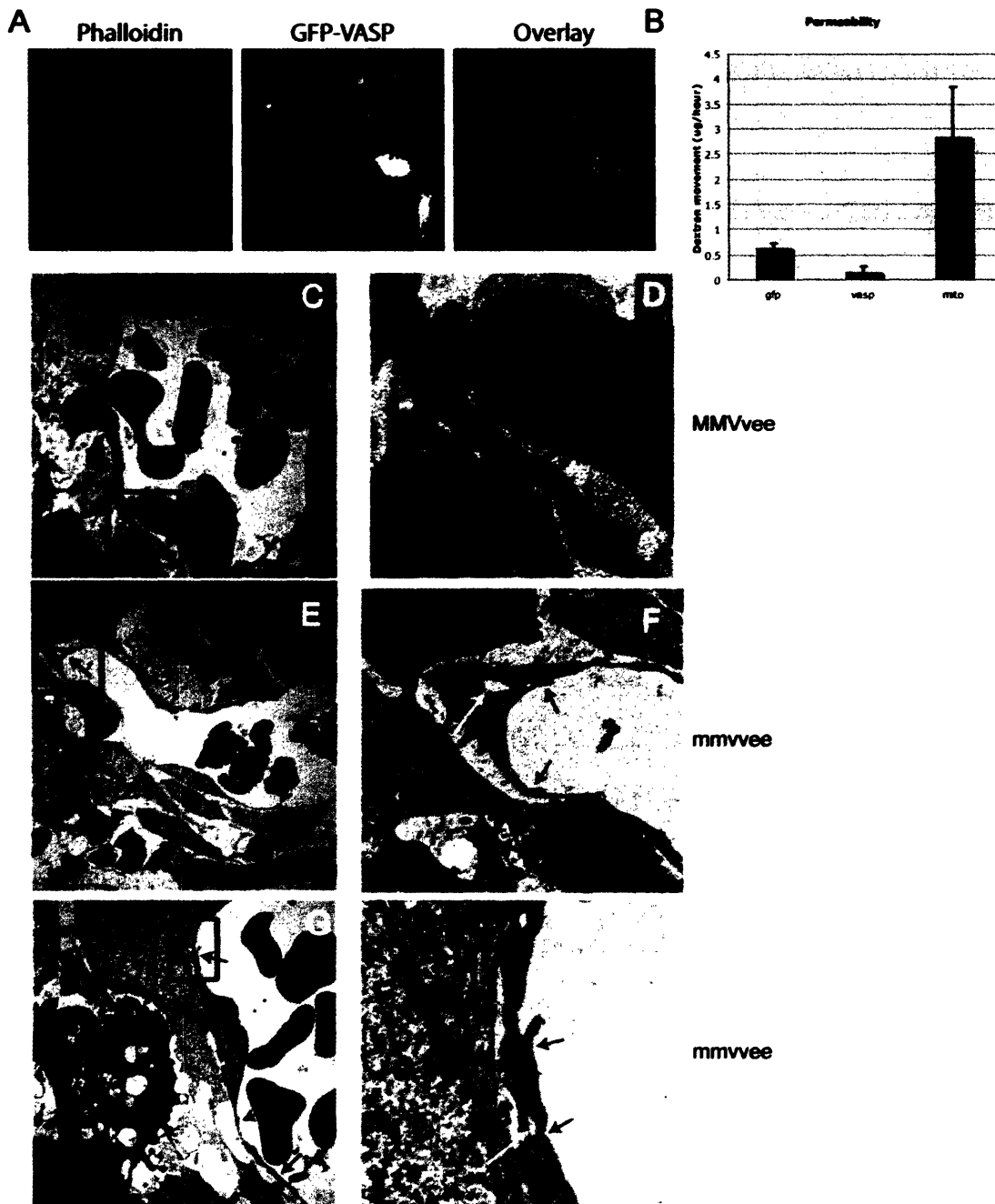


Figure 4: Disruption of Ena/VASP proteins affects endothelial barrier cell function *in vitro* and *in vivo*. A) GFP-tagged VASP expressed in HUVEC monolayers colocalizes to cell:cell contacts and stress fibers. HUVEC cells expressing GFP-VASP (green) were stained with phalloidin to label actin (red) at 60x magnification. B) Expression of the dominant-negative construct (mito) inhibits barrier function in HUVEC monolayers. Movement of dextran was compared between expressing mito, GFP, or overexpressing VASP. C-H) Electron microscopy of mmvvee endothelial cells show disorganized junctions and hemorrhage. Control venules (C-D) contain well formed junctions (black arrows) that extend through the thickness of the cell:cell interface. The boxed area in (C) is shown in higher magnification in D. Ena/VASP deficient venules (E-F) show junction formation

(black arrows in E and G). However, when magnified (F and H) contain gaps (yellow arrows) between areas of apparent desmosomes formation (black arrows). The presence of extravasated red blood cells (pink asterisks in E and F) and fibrin deposits (fibrin in G, and dark ring surrounding extravasated red blood cells in E and F) is indicative of plasma and red blood cell leakage. Note the presence of macrophage (labeled "M" in panel E) and neutrophil (PMN in panel G) engulfing extravasated red blood cells and fibrin respectively.

### **Epitheliogenesis does not require Ena/VASP proteins**

The defect in endothelial barrier function may represent an underlying defect in the formation and integrity of epithelial sheets. Previous reports had described an essential role for Ena/VASP in the formation of adherens junctions, with expression of a putative Ena/VASP dominant negative construct within keratinocytes causing extensive blister formation *in vivo* (Vasioukhin et al., 2000). To determine whether epithelial sheets formed appropriately we analyzed skin (Fig. 3A) and gut epithelia (Fig. 3B) for abnormalities. With the exception of the previously described subdermal edema, the skin showed appropriate histology throughout its thickness with no gross or histologic evidence of blistering (Fig. 3A). Similarly, in the columnar epithelia of the small and large intestines, there was no evidence in monolayer defects.

Whereas the formation of epithelial monolayers appears unaffected in Ena/VASP-deficient mice, we wondered whether the neurulation and craniofacial defects may represent a more general failure in the fusion of epithelial sheets. Epithelial sheet fusion occurs at several sites on the embryo during development. In addition to fusion of the neural folds and the fusion of the medial nasal prominence and the right and left maxillary prominences, the superior and inferior eyelids fuse late in fetal development at E15.5 to protect the developing eye. Other sites of epithelial fusion events include midline fusions at the umbilicus and genitals, and sculpting of the digits. Consistent with a role in epithelial fusion, *mmvvee* mice at E18.5 fail to undergo eyelid fusion (Fig. 5A, second row). The majority of *mmvvee* mice have properly formed pedal



digits and midline fusion at the umbilicus and genitals (Fig. 5C and data not shown).

Several *mmvvee* mice were recovered exhibiting omphalocele, the protrusion of small intestines and liver into the umbilicus (Fig. 5B). However, whether the omphalocele represents a defect in midline fusion, increased intraabdominal pressure due to edema and hepatic congestion, or an unrelated developmental defect can not be distinguished. Interestingly, *Mena*<sup>-/-</sup>;*Profilin*<sup>+/-</sup> mice also show a failure of eyelid fusion in addition to the reported defects in neurulation (Fig. 5A). Failure in fusion may represent a

defect in directed cell motility at the leading edge of the epithelia, a failure of filopodial knitting, or a failure in adherens junction formation. To help differentiate between these possibilities we examined the fusion of the eyelids shortly after the completion of fusion at E16.5. At E15, the eyelid epithelia begin to migrate towards one another preceding the fusion event. The eyelids of a nonexencephalic mouse at E16.5 appeared puckered and are not properly apposed when compared to a littermate control (Fig. 5A). This observation indicates that eyelid fusion is not secondary to exencephaly, and that the failure in fusion may result from a failure in apposition or filopodial knitting.

### VASP and EVL are not required to form an immune system

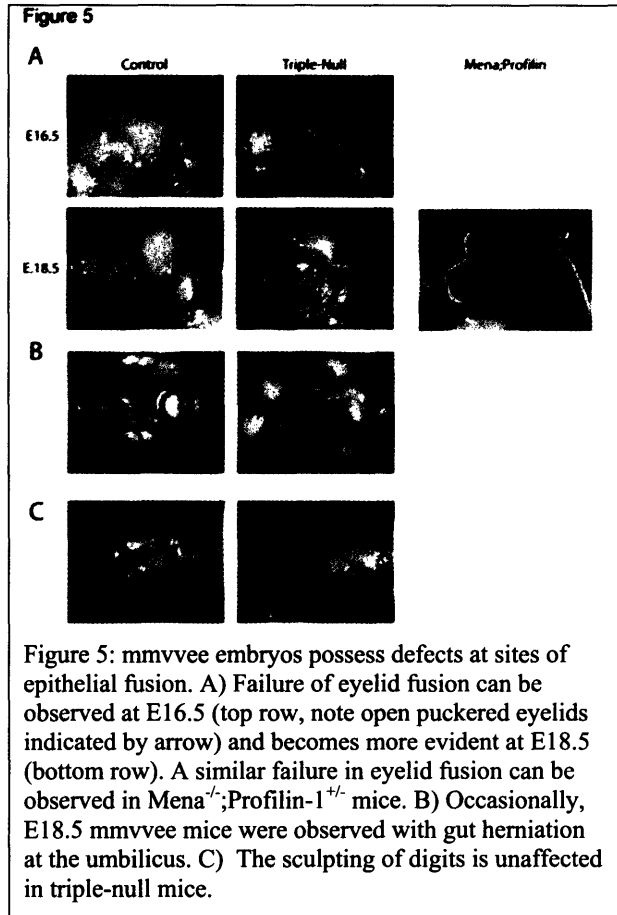


Figure 5: *mmvvee* embryos possess defects at sites of epithelial fusion. A) Failure of eyelid fusion can be observed at E16.5 (top row, note open puckered eyelids indicated by arrow) and becomes more evident at E18.5 (bottom row). A similar failure in eyelid fusion can be observed in *Mena*<sup>-/-</sup>;*Profilin*<sup>+/-</sup> mice. B) Occasionally, E18.5 *mmvvee* mice were observed with gut herniation at the umbilicus. C) The sculpting of digits is unaffected in triple-null mice.

Mice lacking VASP and EVL are viable as adults (Table 1) but are noticeably smaller in size than littermates when observed at four weeks of age (data not shown). Cells of the hematopoietic system express high amounts of VASP and EVL and very low levels of Mena. As much of the immune system develops postnatally we hoped that the MMvvee mice may provide a mode to analyze Ena/VASP proteins' role in the immune system. Previous work has shown a role for Ena/VASP proteins in the function of T cell and macrophage cell lines (Coppolino et al., 2001; Krause et al., 2000). Inhibition of Ena/VASP prevented immune synapse formation in T cells and macrophage phagocytosis of opsonized beads. Several events in immune system development require actin cytoskeleton rearrangement including the homing of T cell precursors to the thymus and emigration of mature immune cells from sites of development to the bloodstream and lymph nodes. We questioned whether Ena/VASP may play a role in immune system development *in vivo*. We analyzed cells from the blood, peripheral lymph nodes, thymus, bone marrow, and spleen by fluorescent activated cell sorting (FACS) using antibodies against mature cell markers for B cells, T cells, and myeloid cells including macrophages and PMNs. Each of these compartments possessed the normal complement and ratios of mature hematopoietic cell types, suggesting that loss of VASP and EVL is tolerated during immune system development (data not shown). Interestingly, we noted the presence of macrophage and neutrophils engulfing extravasated fibrin and red blood cells in the mmvvee electron microscopy sections (Fig. 4, E and G). This indicates that Ena/VASP-deficient inflammatory cells are capable of localizing to sites of vascular damage and engulfing damaged tissue.

## **Discussion**

In the fly and worm the functions of Ena/VASP proteins are largely confined to the nervous system. Previous work with the Mena and VASP knockout mice confirmed the developmental importance of Ena/VASP in the nervous system and suggested additional extraneuronal roles for Ena/VASP. This work demonstrates that Ena/VASP proteins play important roles in fusion of epithelial sheets and in some epithelial functions. However, in contrast with published reports, we have found no evidence for Ena/VASP in the anatomic or cellular architecture of the heart, nor in the integrity of the skin. Nor does the loss of Ena/VASP cause general defects in cell migration necessary for establishing basic embryo topology or organogenesis. Furthermore, recent work has defined new roles for axon guidance molecules in the development of branched structures such as the lungs and blood vessels. While we have observed dysfunction of blood vessels, we have not observed defects in the branching morphogenesis of blood vessels or of lungs. This suggests that the pathways downstream of axon guidance molecules have not necessarily been conserved outside of the nervous system.

### **Ena/VASP in epithelial development and function**

During development, the neural folds are brought into apposition through a series of actin-based alterations in cell morphology and movement. This is followed by filopodial knitting between the adjacent sheets which then fuse through the maturation of adherens junctions. Defects in neurulation are caused by one of three classes of mutations: folate-responsive, inositol-responsive, and non-responsive. The latter group can not be rescued by the dietary administration of folate or inositol. The mechanism by which supplementation with either nutrient ameliorates neurulation has been unclear. Administration of inositol may affect  $\text{Ca}^{2+}$  or phosphoinositol signaling at the membrane, whereas folate functions as an essential cofactor in

several metabolic pathways(Copp and Greene, 2000). The recent identification of RIAM and Lpd as PH-domain containing proteins that interact with Ena/VASP suggested a link between inositol signaling and Ena/VASP, and that Ena/VASP proteins may form part of the inositol-responsive neurulation pathway(Krause et al., 2004; Lafuente et al., 2004). The failure of Ena/VASP-deficient mice to respond to inositol supplementation indicates that Ena/VASP is not part of this pathway.

The *mmvEE* and the *Mena<sup>-/-</sup>;Profilin<sup>+/-</sup>* mice have been previously described to exhibit exencephaly(Lanier et al., 1999; Menzies et al., 2004). However, the mechanism of exencephaly and its relationship to CLP has been unclear. Analysis of these embryos at E8.5, prior to neural tube fusion, showed the neural folds are not properly apposed suggesting that a primary defect may lie in the hinge region(Menzies et al., 2004). However, mutations of *Drosophila Ena* cause defects in dorsal closure, a process analogous to a variety of epithelia fusion events in the developing mouse, including neurulation(Grevengoed et al., 2001). Our work has uncovered failures in a variety of these events including eyelid fusion, and midline fusion at the umbilicus. Further, these fusions are similarly affected in the exencephalic *Mena<sup>-/-</sup>;Profilin<sup>+/-</sup>* mouse. While we can not eliminate a possible contribution of hinge formation, we believe that mutation of Ena/VASP proteins results in a fundamental defect in epithelial fusion.

Work in a variety of cell types, including fibroblasts and primary neurons, have described a role for Ena/VASP proteins in the formation of filopodia. Initiation of epithelial fusion has been reported to require the interdigitation of filopodia. An attractive hypothesis suggested by this work is that failure to elaborate filopodia in apposed epithelia prevents fusion initiation. However, prior to fusion the sheets must migrate towards one another in a process of directed motility mediated by as yet undescribed factors. Ena/VASP proteins function in lamellipodial

dynamics required for directed motility, and loss of Ena/VASP may prevent the proper apposition of epithelia necessary for fusion. Consistently, analysis of eyelid closure at E16.5, when the eyelids should have just completed fusion, shows abnormal puckering, suggesting the lids were not properly apposed. Further work will be necessary to distinguish between these two possible modes of fusion failure.

Previous work had suggested an essential role for Ena/VASP proteins in the generation and maintenance of an epithelial monolayer. The expression of a COOH-terminal portion of mammalian VASP including the coiled-coil region responsible for tetramerization (TD construct) in keratinocytes resulted in skin blistering at birth (Vasioukhin et al., 2000). This result and the prominent localization of Ena/VASP proteins at cell:cell junctions and focal adhesions suggested an integral role for Ena/VASP proteins in the establishment of an intact epithelium. The Ena/VASP-deficient embryos described in this study do not possess a skin phenotype. An ultrastructural analysis by electron microscopy will be necessary to determine if the junctions between adjacent keratinocytes are normal or whether a similar defect may be present as seen in endothelial cells. Furthermore, to test the integrity of the keratinocyte junctions we plan to assay the skin's barrier function.. Regardless, the lack of blistering in the Ena/VASP-deficient mice represents a clear distinction from previously published reports. This discrepancy likely reflects non-specific activities of the TD domain. The expression of TD was predicted to form non-productive tetramers with endogenous Ena/VASP proteins. However, the requirements for Ena/VASP tetramerization for function are unclear. While expression of a mutant Mena allele lacking its coiled-coil domain (Mena<sup>ΔCOCO</sup>) prevents rescue of motility defects in an Ena/VASP-null cell line, expression of Mena<sup>ΔCOCO</sup> in a wildtype cell line induces an overexpression phenotype (Loureiro et al., 2002). Furthermore, the TD construct contains additional sequence

from the EVH2 domain outside of the coiled-coil. The ability of an isolated EVH2 domain to rescue Ena/VASP-null fibroblast motility and to bind actin barbed ends *in vitro* suggests that the N-terminal two-thirds of Ena/VASP likely serve localization and regulatory functions but are not central to Ena/VASP's anti-capping activity(Loureiro et al., 2002). Overexpression of a Mena mutant lacking the F-actin binding region (Mena<sup>ΔFAB</sup>) retains a coiled-coil and can form tetramers but does not have anticapping activity. Expression of this construct in a wildtype cell line does not produce an overexpression phenotype but neither does it interfere with endogenous Ena/VASP function despite its ability to tetramerize with endogenous Ena/VASP(Loureiro et al., 2002). Thus, one can not predict the functional consequences of TD overexpression at the adherens junction, but it is likely not purely inhibitory.

Similarly, *in vivo* myocyte expression of a VASP NH<sub>3</sub>-terminal EVH1 construct caused dilated cardiomyopathy and disorganization of myocyte intercalated disks(Eigenthaler et al., 2003). These defects were not present in the mmvvee embryos. The overexpression of an EVH1 domain within a cell delocalizes endogenous Ena/VASP proteins from proteins containing FPPPP ligands. However, the functional consequence of EVH1 binding to its ligands in the absence of the proline-rich region and EVH2 domain is unclear.

In contrast with the integrity of epithelia, loss of Ena/VASP clearly affects the integrity of endothelia, possibly through its association with tight or adherens junctions. Interestingly, junctions were able to form between adjacent endothelial cells, but these junctions were incomplete and possibly reflected desmosome but not adherens or occludens junction formation. The status of the occludens junction has been reported to determine endothelial cell barrier function. Ena/VASP proteins have been previously reported to interact with the tight junction protein ZO-1 in a phosphorylation-dependent manner(Comerford et al., 2002). Furthermore,

phosphorylation of VASP correlates with increased barrier function of endothelial monolayers(Comerford et al., 2002). Barrier function of epithelial and endothelial sheets is inversely related to tension forces between cells(Stevens et al., 2000), and these properties are normally regulated by cAMP and Ca<sup>2+</sup> levels within endothelial cells(Moore et al., 1998). *In vitro*, inhibition of Ena/VASP function in HUVEC cells causes an increase in cell tension and a decrease in barrier function(this study and Furman et al., unpublished observations). A disruption of barrier function *in vivo* would be predicted to cause edema due to the loss of albumin to the interstitium. Consistently, we have identified subdermal edema as a primary phenotype in Ena/VASP triple-null embryos, and have observed ultrastructural defects in junction integrity between endothelial cells. Ena/VASP-deficient mice also develop hydrocephalus (Chapter 2), one possible cause of which is barrier dysfunction at the blood brain barrier.

#### **Lack of conservation of axon guidance signaling cascades in branching morphogenesis**

Surprisingly, triple-null mice did not show severe defects in either angiogenesis or lung branching morphogenesis. Members of the Slit/Robo, Netrin/DCC/Unc5, and Sema/Plexin families have all been implicated in the generation of these iteratively branched structures(Bedell et al., 2005; Ito et al., 2000; Kawasaki et al., 1999; Liu et al., 2004; Park et al., 2004; Park et al., 2003; Suchting et al., 2005; Torres-Vazquez et al., 2004; Xian et al., 2001). Ena/VASP interacts genetically and/or biochemically with these pathways in axon guidance in worms, flies, and vertebrates. The protrusion of filopodia by primary hippocampal neurons in response to netrin requires PKA phosphorylation of Ena/VASP proteins(Lebrand et al., 2004). Consistently, mice lacking both Mena and VASP fail to form the major forebrain commissures similar to defects in midline guidance caused by Netrin-1 or DCC mutation(Fazeli et al., 1997; Menzies et al., 2004; Serafini et al., 1996). Furthermore, the endothelial-specific Robo, Robo4, has been shown to

interact with Mena(Park et al., 2003). Endothelial tip cells are believed to navigate in a filopodial dependent manner similar to the neuronal growth cone(Gerhardt et al., 2003; Lu et al., 2004). The ability of endothelial tip cells lacking Ena/VASP proteins to respond to axon guidance cues remains to be examined; however, either the extension of filopodia is not required for tip cell navigation or filopodial protrusion and tip cell guidance downstream of axon guidance receptors can occur through an Ena/VASP-independent mechanism.

### **Ena/VASP proteins within the immune system**

The loss of VASP and EVL is tolerated in mouse development and in the generation of the mature cells of the immune system. Previous work had linked Ena/VASP proteins with the T cell adaptor protein Fyb/SLAP/ADAP, and suggested that this interaction was essential for immune functions including immunological synapse formation and macrophage phagocytosis downstream of the Fc $\gamma$  receptor(Coppolino et al., 2001; Krause et al., 2000). Confounding data from the Fyb/SLAP/ADAP knockout mouse failed to identify a role for this protein in actin polymerization in T cells during immunological synapse formation(Peterson et al., 2001). Electron micrographs in this study appear to show Ena/VASP-deficient macrophage and neutrophil engulfment of cellular and proteinaceous debris. The requirement for Ena/VASP in macrophage phagocytosis may be specific to signaling through the Fc $\gamma$  receptor. Alternatively, Ena/VASP may not be required for the actin-based extension of podocytes needed for phagocytosis. Additional work with Ena/VASP-deficient immune cells in vitro will be needed to resolve this discrepancy with the literature.

VASP and EVL are the predominant Ena/VASP proteins expressed in cells of the hematopoietic system(Lanier et al., 1999). However, mice lacking both proteins form an immune system and do not show evidence of immunological compromise. One possibility is that loss of



VASP and EVL can be tolerated in the absence of pathogen exposure. Preliminary experiments suggest that the response to some infectious agents may be compromised in MMvvee mice (Makar et al., unpublished observations). Recently, expression of a 75kDa isoform of Mena has been reported in B cells, where it appears to be tyrosine phosphorylated by Abl (Tani et al., 2003). Thus the expression of Mena within immune cells may compensate for loss of VASP and EVL. Due to the embryonic lethality of the triple-null we were unable to determine the fate of the triple-null immune system (except as described above). The ability of triple-null hematopoietic stem cells (HSCs) isolated from E13.5 livers to rescue the hematopoietic system of a lethally-irradiated mouse is currently under investigation.

## Experimental Procedures

**Animals.** Mena(Lanier et al., 1999), VASP (Aszodi et al., 1999), EVL (constructed by Kwiatkowski AV and Leslie, J, described in Chapter 2), and EVL<sup>flx</sup> (constructed by Kwiatkowski AV and Leslie J) mutant mice were previously generated. To determine the viability of Mena;VASP;EVL (MVE) combinations, the EVL mutant allele was crossed into MenaVASP (MV) mice that were maintained on a mixed background. Triple-heterozygote MVE crosses were established and pups were genotyped between P8.5-P16.5. To generate mmVVEe embryos, mmVVEe mice were crossed with MmVVEe mice to recover ME double-nulls at an expected frequency of 1 of 4. The ME crosses were initially conducted using mice on mixed backgrounds, and later duplicated by intercrossing mice that had been backcrossed at least four times to the inbred strains 129sv, C57BL6, or Balb/c. An equivalent strategy was employed to generate VE double-null mice. For timed-pregnancies, the morning that a vaginal plug was discovered was denoted embryonic day 0 (E0). To generate mmvvee embryos at reasonable frequencies, Mmvvee studs were crossed with Mmvvee, MmVvee and MmVVeE mice. Triple nulls were expected at frequencies of 1 of 4, or 1 of 8 for the latter two crosses. To test the ability of folic acid and inositol to rescue exencephaly we administered folic acid (3 mg/kg body weight in PBS; Sigma) daily by intraperitoneal injection between E0.5-E11.5, and myo-inositol (10mg/kg body weight in PBS; Sigma) daily between E8.5-E10.5.

**Histology and Immunohistochemistry.** Hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) stainings were performed on 5-micron sections using standard techniques. Immunohistochemistry using anti-smooth muscle actin antibody (clone 1A4 ascites, Sigma) was performed at 1:400 dilution and amplified via Tyramide Signal Amplification (Perkin Elmer) and detected with DAB (diaminobenzamidine) (as described in Chapter 2).

**Organ culture.** Embryos from ME crosses were harvested at E11.5 into PBS at 4 degrees Celsius until dissection. The trachea and primordial lungs were carefully dissected under a dissecting microscope onto organ culture inserts (Falcon cell inserts with 0.8-micron pore size). Cell inserts were placed into 6-well dishes containing 0.5 ml of serum-free BGJb medium (Fitton-Jackson modification; GIBCO/BRL). Media was replaced after 48 hours in culture. Lung buds were photographed immediately after dissection, 48 hours post-dissection, and 96-hours post-dissection. Terminal lung buds were counted along the periphery of the samples without knowledge of sample genotypes.

**Endothelial Cells.** HUVECs (Clonetics) were infected with retroviruses containing eGFP, eGFP-VASP or eGFP-Mito-FPPP expression constructs and isolated by FACS. Fluorescence images were obtained of eGFP-VASP expressing HUVEC monolayers on a Deltavision Spectris deconvolution microscope (Applied Precision). Transwell permeability assays were performed by adding  $0.5 \times 10^6$  HUVECs per transwell (Corning, PC membrane, 12MM diameter, 0.3 mM pore) and maintained at confluence for 3 days with daily media changes. Texas Red Dextran (Molecular Probes, 40,000 MW) was added to the top transwell chamber at a final concentration of 2 mg/ml and flux across the endothelial monolayer assessed by hourly sampling from the bottom chamber. Data shows a representative experiment done in triplicate. Error bars are standard deviation.

**Electron microscopy.** Entire embryos were immersed in fixative immediately after dissection in 2.5% Formaldehyde, 2.5% Glutaraldehyde in a 0.1M Sodium Cacodylate Buffer, pH7.4 (Electron Microscopy Sciences). After several days in fixative, tissue was cut into smaller pieces and returned to fixative. Tissue samples were placed in 1% Osmiumtetroxide/1.5% Potassiumferrocyanide (in H<sub>2</sub>O) for 1 hour at room temperature in the dark, washed with water,

and then placed in 1% Uranyl Acetate in H<sub>2</sub>O for 30 minutes. Samples were dehydrated through ethanol into propyleneoxide, and then infiltrated and embedded in Epon/Araldite resin. Ultrathin sections were cut (Reichert Ultracut-S Microtomes) and imaged by transmission electron microscopy (JEOL 1200EX).

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## *Chapter 4*

### **RNAi: Biology and Delivery**

This chapter is adapted from the review:

**RNAi as an experimental and Therapeutic Tool to Study and Regulate Physiological and  
Disease Processes**

by

Christopher P Dillon, Peter Sandy, Allesio Nencioni, Stephan Kissler, Douglas Rubinson and  
Luk van Parijs

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## **Abstract**

The pace and modes of biological research have been revolutionized by the discovery and subsequent development of RNAi as a research tool. Applied to *in vitro* screens, RNAi allows for rapid and powerful forward genetics to identify genes involved in a particular process. The speed and specificity of the RNAi response allows for the rapid construction of cells and animals lacking a protein of interest. This facility has transformed reverse genetics in mammals. It allows researchers to invert the normal path of experimentation and study the phenotype of a hypomorphic cell line or tissue before deciding to proceed with further investigation. In addition to basic research, RNAi has tremendous potential as a therapeutic. This chapter will review the biology of RNAi, discuss its potential as a therapeutic tool, and evaluate the mechanisms for its delivery into cells, tissues and animals.

## Introduction

The mechanisms by which gene function are determined *in vivo* consist of overexpression, mutation, and gene deletion. These processes can be conducted in both tissue culture as well as in animals (transgenics and knockouts). Arguably the most informative of these approaches is gene deletion. However, elimination of gene function has previously required the costly and time-consuming construction of a knockout mouse, a technique that can not be transferred to human biology. As an alternative, dominant-interfering constructs have been employed to abrogate gene function. However, this approach requires controls to demonstrate the effectiveness and specificity of the construct. These controls are difficult to validate and are often ignored by the researcher, undermining the legitimacy of the results. There are numerous examples within the literature of knockout mice that once constructed undermine published phenotypes obtained with dominant interfering constructs (see Chapter 3 of this thesis).

The completion of the human and mouse genome projects has provided researchers with a nearly complete list of genes coded in the genome. Analysis of the genome has uncovered several trends that demonstrate the inadequacy of homologous recombination as the sole means for gene disruption. Many genes are present within the genome as members of gene families. These families can contain several members with overlapping expression and function. As such, phenotypes often emerge only with disruption of multiple family members, and distinct phenotypes may emerge with different combinations of knockouts (see Chapters 2 and 3 of this thesis). The time and expense associated with knockout construction makes the complete characterization of a gene family extraordinarily difficult. Second, the human genome is estimated to possess only 30,000 genes, a surprisingly modest increase in complexity when compared to lower eukaryotes (Lander et al., 2001). However, it has been proposed that

alternative splicing provides significant additional complexity and that 40-60% of human genes are alternatively spliced (Modrek and Lee, 2002). Our ability to determine the functional significance of these splice isoforms requires a method to eliminate isoform-specific gene products.

The introduction of long double-stranded RNAs (dsRNA) abrogates gene expression in primitive organisms through a process known as RNA interference (RNAi). Initially described in *Caenorhabditis elegans* and *Drosophila melanogaster*, RNAi is highly conserved in all multicellular organisms (Hannon, 2002; Sharp, 1999) including plants (Baulcombe, 1999). The potential use of RNAi in mammalian cell culture and animals was initially stymied by the dsRNA-dependent induction of a potent and non-specific anti-viral response mediated by the dsRNA-dependent protein kinase (PKR) (Caplen et al., 2000; Ui-Tei et al., 2000). This response, also known as the interferon response, inhibits protein translation and causes apoptosis (Stark et al., 1998). Most RNA viruses generate a dsRNA form during their life-cycle. The interferon response has two arms: a cell autonomous suicide mechanism and a paracrine signal through secreted interferon to inhibit protein synthesis in neighboring cells (Williams and Haque, 1997). The breakthrough discovery by the Tuschl group demonstrating that 21 nucleotide (nt) dsRNAs could induce RNAi without triggering the interferon response has allowed RNAi technology to be applied to mammals (Elbashir et al., 2001a).

## **The biology of RNAi**

### **RNAi as a Novel Mechanism that Regulates Development, Physiology, and Disease**

The initial description of RNAi resulted from the discovery that introduction of exogenous long dsRNAs in *C. elegans* caused sequence-specific loss of expression of mRNAs (Fire et al., 1998). Since then, RNAi has been observed in most eukaryotes, with the

notable exception of *Saccharomyces cerevisiae*(Hutvagner and Zamore, 2002b). The evolutionary conservation of this process is thought to reflect the importance of a class of short noncoding RNAs, termed microRNAs (miRNAs). miRNAs were initially discovered in a screen for genes required for larval development in *C. elegans*(Lee et al., 1993). More recently, several groups have identified hundreds of miRNAs in species ranging from *C. elegans* to humans through experimental and computational strategies(Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003). A recent study has shown that overexpression of miRNAs alters the development of immune cells in mice(Chen et al., 2004), and that deletion of RNAi machinery (discussed below) disrupts both mouse and *Xenopus* development(Bernstein et al., 2003; Wienholds et al., 2003) indicating that miRNAs are critical for normal development and tissue physiology in mammals. Genomic microarray analysis has demonstrated a startling abundance of transcribed non-polyadenylated RNA throughout the genome, many of which likely function as miRNAs(Cheng et al., 2005). A computational approach to identify potential miRNA targets indicates that hundreds of genes within the human genome are likely regulated by miRNAs(Lewis et al., 2005). Intriguingly, misexpression of miRNAs has been reported in a number of cancers, suggesting that miRNAs may contribute to disease processes (Calin et al., 2002; Calin et al., 2004). To date little is known about the targets of most miRNAs(Bartel, 2004). In large part this is because these RNAs show imperfect homology with the mRNAs that they regulate(Doench and Sharp, 2004).

### **Biochemistry of RNAi**

Significant strides have been made in our understanding of the biochemical mechanisms by which endogenous miRNAs silence gene function. Initially, miRNAs are transcribed as precursors up to 2 kb in length that exhibit significant secondary structure owing to the presence



of stretches of bases that can undergo extensive base pairing followed by stretches that adopt loop structures(Lagos-Quintana et al., 2002; Lee et al., 2002). Importantly, the pairing regions, or stems, present in miRNAs often contain a small number of mismatched bases that create “bubbles“ in the miRNA structure(Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

Primary miRNA transcripts are processed in the nucleus by the RNase III enzyme, Drosha, into approximately 70-nt-long precursors, known as pre-miRNAs(Lee et al., 2003). These are exported to the cytoplasm, where they are cleaved by a second RNase III enzyme, Dicer. Dicer converts pre-miRNAs into double-stranded 21- to 23-nt-long mature miRNAs(Bernstein et al., 2001). Mature miRNAs associate with an enzymatic machine known as the RNA-induced silencing complex (RISC). The composition of the RISC is not completely defined, but includes Argonaute family proteins(Hammond et al., 2001; Hutvagner and Zamore, 2002b; Morel et al., 2002; Mourelatos et al., 2002; Sasaki et al., 2003; Sontheimer, 2005; Tabara et al., 1999; Williams and Rubin, 2002). The RISC unwinds miRNAs and associates stably with the (antisense) strand that is complementary to target mRNA(Martinez et al., 2002; Schwarz et al., 2003; Tomari et al., 2004).

Depending on the degree of homology between a miRNA and its target mRNA, the miRNA-RISC complex inhibit gene function by one of two distinct pathways(Bartel, 2004). Most miRNAs pair imperfectly with their targets and silence gene expression by translational repression(Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). This RNAi mechanism appears to operate most efficiently when multiple miRNA-binding sites are present in the 3'UTR of the target mRNAs(Bartel and Chen, 2004; Doench et al., 2003). In some cases, miRNAs exhibit perfect sequence identity with the target mRNA and inhibit gene function by

triggering mRNA degradation(Hutvagner and Zamore, 2002a). As discussed below, this appears to be the dominant mechanism by which synthetic siRNAs and plasmid-expressed shRNAs silence gene expression.

## **RNAi as an experimental tool**

### **Discovery and Design of siRNAs**

The bubbles and loops that intersperse miRNAs presumably allow these transcripts to avoid evoking the PKR/interferon response. These transcripts are processed by DICER to yield 21-23nt dsRNA intermediates with 3' overhangs(Zamore et al., 2000). These intermediates then guide the RISC machinery in degrading the corresponding mRNA(Elbashir et al., 2001b). The experimental application of RNAi in mammalian cells was reliant upon this understanding of RNAi biochemistry. Chemically synthesized dsRNAs that mimic these DICER products (called siRNAs) could induce RNAi without activating PKR/interferon.(Elbashir et al., 2001a). The specificity of the approach (discussed below) was suggested by the exquisite sensitivity to single basepair mismatches in the center of the dimer(Elbashir et al., 2001a).

Initial efforts to apply this technology were partly frustrated by the failure of a majority of siRNAs to function in directing mRNA cleavage. Guidelines for the design of siRNA sequences relied upon empirical observations that were minimally useful and often contradictory(Elbashir et al., 2001a; Elbashir et al., 2001b; Tuschl et al., 1999). These rules governed such properties as GC-composition, and the region of the mRNA targeted (5' UTR, coding sequence, 3' UTR).

Recently, two approaches have converged on a unified set of rules governing siRNA design. The first approach was based upon the apparent symmetry of an siRNA. An siRNA is made up of two single-stranded RNAs, only one of which (antisense) complements the targeted mRNA. While the RISC complex initially binds the double-stranded siRNA, it only stably associates

with a single strand. Strand choice was determined by the comparative melting temperature of the two ends of an siRNA. The RISC complex preferentially associated with the antisense strand only if the melting temperature of the 5' end of the antisense strand (to the sense strand) was lower than the 3' end (Khvorova et al., 2003; Schwarz et al., 2003). The introduction of mutations in the sense strand to decrease the melting temperature at the 5' end of the antisense strand resulted in more effective silencing. Independently, researchers at Dharmacon employed a systematic approach to delineate the properties of an effective siRNA. A large panel of siRNAs was generated against two genes and the effectiveness of each siRNA was analyzed. Shared traits that associated with effective siRNAs were identified to generate a scoring system for siRNA design(Reynolds et al., 2004). The most important attribute identified by Dharmacon was the melting temperature at the 5' end of the antisense strand.

### **Stable Induction of RNAi in Mammalian Cells Through Expression of shRNAs**

In some organisms, RNA-dependent RNA polymerases exist that are able to amplify siRNAs and even pass them on through the germ line(Sijen et al., 2001; Smardon et al., 2000). As a consequence, introduction of dsRNAs triggers long-lived, organismal, stable gene silencing in these organisms(Sijen et al., 2001). These polymerases do not exist in mammalian cells and, consequently, both the degree and longevity of gene silencing induced by siRNAs is limited by the number of RNA molecules introduced into a cell. In many cell culture systems, gene silencing is seen for only a few days after siRNAs are administered(Elbashir et al., 2001a). This is not true for all cell types, especially those that do not proliferate. Stable gene silencing induced by siRNAs has been observed for weeks in macrophages and hepatocytes(Song et al., 2003a; Song et al., 2003b). Chemical modification of the RNA backbone to increase intracellular stability also enhances siRNA longevity(Chiu and Rana, 2003).

As opposed to siRNAs, short hairpin RNAs (shRNAs) can be transcribed from a DNA template from a single promoter. shRNAs are composed of complementary RNAs of 19-30nt with an intervening loop region. This sequence folds into a stem-loop structure that resembles endogenous pre-miRNAs, and as such, shRNAs are processed by DICER to produce siRNAs within the cell (Brummelkamp et al., 2002b; Paddison et al., 2002; Paul et al., 2002). The RNA polymerase III promoters U6 or H1 are used to drive expression of shRNAs. RNA polymerase III (Pol III) directs transcription of tRNAs, snRNAs, the RNaseP RNA, and other small RNAs within the cell. Pol III transcription is well-suited for shRNA production. Transcription initiation and termination sites are well-defined, and transcription is highly efficient- polIII recycles to complete several rounds of transcription without leaving the DNA template (Dieci and Sentenac, 1996; Paule and White, 2000). The U6 and H1 promoters are unique from other pol III promoters in that the promoter sequences are located upstream of their start sites (Chong et al., 2001).

### **Regulated and Tissue-Specific Gene Silencing by RNAi**

The development of shRNA expression-based RNAi suggested the development of techniques to control the timing and/or tissues in which the shRNA was expressed. Several approaches have been developed for this purpose. Hybrid promoters have been constructed to create tetracycline or ecdysone responsive H1 or U6 promoters (Chen et al., 2003; Czauderna et al., 2003b; Gupta et al., 2004; Matsukura et al., 2003; van de Wetering et al., 2003; Wang et al., 2003; Wiznerowicz and Trono, 2003). A second approach makes use of a modified U6 promoter that is incompetent for transcription due to the inclusion of a floxed cassette. Cre recombinase mediated recombination regenerates a competent U6 promoter to drive shRNA expression (Fritsch et al., 2004; Kasim et al., 2003; Tiscornia et al., 2004; Ventura et al., 2004). Pol II promoters, which drive the expression of polyadenylated mRNAs, provide an alternative

means to achieve tissue-specific shRNA production. With few exceptions, most of the early attempts to drive shRNA production directly from a pol II promoter were not successful due to the imprecise transcription start site and post-transcriptional modifications (polyadenylation, 5' methylguanine cap)(Shinagawa and Ishii, 2003; Xia et al., 2002). More recently, shRNA constructs have been buried within the 3'UTR of genes, to resemble naturally occurring miRNAs(Zeng et al., 2002).

### **RNAi Specificity**

The most contentious issue in the experimental application of RNAi concerns the specificity of gene silencing. The initial description of siRNAs claimed the exquisite sensitivity to single base pair mutations in the middle of the siRNA sequence(Elbashir et al., 2001a). This argues that perfect homology (at least in the middle of the siRNA) is required for gene silencing. Several groups have claimed marked alterations in the protein levels of nontargeted genes following RNAi administration. Other groups have employed microarrays to directly probe to specificity of RNAi(Bilanges and Stokoe, 2005). The concentration of siRNA(Persengiev et al., 2004), off-target homology with the 5' end of the siRNA(Jackson et al., 2003; Saxena et al., 2003), and even the mechanism of siRNA delivery (Fedorov et al., 2005) have all been shown to affect the spectrum and degree of RNAi specificity. As noted above, miRNAs frequently contain mismatches from their mRNA targets and result in gene silencing via translational repression(Doench and Sharp, 2004). Thus, the off-target effects caused by siRNAs could result from its acting similarly to miRNAs. As the mechanisms for generating off-target silencing are further delineated rules can be generated to predict potential non-specific targets(Qiu et al., 2005). These can be combined with the sequences from the genome project and the guidelines for siRNA construction to generate a heuristic to allow the use of effective and specific siRNAs.

## **PKR/Interferon Induction**

A second major concern among researchers using RNAi in mammals is the possibility that exogenous dsRNAs, despite their short length, may trigger an antiviral interferon response mediated by the PKR. Indeed, many early attempts at silencing gene expression using dsRNAs using strategies analogous to those developed for primitive organisms failed because they triggered the production of interferon, nonspecific gene silencing, and apoptosis in mammalian cells. Early work by Tuschl and colleagues suggested that dsRNAs that were less than 30 bases in length were able to silence gene expression in a specific manner, while eluding the molecular machinery responsible for triggering the interferon response(Elbashir et al., 2001a). This finding has been corroborated by the successful use of siRNAs as reagents to interfere specifically with gene function in a wider variety of different mammalian systems. The successful production of transgenic knockdown mice expressing shRNAs (discussed below) strongly suggests that PKR/interferon induction is not a general property of shRNAs. However, a number of studies suggest that short dsRNAs can trigger the expression of some of the target genes of the interferon response and, in some cases, can induce the cellular changes associated with this process(Bridge et al., 2003; Kim et al., 2004; Sledz et al., 2003). It is not clear how often siRNAs and shRNAs trigger the interferon pathway and which conditions favor this response to these RNAs. It may be that chemical features of dsRNAs, as well as their expression levels and delivery routes, may determine whether they become visible to the interferon response machinery(Bridge et al., 2003; Kim et al., 2004; Sledz et al., 2003).

Similar to the interferon response, evidence exists that siRNAs and shRNAs can activate dendritic cells and other cells of the immune system through a much more specific and restricted class of receptors, the Toll-like receptors (TLRs), that can recognize foreign nucleic acids

including dsRNAs(Alexopoulou et al., 2001; Kariko et al., 2004). Analysis of viral genomes has uncovered an unexpected abundance of virally encoded miRNA sequences(Bennasser et al., 2004; Pfeffer et al., 2005). Therefore, an immune mechanism to differentiate between endogenous miRNA and virally encoded miRNAs may have evolved. It is possible that there are structural requirements to experimentally introduced shRNAs to avoid triggering this immune response. While the consequence of this remains to be determined, these findings do raise the possibility that RNAi reagents may trigger adverse immune responses in vivo.

### **RNAi delivery: Hail to the vector**

#### **In Vivo Delivery of siRNAs to Induce RNAi**

By allowing efficient and cheap silencing of gene expression, RNAi promises to provide a significant boost to research of the genetic basis of normal tissue physiology, as well as disease process in animal models. For this reason, many groups have worked on developing strategies to deliver siRNAs or shRNAs to cells and tissues of experimental animals. Early efforts focused on direct administration of synthetic siRNAs, and four major delivery methods have been shown to be successful. The first of these, intravenous injection of siRNAs in a large volume (1 ml) of saline solution, works by creating a back-flow in the venous system that forces the siRNA solution into several organs (mainly the liver, but also kidneys and lung with lesser efficiency)(Lewis et al., 2002; McCaffrey et al., 2003).

Gene silencing has also been achieved in vivo by injecting smaller volumes of siRNAs that are packaged in cationic liposomes. When siRNAs are administered intravenously using this strategy, silencing is primarily seen in highly perfused tissues, such as the lung, liver, and spleen(Sorensen et al., 2003). Local delivery of siRNAs has been shown to be successful in the central nervous system(Baker-Herman et al., 2004). Gene silencing has also been achieved by

electroporation of siRNA duplexes directly into target tissues and organs, including muscle, retina, and the brain(Kishida et al., 2004; Kong et al., 2004; Konishi et al., 2004).

Although successful, it is likely that these strategies to silence genes are limited by the stability of siRNAs molecules in vivo and the efficiency by which they are taken up target cells and tissues. Much effort has been directed to increasing the half-life of the siRNAs by modifying the chemistry of the RNAs used(Braasch et al., 2003; Braasch et al., 2004; Chiu and Rana, 2003; Czauderna et al., 2003a). A number of groups have also used plasmid-based shRNAs, instead of siRNAs, to obtain relatively long-lived gene silencing in vivo(Zhang et al., 2003). A number of approaches have also been shown to improve cell and tissue delivery of siRNAs and shRNAs, including conjugating RNAs to membrane-permeant peptides and by incorporating specific binding reagents such as monoclonal antibodies into liposomes used to encapsulate siRNAs(Muratovska and Eccles, 2004; Zhang et al., 2003).

The therapeutic potential of siRNA delivery was stunningly demonstrated by a group at Alnylam Pharmaceuticals. siRNAs against Apolipoprotein B (Apo B) were chemically modified to increase stability and to include a conjugated cholesterol moiety. The included cholesterol moiety caused the siRNAs to reside within plasma lipoproteins that were then efficiently endocytosed by cells of the liver and small intestines. The silencing of ApoB was monitored by a reduction in serum ApoB and cholesterol levels(Soutschek et al., 2004).

### **Retroviral and Lentiviral Vectors for RNAi and Gene Therapy**

To obtain efficient and long-lived gene silencing using RNAi in cells and tissues, many groups have developed a variety of viral vectors to deliver siRNAs both *in vitro* and *in vivo*. Retrovirus-based vectors that permit stable introduction of genetic material into cycling cells(Lois et al., 2001) have been engineered to express shRNAs and to trigger RNAi in



transformed cells, as well as in primary cells(Abbas-Terki et al., 2002; Brummelkamp et al., 2002a; Dirac and Bernards, 2003; Hemann et al., 2003; Hommel et al., 2003; Qin et al., 2003; Rubinson et al., 2003; Stewart et al., 2003; Tiscornia et al., 2003). Because they infect and are expressed in certain adult stem cells, notably hematopoietic stem cells, retrovirus-based vectors have also been used to create “knockdown” tissues in mice(Hemann et al., 2003).

Lentiviruses, a class of retroviruses that includes HIV, provide even more wide-ranging applications of RNAi. Lentiviruses provide several advantages over murine retroviruses. The presence of a nuclear localization sequence (NLS) on several viral proteins allows import of the pre-integration complex into the nucleus of non-cycling cells(Bukrinsky and Haffar, 1997). This extends the range of infectible cells to include noncycling and postmitotic cells such as neurons and naïve immune cells(Abbas-Terki et al., 2002; Dirac and Bernards, 2003; Hommel et al., 2003; Rubinson et al., 2003). Attempts to use murine retroviruses to express exogenous genes in transgenic animals fail due to methylation and silencing of the viral LTR during development(Cherry et al., 2000). In contrast, lentiviral integrants are not susceptible to methylation and lentiviruses can be used to generate transgenic mice(Lois et al., 2002; Pfeifer et al., 2002) and other animals(Hofmann et al., 2003; Hofmann et al., 2004). Finally, lentiviruses better tolerate the expression of genes from one or more internal promoters(Mitta et al., 2004; Reiser et al., 2000). This allows for the generation of bifunctional lentiviral vectors that are capable of co-expressing shRNAs as well as either marker proteins (GFP) or other genes of interest. For these reasons, lentiviral RNAi vectors have been developed to generate transgenic knockdown animals by infecting embryonic stem cells or single-cell embryos(Rubinson et al., 2003; Tiscornia et al., 2003). These animals display expected loss-of-function phenotypes and transmit the integrated shRNA construct and resulting phenotype to their offspring, suggesting

that this technique represents an efficient, low-cost alternative to knockout technologies to study normal tissue physiology and disease processes in a variety of experimental animal systems(Rubinson et al., 2003; Tiscornia et al., 2003).

In addition to their use in basic research, lentiviruses have been proposed as a vector for gene therapy. *Ex vivo* lentiviral infection of hematopoietic stem cells can deliver wildtype copies of mutant genes. Efforts to use lentiviruses to deliver the wildtype  $\gamma$ c chain gene into hematopoietic stem cells from patients suffering from X-linked Severe Combined Immunodeficiency (X-SCID) successfully generated a functional immune system(Hacein-Bey-Abina et al., 2002). Similar strategies have been proposed for treatment of hemoglobinopathies(Puthenveetil et al., 2004). The use of lentiviral delivery of RNAi has been proposed as a gene therapy agent. RNAi knockdown of the HIV co-receptors CCR5 and CXCR4 within the hematopoietic stem cells of an HIV-positive patient should produce an uninfected immune cell population(Anderson and Akkina, 2005; Anderson et al., 2003; Zhou et al., 2004). Researchers have used RNAi to target the HIV mRNA directly{Novina, 2002 #318. However, the rate of HIV mutation and the sensitivity of RNAi to mismatched basepairs suggest that this technique may have limited efficacy *in vivo*.

The potential therapeutic use of lentiviruses has recently encountered a significant stumbling block. All retroviruses integrate randomly into the genome with integration hotspots at sites of active transcription{Schroder, 2002 #352}. Several of the X-SCID patients treated with *ex vivo* lentiviral therapy subsequently developed leukemia. The leukemic cells represented a clonal population of lentivirus infected cells in which the viral integration disrupted a tumor suppressor gene LMO-2(Hacein-Bey-Abina et al., 2003). Changes to the treatment protocol that rely on a

modified vector and significantly smaller pools of infected cells should minimize the risk of an integration resulting in tumorigenesis.

### **Adenovirus and Adenovirus-associated viruses for RNAi delivery**

Highly effective siRNA delivery systems have also been created that are based on adenoviruses and adenovirus-associated viruses (AAV). Adenoviruses can infect a wide range of cells, and have been shown to silence gene expression *in vivo* (Arts et al., 2003; Shen et al., 2003; Shen and Reske, 2004; Zhao et al., 2003). However, as opposed to retroviruses, adenoviral vectors do not integrate into the genome and tend to induce strong immune responses. In contrast, AAV does not cause disease in humans (Hildinger and Auricchio, 2004) and can integrate into the genome of infected cells. Unlike retroviruses and lentiviruses, AAV tends to integrate at a defined location in the genome, thus minimizing the chance of a mutagenic effect of the integrated virus (Kay and Nakai, 2003; Thomas et al., 2003). Effective gene silencing mediated by AAV-based vectors has been demonstrated following systemic or tissue-specific injection of viral particules (Boden et al., 2004; Xia et al., 2004; Xia et al., 2002). The unexplained death of a patient undergoing AAV-delivered gene therapy at the University of Pennsylvania has dampened some of the enthusiasm for this vector.

### **Creation of Transgenic Animal Models Using RNAi**

In addition to use of lentiviral vectors, more traditional transgenesis strategies have been used to successfully create loss-of-function models to study gene function in rodents using RNAi, thus providing another strategy by which RNAi might provide an alternative to creating gene knockout animals (Fedoriw et al., 2004; Hasuwa et al., 2002; Shinagawa and Ishii, 2003; Stein et al., 2003). Inheritable RNAi transgenesis has been achieved both through expression of shRNAs and long dsRNAs whose expression is restricted to the nucleus or oocyte (Fedoriw et al., 2004;

Hasuwa et al., 2002; Shinagawa and Ishii, 2003; Stein et al., 2003). On the basis of this small number of pioneering studies, it appears that RNAi is effective at silencing gene expression in many, if not all, tissues(Hasuwa et al., 2002). In some instances, attempts to create RNAi transgenic animals by injection of plasmids encoding shRNAs into single cell embryos have been unsuccessful, whereas injection of DNA into blastocysts has succeeded(Carmell et al., 2003). This may reflect a toxic effect of overexpression of siRNAs during early development, possibly due to competition with miRNA pathways.

Whereas RNAi in transgenic animals has been shown to recapitulate some loss-of-function phenotypes established in knockout animals, there is mounting evidence that the RNAi phenotype will often appear more variegated than the knockout phenotype(Kunath et al., 2003)(see Chapter 5 of this thesis). This is probably due to the fact that RNAi does not abrogate gene expression, but rather reduces it to varying levels. Although this may in some cases limit the use of RNAi in vivo, it is also likely to provide important new insights into the genetic basis of normal tissue physiology, disease processes, and therapeutic strategies by demonstrating the effects of altering gene expression to varying degrees. In particular, RNAi may prove especially important in the creation of animal models of human diseases in which susceptibility and resistance are encoded by alleles that show relative, rather than absolute, differences in expression levels(Hemann et al., 2003).

## **Summary and prospects**

In the past few years, RNAi has come to prominence as a novel and essential biological process, as well as a powerful experimental tool and a potential therapeutic strategy. New discoveries in the field of RNAi biochemistry, coupled with technological break-throughs, have permitted the creation of effective RNAi reagents that can be used to study normal tissue

physiology and disease processes in a range of settings, including experimental animals. By further exploring the biology of RNAi and improving delivery and evaluation technologies for RNAi reagents, these strategies will become more effective and more generally available. Now that the first phase I clinical studies of RNAi are on the horizon, several questions related to the safety and efficacy of using RNAi as a therapeutic strategy must be addressed. Ongoing and future preclinical studies in animal models will hopefully help optimize RNAi therapeutics for applications in humans.

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## *Chapter 5*

### **A lentivirus-based system to silence genes in primary mammalian cells and transgenic mice by RNAi**

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Christopher Dillon and this author contributed equally to this work. This author designed the vector and contributed the data with Christopher Dillon to Figures 1A-D, 2A, 3A-D, 4A-B,D,E, and components of Tables 1 and 2. Adam Kwiatkowski provided ES cell work. Lili Yang contributed Figure 2D,E. Luk van Parijs contributed Figure 2B and components of Table 1 and Table 2. Christopher Dillon contributed Figure 2C. Johnny Kopinja performed all embryo injections. Dina Rooney performed all blastocyst injections. Michael McManus contributed Figure 1F and 4C. Martin Scott contributed components of Table 1 and Table 2.

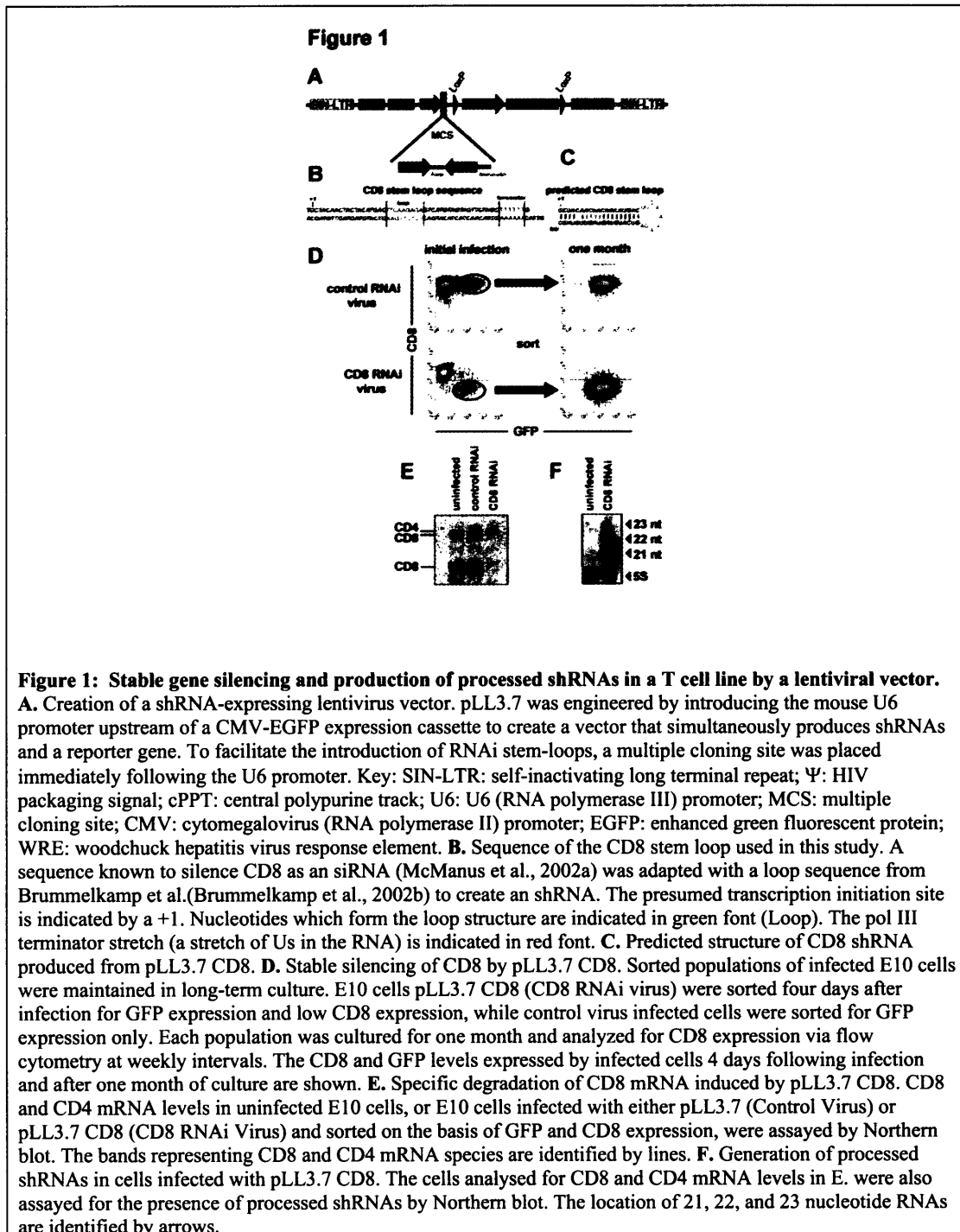
The text was written by this author and Luk van Parijs. The figures were assembled by Christopher Dillon. Work was conducted in the Van Parijs and Gertler labs at MIT, the Baltimore lab at California Institute of Technology, and the Martin Scott group at Biogen Inc..

## Abstract

RNA interference (RNAi) has recently emerged as a specific and efficient method to silence gene expression in mammalian cells either by transfection of short interfering RNAs (siRNA)(Elbashir et al., 2001) or, more recently, by transcription of short hairpin RNAs (shRNA) from expression vectors and retroviruses(Brummelkamp et al., 2002a; Brummelkamp et al., 2002b; Lee et al., 2002; McManus et al., 2002b; Miyagishi and Taira, 2002; Paddison et al., 2002a; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). However, the resistance of important cell types to transduction by these approaches, both *in vitro* and *in vivo*(McCaffrey et al., 2002), has limited the use of RNAi. Here, we describe a lentiviral system for delivery of shRNAs into cycling and non-cycling mammalian cells, stem cells, zygotes, and their differentiated progeny. We demonstrate that lentivirus-delivered shRNAs are capable of specific, highly stable, and functional silencing of gene expression in a variety of cell types and also in transgenic mice. Our lentiviral vectors should permit rapid and efficient analysis of gene function in primary human and animal cells and tissue, and the generation of animals that show reduced expression of specific genes. They may also provide new approaches for gene therapy.

Rapid progress in sequencing genes and characterizing their expression patterns has resulted in a growing list of coding regions that are predicted to contribute to normal mammalian tissue function and to the development of disease. Current approaches to study gene function, such as generating “knockout” mice, are time-consuming, expensive to perform, and cannot be directly applied to human tissues. RNA interference (RNAi) has recently emerged as a rapid and efficient means to manipulate and investigate gene function in mammalian cells (McManus and Sharp, 2002). Viral vectors, particularly retroviral vectors, are efficient, stable gene delivery tools in mammalian cells (Lois et al., 2001; Scherr and Eder, 2002), and recent studies suggest that they can stably express shRNAs in transformed and primary cells (Brummelkamp et al., 2002a). We have developed a lentivirus-based vector called pLL3.7 that expresses RNAi-inducing shRNAs under the control of the U6 promoter (Tuschl, 2002) (Fig. 1A). The pLL3.7 vector was also engineered to express EGFP as a reporter gene, permitting infected cells to be tracked by flow cytometry (Fig. 1A, 1D). Lentiviruses have two key advantages over other gene delivery systems. First, they can infect many non-cycling and post-mitotic cells (Naldini, 1998; Naldini et al., 1996). Second, transgenes expressed from lentiviruses are not silenced during development. Therefore lentiviruses can be used to generate transgenic animals through infection of embryonic stem cells or embryos (Lois et al., 2002; Pfeifer et al., 2002).

Initial experiments indicated that pLL3.7 vectors could be used to generate high titre infectious lentivirus and could express shRNAs and silence gene expression upon infection of mammalian cell lines (Fig. 1D, 1E, 1F, see Methods for details). To test whether pLL3.7 could be used to silence gene expression in primary mammalian cells, CD8<sup>+</sup> T cells derived from the spleens of OTI T cell receptor (TCR) transgenic mice

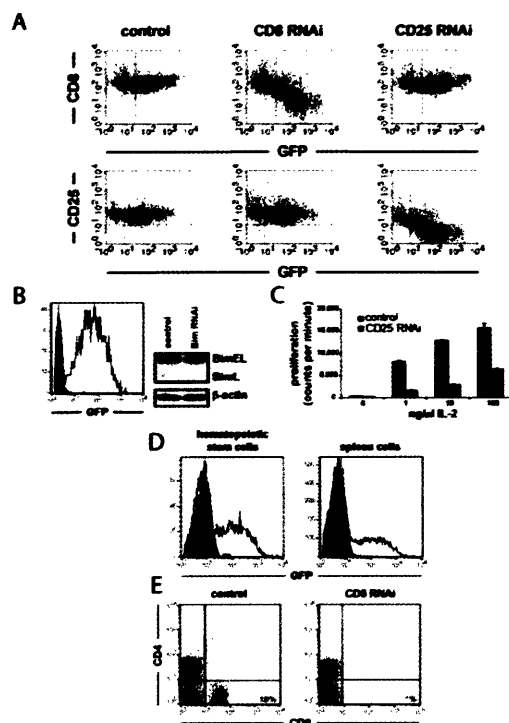


were activated with cognate peptide *in vitro* and infected with a version of pLL3.7 engineered to express a shRNA that silences expression of murine CD8 (pLL3.7 CD8) (Fig. 1B,

1C)(McManus et al., 2002a; McManus et al., 2002b). After three days, cells were harvested and analysed by flow cytometry. Between 68 and 82% of the cells were infected and reproducibly showed approximately a 14-fold reduction in CD8 expression, demonstrating that lentivirus-driven expression of shRNAs efficiently silenced gene expression in primary T cells (Fig. 2A). This effect of pLL3.7 CD8 was specific since infected cells showed normal expression of other T cell surface markers (Fig. 2A, and data not shown). To determine whether pLL3.7-based vectors could also silence gene expression in non-cycling cells, we infected murine dendritic cells with RNAi lentiviruses that expressed shRNAs against the pro-apoptotic molecule, Bim(Strasser et al., 2000) (pLL3.7 Bim). Infection of these cells with pLL3.7-based lentiviruses was also efficient, as gauged by GFP expression, and resulted in a significant reduction of Bim expression in cells that received pLL3.7 Bim (Fig. 2B).

To determine whether lentivirus-mediated expression of shRNAs could induce functional silencing of a gene in primary cells, OTI T cells(Hogquist et al., 1994) were infected with an RNAi lentivirus that targeted CD25, the alpha chain of the IL-2 receptor (pLL3.7 CD25) (Fig. 2A). IL-2 is an important growth factor for T cells, and T cells derived from mice that lack the receptor for this cytokine fail to proliferate *in vitro*(Willerford et al., 1995). Activated OTI T cells infected with pLL3.7 CD25 showed on average a 25-fold reduction in IL-2R $\alpha$  chain expression, but expressed normal levels of other surface markers (Fig. 2A, and data not shown). These cells were challenged with increasing concentrations of IL-2, resulting in a 4- to 5-fold reduction in the response to this cytokine (Fig. 2C). Since not all cells were infected in these

**Figure 2**

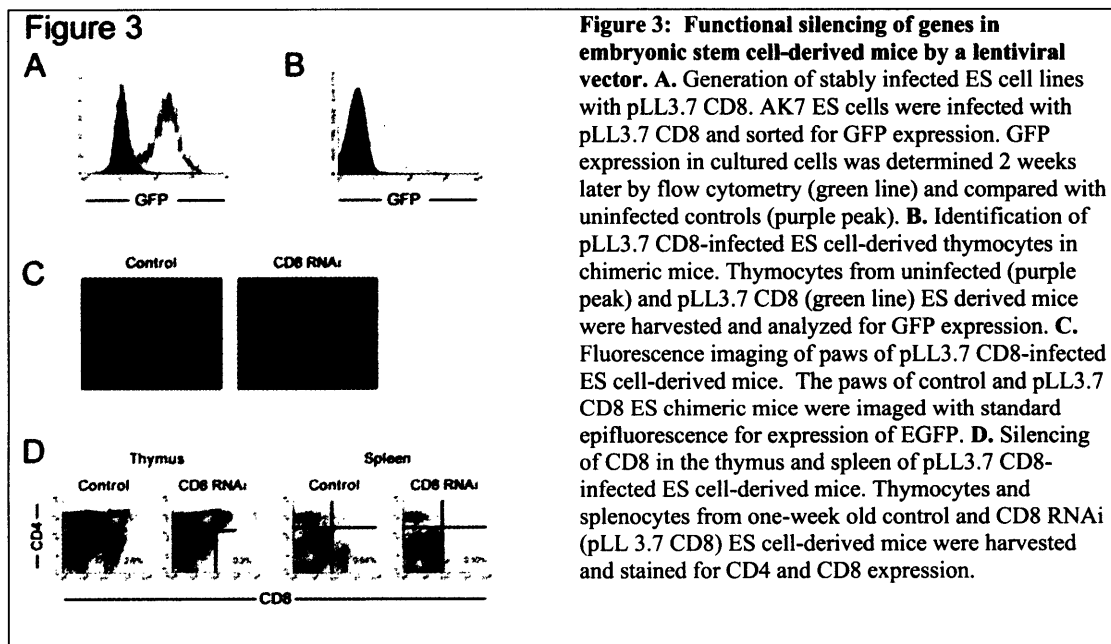


**Figure 2: Functional silencing of genes in cycling and non-cycling primary immune cells in vitro and in vivo by a lentiviral vector.** **A.** Specific silencing of genes in cycling T cells by pLL3.7 CD8 and pLL3.7 CD25. OTI CD8+ TCR transgenic T cells were activated for 3 days with cognate peptide and then infected with pLL3.7, pLL3.7 CD8, or pLL3.7 CD25. The efficiency of infection was determined by assaying GFP expression by flow cytometry. The expression of CD8 and CD25 on infected T cells was assayed by staining with specific antibodies that bind these surface markers. **B.** Efficient infection and gene silencing in non-cycling dendritic cells with pLL3.7 Bim. Bone marrow derived dendritic cells were infected with pLL3.7 or pLL3.7 Bim. The efficiency of infection was determined by assaying GFP expression by flow cytometry (green line) and comparing to uninfected control cells (purple peak). The expression of Bim in pLL3.7 CD8 infected and control dendritic cells was assayed by Western blot. **C.** Functional silencing of genes in primary T cells with pLL3.7 CD25. OTI CD8+ TCR transgenic T cells were infected and activated as in A. and then cultured for 48 hours in the presence of increasing concentrations of IL-2. Proliferation was assessed by <sup>3</sup>H-thymidine incorporation. **D.** Efficient infection of HSCs and gene expression in differentiated progeny with pLL3.7 CD8. HSCs were purified from the bone marrow of wild type mice, infected with pLL3.7, pLL3.7 CD8, or pLL3.7 CD25, and then cultured for 3 days with IL-3, IL-6, and SCF. The efficiency of infection was determined by assaying GFP expression by flow cytometry (left histogram, green line) and compared to uninfected cells (purple peak). Bone marrow chimeras were generated by injecting sorted (GFP+) HSCs into lethally irradiated recipients. 6 to 8 weeks later the contribution of these cells to the mature spleen cells of the chimeras was determined by staining with the congenic marker CD45.2, analysing GFP expression by flow cytometry (right histogram, green line), and comparing to mice that received uninfected HSCs (purple peak). Histograms show GFP expression of HSC-derived (CD45.2+) cells. **E.** Functional gene silencing in T cells derived from HSCs infected with pLL3.7 CD8. The percentage of CD8+ T cells in the spleen of bone marrow chimeras from pLL3.7 (left dot plot) and pLL3.7 CD8 (right dot plot) was determined by staining with antibodies to CD4 and CD8, as well as the congenic marker CD45.2, and flow cytometry. Dot plots show CD4 and CD8 expression of HSC-derived (CD45.2) cells.

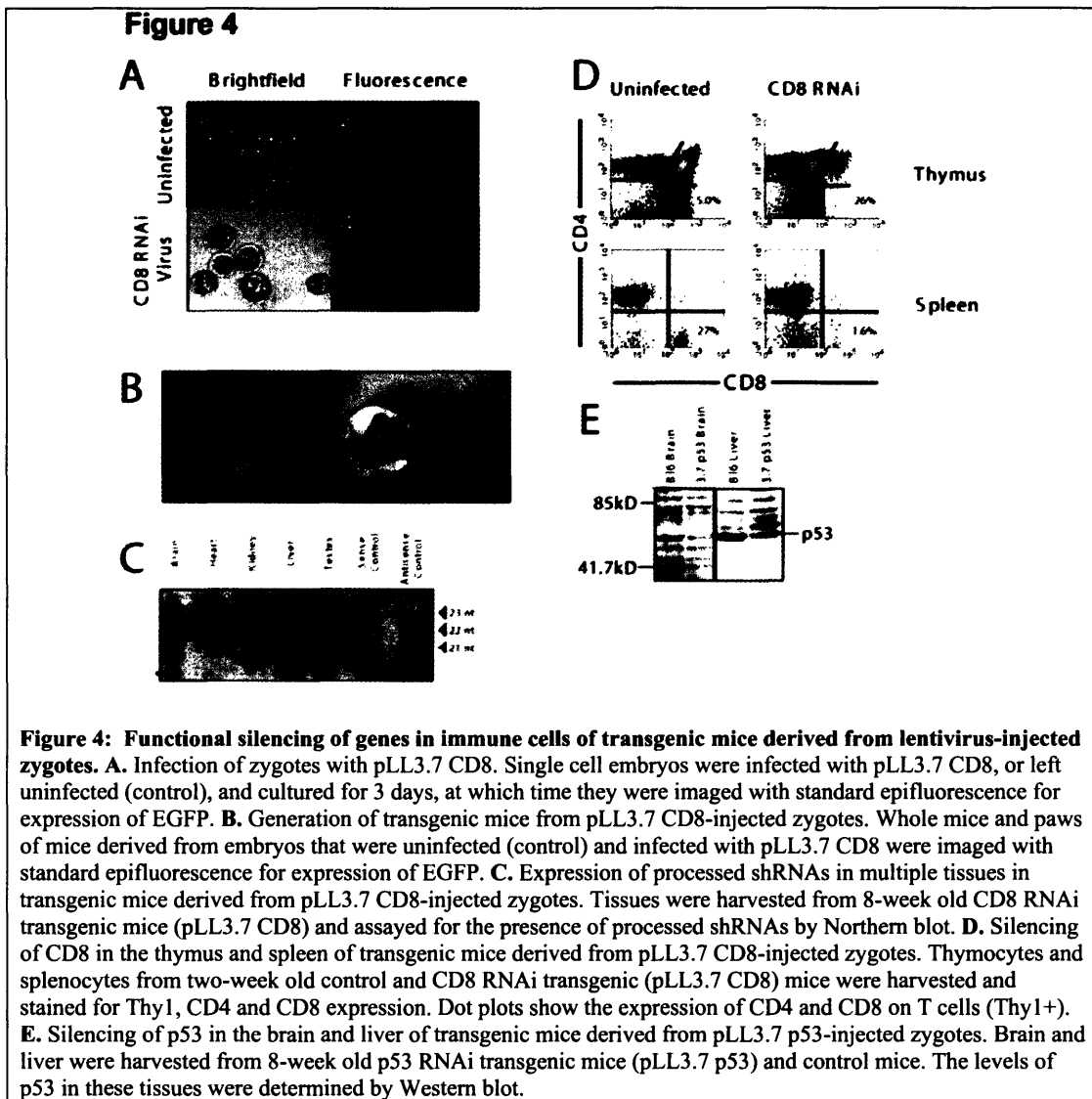
experiments (typically between 70 and 85% of cells were GFP+), it is likely that the actual inhibition of IL-2-induced proliferation by RNAi was even more significant.

Lentivirus-based vectors are capable of stably expressing transgenes in stem cells, and are not silenced during development (Pfeifer et al., 2002; Scherr and Eder, 2002). We tested whether pLL3.7 could be used to silence gene expression in murine haematopoietic stem cells (HSCs) and their progeny. To accomplish this, HSCs were purified from whole bone marrow by cell sorting, infected with pLL3.7 or pLL3.7 CD8, and then cultured for 2 days in the presence of cytokines. This protocol led to infection of 30 to 60% of HSCs (Fig. 2D). Next, GFP+ cells were sorted and injected into lethally irradiated congenic mice. After 8 weeks, the infected HSCs had contributed to all blood cell lineages in reconstituted mice as determined by staining for the congenic CD45 allele, and about 20 to 40% of HSC-derived lymphocytes were GFP+ (Fig. 2D). The presence of GFP- cells is probably the consequence of the low activity of the CMV promoter in these cells (Schmidt et al., 1990). To examine whether lentivirus-mediated expression of shRNAs in T cells resulted in gene silencing *in vivo*, splenocytes from reconstituted mice were stained and analyzed by flow cytometry. The frequency of CD8+ T cells present in mice that received HSCs infected with pLL3.7 CD8 were reduced at least 10-fold compared with those receiving cells infected with pLL3.7 (Figure 2e). No effect was seen on CD4+ T cell levels and other immune cell populations, indicating that lentivirus-induced gene silencing was both functional and specific. Confirming that we had infected true haematopoietic stem cells, we were able to serially passage bone marrow cells from reconstituted mice and still observe lentivirus-driven expression of GFP in haematopoietic cells (data not shown).

Lentiviruses have also been reported to infect mouse embryonic stem (ES) cells and to maintain their expression in transgenic mice generated from these cells (Pfeifer et al., 2002). We were able to generate and maintain stable lines of ES cells that were infected with pLL3.7 CD8, or with versions of pLL3.7 that expressed shRNAs against p53 (Paddison et al., 2002b), pLL3.7 p53, or a neuron-specific isoform of Mena (Gertler et al., 1996), pLL3.7 Mena<sup>+</sup> (Fig. 3A, and data not shown). To test whether gene silencing could be maintained throughout organogenesis, uniformly GFP<sup>+</sup> ES cell populations that had been infected with RNAi lentiviruses were purified by cell sorting and injected into RAG-deficient blastocysts, which were then implanted into pseudopregnant foster mothers. Because RAG-deficiency blocks T and B lymphocyte development in the bone marrow, any peripheral T and B lymphocytes present in the chimeric progeny must be derived from the injected (wild type) ES cells (Chen et al., 1993). The degree of chimerism in animals derived from cells infected by each virus was between 50 and 90% as gauged by GFP fluorescence analysis of whole mice and dissected organs (Fig. 3C, and data not shown). About 20 to 40% of immune







cells in these mice were GFP+ (Fig. 3B and data not shown). To examine whether lentivirus-mediated expression of shRNAs resulted in the silencing of CD8 *in vivo*, thymus and spleen cells of 7 day-old chimeric mice were stained with antibodies against CD8 and CD4, and then analysed by flow cytometry. Developing T cells in the thymus of pLL3.7 CD8 mice showed approximately a 9-fold reduction in CD8 expression (Fig. 3D). Furthermore, very few CD8+ T cells were detected in this organ or in the spleen (Fig. 3D). In contrast, thymocytes from these mice showed normal expression of CD4, and normal percentages of mature CD4+ T

cells in their lymphoid organs (Fig. 3D). No effects were observed on T cell differentiation and numbers in mice derived from pLL3.7 Mena+ and pLL3.7 infected ES cells (Fig. 3D and data not shown).

<b>Table 1: EFFICIENT GENERATION OF RNAi TRANSGENIC MICE THROUGH INFECTION OF ZYGOTES WITH LENTIVIRUSES</b>				
<b>RNAi Lentivirus</b>	<b>Litters</b>	<b>Total mice born</b>	<b>Number of transgenic offspring (number/percentage)</b>	<b>Number of lentiviral integrants (range/average)</b>
<b>Control</b>	2	15	8/53%	2 to 5/3.4
<b>CD8</b>	4	32	16/50%	2 to 6/3.1
<b>CD25</b>	7	42	11/26%	N.D.
<b>P53</b>	4	22	5/23%	N.D.
Legend: RNAi transgenic mice were generated with pLL3.7 (Control), pLL3.7 CD8, pLL3.7 CD25, and pLL3.7 p53 as described in the text. Transgenic offspring were identified based on their expression of GFP in the skin at 2 to 4 days of age using a 100W UV light source. The number of lentiviral integrants present in the genome of transgenic (GFP+) mice was determined by Southern blot. No lentiviral integrants were detected in 5 non-transgenic (GFP-) mice analyzed. N.D. Not determined.				

An efficient and broadly applicable approach to generate transgenic animals is direct lentiviral infection of single cell embryos (Lois et al., 2002). To determine whether this methodology could be used to bypass the use of ES cells and to directly generate transgenic mice that show specific silencing of genes, we infected single cell embryos with RNAi lentiviruses. Embryos infected with pLL3.7, pLL3.7 CD8, pLL3.7 CD25, pLL3.7 p53, or pLL3.7 Mena+ expressed GFP after 3 days of culture (Fig. 4A, and data not shown), and produced offspring that showed expression of GFP and siRNAs in all tissues tested (Table 1, Fig. 4B, 4C, and data not shown). Identical to ES cell chimeric mice, two-week old RNAi transgenic mice generated from zygotes that were infected with pLL3.7 CD8 showed approximately a 10-fold reduction in CD8 expression in developing thymocytes (Table 2 and Fig. 4D). The numbers of mature CD8+ T cells in the peripheral lymphoid organs of these mice was also reduced (Table 2 and Fig. 4D). Importantly, gene silencing was maintained in adult mice (Table 2). As in HSC-reconstituted and ES cell-chimeric mice, the expression of

GFP in immune cells of RNAi transgenic mice generated with pLL3.7 vectors was low (between 5 and 60%).

<b>Age of RNAi transgenic mice (weeks)</b>	<b>2</b>	<b>8</b>	<b>8</b>	<b>8</b>	<b>8</b>	<b>8</b>	<b>9</b>	<b>9</b>
<b>Decrease in % of mature CD8+ cells</b>	56%	20%	35%	44%	99%	100%	46%	56%
<b>% Decrease in CD8 expression</b>	93%	93%	93%	88%	87%	92%	94%	94%
<b>Number of lentiviral integrants</b>	N.D.	2	3	2	6	5	4	3

Legend: The frequency of CD8+ T cells present in neonatal and adult CD8 RNAi transgenic mice and age-matched controls and the reduction of CD8 expression in cells showing gene silencing was determined by staining lymphocytes with antibodies against Thy1, CD4, and CD8. The percent reduction in CD8+ T cells seen in CD8 RNAi transgenic mice was determined by dividing the frequency of CD8+ T cells in CD8 RNAi transgenic and control mice. The percent reduction in CD8 expression was determined by dividing the mean fluorescence intensity of CD8 staining on T cells (Thy1+) from CD8 RNAi transgenic mice that showed silencing by the mean fluorescence intensity of CD8 on T cells from control mice. The number of lentiviral integrants present in the genomes of the CD8 RNAi transgenic mice analyzed was established by Southern blot. N.D. Not determined.

Although all mice showed an equal degree of CD8 gene silencing in cells exhibiting silencing (Table 2), the percentage of cells showing gene silencing differed between transgenic strains (Table 2). This variation may be a function of the number of integrated lentiviral genomes present in different transgenic lines (Table 2). Gene silencing was not restricted to immune cells, since brain and liver cells derived from transgenic mice that expressed pLL3.7 p53 showed markedly reduced expression of the p53 tumor suppressor protein (Fig. 4E).

The promise of RNA interference as a means to efficiently silence genes in mammalian cells has become widely recognized (Hannon, 2002; McManus and Sharp, 2002; Paddison et al., 2002a; Tuschl, 2002). The results presented here demonstrate that lentiviruses can be used to express shRNAs and reduce gene expression in cycling and non-cycling cells from different tissues, as well as in chimeric and transgenic mice. This technology should allow

systematic genetic analysis in most cell types and tissues, including those of human origin, and should facilitate comprehensive studies of gene function in mice, as well as species that are not traditionally amenable to genetic manipulation. Lentiviral expression vectors might be used therapeutically to silence disease-causing genes, render cells resistant to infectious organisms, and to facilitate the creation of tissues deficient in specific antigens as source of transplant organs. Future modifications to lentiviral expression vectors, such as the inclusion of inducible polIII promoters, will likely extend the range of cells and situations in which they can induce RNAi.

## Methods

**Construction and validation of an RNAi lentivirus system:** The complete details of the construction of pLL3.7 and other “LentiLox” vectors are described online at <http://web.mit.edu/ccrhq/vanparijs/>. In brief, the pBFGW plasmid(Lois et al., 2002) was extensively modified to carry loxP sites, a CMV promoter driving expression of EGFP, and the mouse U6 promoter with downstream restriction sites (HpaI and XhoI) that allow the efficient introduction of oligonucleotides that code for shRNAs (Fig. 1A, 1B, 1C). The U6 promoter was chosen to drive expression of shRNAs since it had been shown by other groups to efficiently transcribe small RNAs that silence gene expression(Tuschl, 2002). Because very little is known about the effects of placing a strong RNA polymerase (pol) II promoter (CMV) close to a pol III promoter (U6), some degree of promoter interference was anticipated that might decrease expression of shRNAs in infected cells. The CMV promoter was therefore placed between LoxP sites to allow its removal if necessary.

To confirm that pLL3.7 could be used to silence gene expression in mammalian cells, a shRNA predicted to target CD8 was used to generate pLL3.7 CD8 (Fig/ 1b , 1c). The CD8 shRNA used was based on sequences that had previously been shown to downregulate this molecule in T cells(McManus et al., 2002a; McManus et al., 2002b). Lentivirus particles were generated as described below and used to infect E10 cells. Infected (GFP+) cells on average showed a 16-fold reduction of CD8 expression (Fig. 1D). Inhibition of CD8 expression was specific since the levels of other surface proteins were not altered (Fig. 1D, and data not shown). Furthermore, in a subline of E10 cells engineered to express human CD8, which differs from mouse CD8 by 4 of 19 nucleotides in the targeted region, only the murine version of the gene was silenced (data not shown). Cells infected with control virus (pLL3.7) or viruses expressing shRNAs against other genes showed no decrease in CD8 levels (Fig. 1D and data not shown). To confirm that the decrease in surface expression of

CD8 seen in infected E10 cells resulted from mRNA degradation, CD8 transcript levels in sorted (GFP+) cell populations infected with either pLL3.7 CD8 or a control virus was quantified by Northern blot (Fig. 1E). E10 cells that exhibited silencing expressed short RNAs of approximately 21 nucleotides that were complementary to an anti-sense strand of the CD8 stem loop, demonstrating that lentivirus-encoded shRNAs were being expressed and processed into siRNAs (Fig. 1F). To test the stability of lentivirus-induced RNAi in mammalian cells, we followed expression of CD8 in long-term cultures of E10 cells infected with pLL3.7 or pLL3.7 CD8 and sorted for expression of GFP (Fig. 1D). No change in expression of CD8 was observed over the course of a month, and these cells remained uniformly GFP positive (Fig. 1D). However, in each experiment a small fraction (2 to 10%) of infected (GFP+) E10 cells showed no evidence of gene silencing. These cells were shown to express no shRNAs (data not shown), suggesting that the activity of the U6 promoter was reduced, possibly due to positional effects on the inserted transgenes (Lois et al., 2001), (Lois et al., 2002).

**Design and sequences of shRNAs used in this study:** The pLL3.7 vector was engineered to allow efficient and directional introduction of oligonucleotides that encode shRNAs downstream of the U6 promoter. To this end an HpaI site was introduced such that digestion with this restriction enzyme leaves a blunt end at the -1 position of the promoter. An XhoI site was introduced further downstream (Fig. 1A). To design shRNAs, we have developed a set of criteria based both on the known specificity requirements for RNAi (Elbashir et al., 2001) and the structure of pLL3.7. In brief, we search the whole mRNA of target genes, including untranslated regions, for sequences that display the consensus sequence; AAGN<sub>18</sub>TT. The GN<sub>18</sub> component of this sequence should ideally have a GC content of approximately 50%, lack stretches of 4 or more As or Ts, which act as termination sequences for RNA polymerase III, and be devoid of substantial secondary structure. This sequence

should also be screened for significant homology to other genes. Once an acceptable GN<sub>18</sub> sequence is identified, a 5' thymidine is added to reconstitute the U6 promoter following HpaI digestion of pLL3.7 and a loop sequence is added to the 3' end of the sequence (TTCAAGAGA)(Brummelkamp et al., 2002b), followed by the reverse complement of the GN<sub>18</sub> sequence and a polymerase III termination sequence (TTTTTT). Once both the sense and anti-sense strands have been designed, sequences are added that allow the oligonucleotide to be annealed to an XhoI site. It should be noted that these design criteria do not guarantee that a sequence will be created that silences gene expression. More complete and up-to-date information on shRNA design, which incorporates new information on RNAi specificity and shRNA design as this becomes available, is available online (see above).

***CD8 oligonucleotide sequences:***

Sense: TGCTACAACACTACTACATGACTTCAAGAGAGTCATGTAGTAGTTGTAGCTTTTTTG

Antisense: GTTACAAAAAAGCTACAACACTACTACATGACTCTCTTGAAGTCATGTAGTAG  
TTGTAGCA

***CD25 oligonucleotide sequences:***

Sense: TGCATTACCTAATCGGCTGTTCAAGAGACAGCCGATTAGGTGAATGCTTTTTTG

Antisense:

GTCACCAAAAAAGCATTACCTAATCGGCTGTCTTGAACAGCCGATTAGGTGAATGCA

***Bim oligonucleotide sequences:***

Sense: TGGAGGGTGTGTTGCAAATGATTCAAGAGATCATTGCAAACACCCTCCTTTTTTG

Antisense: GTCACCAAAAAAGGAGGGTGTGTTGCAAATGATCTCTTGAATCATTGCAAACACCC

***p53 oligonucleotide sequences:***

Sense:

TGGTCTAAGTGGAGCCCTTCGAGTGTTAGAAGCTTGTGACACTCGGAGGGCTTCACTTGGGCCTTT  
TTGGAA

Antisense:

TCGATTTCAAAAAGGCCCAAGTGAAGCCCTCCGAGTGTCACAAGCTTCTAACACTCGAAGGGCT  
CCACTTAGACCA

***Mena+ oligonucleotide sequences:***

Sense: TGTCTGTGCCTGGCCTACTTTCAAGAGAAGTAGGCCAGGCACAGGACTTTTTGGAAAC

Antisense:

CGAGTTCCCAAAAAGTCCTGTGCCTGGCCTACTTCTTGTAAAGTAGGCCAGGCACAGGACA

**Generation and titre of lentivirus:** Lentiviral production was performed as described (Lois et al., 2002). Briefly, pLL3.7 and packaging vectors were co-transfected into 293T cells and the resulting supernatant was collected after 36 h. Virus was recovered after concentrating by ultracentrifugation for 1.5 hours at 25,000 rpm in a Beckman SW28 rotor and resuspended in PBS (15-200 $\mu$ L). Lentiviral titres were determined by culturing 3T3 cells with serial dilutions of concentrated lentivirus preparations. The percentage of infected cells was determined by assaying GFP expression by flow cytometry after 48 hours and for a typical preparation was approximately  $1 \times 10^8$  infectious units (IFU)/ml.

**T cell and dendritic cell infection:** CD8+ T cells were activated by culturing splenocytes derived from OTI TCR transgenic mice at a density of  $2 \times 10^6$ /ml in the presence of 1 $\mu$ g/ml of OVA peptide and 100ng/ml IL-2. After 24 and 48 hours cultures were supplemented with 20 and 100 $\times 10^6$  lentiviral particles (MOI of 10 to 50), 10 $\mu$ g/ml Polybrene, and spun for 1 hour at 30°C at 1,200 RPM in a Beckman Allegra 6R centrifuge. Supernatant was removed following infection and replaced with growth medium containing 1 $\mu$ g/ml OVA and 100ng/ml IL-2. T cells were harvested for experiments after 72 hours. Dendritic cells were generated by culturing whole bone marrow cells for 7 days at a density of  $2 \times 10^6$  per ml in the presence of 20ng/ml GM-CSF. During this culture period, fresh cytokine was provided every other day. The resulting cell populations were typically greater than 60% CD11c+ as determined by flow cytometry (data not shown). On day 7 dendritic cells were transferred to a new plate, cultured at a density of  $2 \times 10^6$  cells per ml with medium containing no GM-CSF, and infected



on day 8 and 9 with approximately  $100 \times 10^6$  (MOI of 50) lentiviral particles. Cells were harvested for analysis on day 10.

**Haematopoietic stem cell infection:** Whole mouse bone marrow cells were depleted for cells expressing B cell (B220) and granulocyte lineage markers (CD11b) using magnetic beads (Miltenyi) and then sorted for Sca1<sup>+</sup> and c-Kit<sup>+</sup> cells. These cells were spin infected with RNAi lentiviruses at an MOI of approximately 10 to 50, and then cultured at a density of  $2 \times 10^6$  cells per ml for 2 days in the presence of IL-3 (20ng/ml), IL-6 (50ng/ml), and SCF (50ng/ml). Infected HSCs were sorted for GFP expression, after which approximately  $2 \times 10^5$  cells were injected into lethally (1200 rads)  $\gamma$ -irradiated recipient mice that were congenic for the CD45 antigen to allow us to distinguish host and donor cells (HSCs were derived from a mouse strain that expresses CD45.2 on hematopoietic stem cells, recipient mice expressed CD45.1 on these cells). Most reconstituted mice also received  $2 \times 10^6$  host-derived (CD45.1) whole bone marrow cells. Bone marrow chimeras were analysed between 6 and 8-weeks after reconstitution for GFP expression in splenocytes and CD8 gene silencing by staining and flow cytometry. In all experiments, progeny of infected HSCs were identified by staining for the congenic marker, CD45.2.

**ES cells infection:** AK7 ES cells were maintained and infected as described (Pfeifer et al., 2002). Clones of ES cells were picked, expanded, and analyzed by flow cytometry for GFP expression. If the clone contained a mixed population of infected and uninfected cells, the GFP<sup>+</sup> population was purified by fluorescence activated cell sorting prior to blastocyst injection.

**Generation of RNAi chimeric and transgenic mice:** For ES cell-derived mice, approximately 10-12 pLL3.7-infected GFP<sup>+</sup> ES cells were injected into Rag2<sup>-/-</sup> blastocysts, which were then implanted into a pseudo-pregnant female recipient mouse (Chen et al., 1993).

Neonates resulting from these injections were screened for chimerism by determining the level of GFP fluorescence of their skin and paws. Highly chimeric (>50%) neonates were used for analysis. To generate lentiviral transgenics, approximately a small volume of high-titre LentiLox lentivirus ( $10^8$  IU/ $\mu$ l) was injected into the perivitelline space of single-cell mouse embryos, which were then implanted into pseudo-pregnant female recipient mice (Lois et al., 2002). The resulting neonates were screened for lentiviral integration by Southern blot and expression by GFP fluorescence.

**Flow cytometry:** For flow cytometric analysis, the following phycoerythrin (PE) conjugated antibodies were used: anti-CD4 (clone RM4-5), anti-CD8 $\alpha$  (clone 53-6.7), anti-CD25 (clone PC81), anti-CD45.2, anti-CD95.2 (Thy1.2) and strepavidin. Allophycocyanin (APC)-conjugated anti-CD8 $\alpha$  and biotin-conjugated anti-Thy1.2 were also used for analysis. All antibodies were from BD Pharmingen (San Diego, California). All plots shown are gated for viable cells, which were isolated by selecting PI<sup>-</sup> cells.

**Northern blot analysis:** For Northern blot analysis, cells were lysed with Trizol reagent (Invitrogen), and total cellular RNA was prepared according to the manufacturer's instructions. RNA was prepared from tissues using RNAlater (Ambion Diagnostics) according to manufacturer's instructions. CD4/CD8 probe hybridization was performed as described (McManus et al., 2002b). For the small RNA Northern, total RNA (60  $\mu$ g) was fractionated on a 10% denaturing polyacrylamide gel and transferred to nylon membrane. The membrane was hybridized to a probe consisting of a 21nt CD8 siRNA sense strand 5' end-labeled with  $^{32}$ P. A 5' radiolabeled oligonucleotide probe to 5S RNA was used to determine equal loading of RNA.

**Western blot analysis:** For Western blot analysis, mouse tissues were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% Sodium Dodecylsulfate) supplemented with Complete Protease Inhibitor Tablets (Roche).

Protein concentrations were determined using BCA Protein Assay (Pierce). Equal amounts of protein (100µg) were loaded per lane and run on a 10% SDS-PAGE gel. Protein was transferred to a PVDF membrane. p53 was detected using anti-p53 Ab-3 (Novagen) diluted 1:1,000 and Donkey anti-mouse conjugated with horse radish peroxidase diluted 1:10,000. The blot was developed with ECL+ reagent (Amersham Biosciences).

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## *Chapter 6*

### **Conclusions**

The broad and overlapping expression patterns of the vertebrate Ena/VASP proteins have limited our understanding of their functions during development. Previous work had established Ena/VASP proteins as regulators of directed cell migration and axon guidance through their effects on actin cytoskeleton architecture. This study provides the first comprehensive analysis of Ena/VASP protein function during vertebrate development. In chapter two, we established a role for Ena/VASP proteins in axogenesis and axon extension in addition to the previously described role in axon guidance. In addition, we found surprising evidence for a role in the integrity of the pial membrane. Both the defects in axogenesis and pial integrity have intriguing implications for Ena/VASP function at integrin-mediated adhesive structures. In chapter three, we found that Ena/VASP has functions in the fusion and integrity of some epithelial sheets. Surprisingly, Ena/VASP function was dispensable for the complex cell migrations necessary to generate basic embryo topology and organogenesis. Thus, Ena/VASP function in the formation and integrity of adherens and tight junctions play an unexpectedly important role in vertebrate development. This study has extended our understanding of Ena/VASP biology and forced us to reconsider its roles beyond those of regulating the protrusion of lamellipodia and filopodia.

The inefficiency of mouse knockout construction has proved an impediment to biological progress. As example, the initial descriptions of both the *Mena* and *VASP* knockout mice were published by independent groups in 1999 (Aszodi et al., 1999; Lanier et al., 1999) and the *Mena;VASP* double-knockout was published in 2004 (Menzies et al., 2004). The *Mena;VASP;EVL*-deficient mice are described for the first time in this study, nearly a decade after construction of the *Mena* knockout began. The Ena/VASP protein family is typical of many gene families in vertebrates, with the complete elucidation of their function requiring the elimination of all the family members. In chapter five, we have described a novel method for the



construction of hypomorphic alleles in mice using lentiviral delivery of RNAi. This method will not supplant the creation of knockouts by homologous recombination. However, as an adjunct, it allows for the rapid, inexpensive and effective knockdown of gene function in mice. This technique can be used to accelerate the analysis of gene function in the mouse, and can easily be applied iteratively to disrupt entire gene families. The lentiviral system is versatile and can be applied towards gene knockdowns in cell lines, primary cells, including post-mitotic or non-cycling cells, as well as in the construction of mice.

### **Ena/VASP proteins in the nervous system**

We have analyzed the role of Ena/VASP in the development of the mouse nervous system. Loss of all three alleles of Ena/VASP caused a failure in axogenesis by neurons within the cortical plate. The ECM of the cortical plate is rich in fibronectin and collagen but lacks laminin. Surprisingly, cortical neurons that had invaded past the laminin-rich pial membrane were able to form axons. Elsewhere in the developing nervous system, neurons that developed in the presence of laminin generated axons. This supports a model in which axogenesis requires either Ena/VASP proteins or laminin to occur. Consistent with this model, neuritogenesis of cortical neurons *in vitro* could be rescued by the addition of laminin. At this time the molecular mechanisms by which either Ena/VASP or laminin support axogenesis is unclear. Analysis of Ena/VASP-deficient cortical neurons *in vitro* show clear disruptions of the actin and microtubule cytoskeletons, and a complete absence of filopodial or lamellipodial protrusions. As far as we are aware, Ena/VASP proteins are the first molecules implicated in the stage 1 to stage 2 transition of axogenesis. Ena/VASP has been implicated in the generation of filopodia by binding to actin barbed ends and protecting them from capping protein (CP)(Bear et al., 2002; Lebrand et al., 2004; Mejillano et al., 2004). One possibility is that Ena/VASP may direct the formation of

filopodia, and that filopodia initiate the formation of neuritic processes. Alternatively, Ena/VASP localizes via its interactions with Vinculin and Zyxin to the intracellular domain of integrins. Cortical neurons express two laminin receptors,  $\alpha_3\beta_1$  and  $\alpha_6\beta_1$ . Potentially, in the absence of laminin, Ena/VASP activity at these or other integrin receptors is sufficient for neuritogenesis. In the absence of Ena/VASP, intracellular signalling at  $\alpha_3\beta_1$  and/or  $\alpha_6\beta_1$  requires engagement of laminin binding. Signaling through integrin receptors may activate proteins that can perform analogous functions to Ena/VASP at the leading edge. The formins, like Ena/VASP proteins, bind to actin barbed ends and support the continued addition of actin monomers (Zigmond et al., 2003). To demonstrate that these integrin receptors are employed in neuritogenesis we could employ the snake venom toxin disintegrin, which specifically blocks integrin heterodimers containing  $\beta_1$ . Assuming that laminin rescue requires integrin activity, we could discriminate between Ena/VASP function in filopodial extension and integrin signaling through the transfection of cortical neurons with either Ena/VASP structural variants (discussed in Appendix A), or a construct that specifically delocalizes endogenous Ena/VASP proteins from focal adhesions (Bear et al., 2000). The COOH-terminal EVH2 domain is sufficient to mediate barbed-end binding, filopodia protrusion, and rescue cell motility defects of Ena/VASP deficient fibroblasts (Appendix A). This mutant appears to be constitutively active. Expression of the EVH2 domain in Ena/VASP-deficient neurons would test whether barbed-end anticapping activity was sufficient to rescue neuritogenesis. Alternatively, expression of a short peptide encoding the EVH1-binding motif, FPPPP, delocalizes Ena/VASP proteins from focal adhesions by disrupting their interactions with Vinculin and Zyxin. Expression of this construct in wildtype cortical neurons would test whether Ena/VASP activity at the intracellular domain of integrins is required for neuritogenesis in the absence of laminin. However, the identification of FPPPP-

containing proteins at the leading edge indicates that this approach may lack the specificity necessary to address this question(Krause et al., 2004; Lafuente et al., 2004). To determine the nature of the intracellular signaling pathways occurring at integrins we could use a combination of pharmacologic and RNAi inhibition of candidate downstream molecules. Lentiviruses can infect post-mitotic primary neurons and could be used to delivery shRNA constructs against candidate molecules such as specific formins or capping protein. Numerous pharmacologic compounds exist with specific activity against Rho GTPases, allowing us to probe their specific involvement.

The second major finding within the nervous system was the formation of a cobblestone cortex. As discussed extensively in Chapter 2, the formation of cobblestone cortex has previously been associated with disruptions of the basal lamina due to mutations of laminin, its integrin receptors, or the intracellular signaling molecule FAK(De Arcangelis et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001). The requirement for these proteins is exclusively in the radial glia or meningeal fibroblasts, but not within neurons(Beggs et al., 2003). Thus, it is likely that the generation of ectopias in the *Ena/VASP* deficient brain reflects a requirement of *Ena/VASP* downstream of adherens junctions (between radial glia endfeet) or adhesive contacts between glial endfeet and the laminin-rich basal lamina. As expression from a single *EVL* allele rescues this phenotype, we could test the cell autonomy of neuronal ectopias through the cell type-specific delivery of Cre recombinase to eliminate a floxed allele of *EVL*. The use of Nestin-Cre to eliminate *EVL* from both neurons and radial glia(Betz et al., 1996), and Nex-Cre to eliminate *EVL* solely in migrating neurons(Beggs et al., 2003) should allow us to determine whether *Ena/VASP* function in radial glia is required for proper cortical organization. To determine the nature of *Ena/VASP* function in the establishment of the pial basal lamina we

could culture Ena/VASP-deficient meningeal fibroblasts *in vitro* and assay their ability to produce an organized laminin ECM(Beggs et al., 2003).

A more detailed analysis of brain development was precluded in this study because of the prevalence of exencephaly in mice lacking both Mena and VASP. The construction of a conditional allele of Mena would facilitate the production of Ena/VASP deficient brains. Typically this would require the introduction of a *Mena* targeting construct into a wildtype ES cell line. However, this new allele of *Mena* would then need to be crossed into the *VASP* and *EVL* mutant backgrounds. To accelerate this process we could generate an ES line from blastocysts having an *Mmvvee* genotype, and target the remaining allele of *Mena*. This ES line would allow the analysis of Ena/VASP brains in the F<sub>1</sub> generation by crossing chimeras with *Mmvvee* mice that also carry a Cre transgene. The ES line could also be readily differentiated into neurons or endothelial cells *in vitro*, and transfected with Cre recombinase to simplify the analysis of these cell types. Alternatively, lentiviral delivery of conditional shRNA can be achieved directly into single-cell embryos allowing for the analysis of mice in the first generation. Several groups have published methods for the conditional expression of shRNAs from lentiviral vectors, including a direct modification of the system described in chapter five(Ventura et al., 2004). This would circumvent the construction of a targeting vector, and allow for the conditional ablation of Mena activity through the introduction of tissue-specific Cre transgenes. Finally, shRNAs under the control of tetracycline-inducible promoters would allow for the analysis of Ena/VASP-deficiency at specific developmental time-points, or postnatally.

#### **Ena/VASP proteins outside the nervous system**

In our analysis of Ena/VASP function outside of the nervous system we found defects in the generation of cell:cell junctions in specific situations. While we failed to recapitulate the

previously reported defect in cell:cell junctions between keratinocytes(Vasioukhin et al., 2000), we encountered defects in the fusion of epithelial sheets and the integrity of the endothelial monolayer. Endothelial barrier function has been attributed to the function of occludens junctions. Consistently, we have found evidence for the successful formation of desmosomes with gaps that may represent occludens junction defects. Immuno-EM will be necessary to determine conclusively whether the junctional defects represent occludens or adherens junction disruption. The proper formation of adherens junctions in the skin and gut epithelia is suggested by the lack of blistering or histologic deficit. By contrast, the fusion of epithelial sheets requires adherens junction assembly after knitting of filopodia from apposed epithelial sheets. This process appears to be defective in the fusion of the upper and lower eyelid, neural tube fusion, and occasionally at the periumbilicus. Developmentally, fusion requires a number of activities, and can not be easily observed or teased apart. The process of wound healing in the adult recapitulates embryonic epithelial fusion(Martin and Parkhurst, 2004). To discriminate between a requirement of Ena/VASP in filopodial extension and adherens junction assembly we could examine the process of wound healing *ex vivo* or in the adult mouse. Two alternative approaches can be used as experimental modalities; the surgical application of Ena/VASP-deficient skin grafts to recipient adult mice allows for the analysis of wound healing in the absence of Ena/VASP proteins. Alternatively, limb buds from Ena/VASP-deficient embryos can be maintained in culture and used to model wound healing. Scanning electron microscopy of wounds during closure will allow us to assess the formation and knitting of filopodia from epithelial sheets. The delivery of fluorescently-tagged markers of adherens junctions will allow us to observe the formation adherens junctions during epithelial fusion in real time.

## **Summary**

Five years ago, our lab published a paper that indicated that Ena/VASP function at focal adhesions was dispensable for fibroblast adhesion and motility (Bear et al., 2000). This result was easily incorporated in a model of Ena/VASP function that emphasized their role to the response of neuronal growth cones to guidance cues by modulating actin dynamics at protrusive membrane structures. The work described in this thesis clearly demonstrates that much of Ena/VASP proteins' function in development requires its function at sites of cell:cell and cell:matrix adhesions. The development of in vitro systems to model Ena/VASP function at adhesions will be essential in describing molecular mechanism of the developmental defects.

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## *Appendix A*

### **Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/VASP function during cell migration**

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The author contributed Figure 1C, 3B, 7B. Supplementary movies are available online at the *Molecular Biology of the Cell* website

## **Abstract**

The Ena/VASP protein family is implicated in the regulation of a number of actin-based cellular processes including lamellipodial protrusion necessary for whole cell translocation. A growing body of evidence derived largely from in vitro biochemical experiments using purified proteins, cell-free extracts and pathogen motility has begun to suggest various mechanistic roles for Ena/VASP proteins in the control of actin dynamics. Using complementation of phenotypes in Ena/VASP-deficient cells and overexpression in normal fibroblasts, we have assayed the function of a panel of mutants in one member of this family, Mena, by mutating highly conserved sequence elements found in this protein family. Surprisingly, deletion of sites required for binding of the actin monomer-binding protein profilin, a known ligand of Ena/VASP proteins, has no effect on the ability of Mena to regulate random cell motility. Our analysis revealed two features essential for Ena/VASP function in cell movement, cyclic-nucleotide dependent kinase phosphorylation sites and an F-actin binding motif. Interestingly, expression of the C-terminal EVH2 domain alone is sufficient to complement loss of Ena/VASP function in random cell motility.

## Introduction

Cell motility is a complex and highly regulated process. Many aspects of organismal development and physiology require that cells control their movement in response to diverse arrays of environmental signals. To move, cells must maintain polarity while coordinating membrane extension, changes in adhesiveness and contractile mechanisms. These processes all depend upon dynamic remodeling of the actin cytoskeleton. While the basic biochemistry of actin polymerization has been extensively studied, the mechanisms by which cells orchestrate assembly, organization and disassembly of actin networks during cell movement remain poorly understood.

The Ena/VASP proteins are a conserved family of molecules known to regulate cell movement and shape change (Gertler et al., 1996; Reviewed in Bear et al., 2001). *Drosophila* *Ena* regulates axonal growth cone migration in response to several types of signaling pathways (Bashaw et al., 2000; Lanier and Gertler, 2000). The three vertebrate Ena/VASP proteins, Vasodilator-stimulated phosphoprotein (VASP), Mammalian Enabled (Mena), and Ena-VASP-like (EVL) negatively regulate fibroblast motility by modulating lamellipodial behavior (Bear et al., 2000; Bear et al., submitted). Other data implicates Ena/VASP proteins in actin-dependent processes including Jurkat T-cell polarization, inhibition of platelet aggregation and the motility of the intracellular pathogen *Listeria monocytogenes* (Smith et al., 1996; Niebuhr et al., 1997; Aszodi et al., 1999; Krause et al., 2000; Skoble et al., 2001). Biochemical data support a role for Ena/VASP proteins in actin dynamics ( Huttelmaier et al., 1999; Harbeck et al., 2000; Lambrechts et al., 2000) and the proteins localize to cellular structures rich in actin assembly such as protruding lamellipodial and filopodial tips (Reinhard et al., 1992; Gertler et al., 1996; Lanier et al., 1999; Rottner et al., 1999).

Ena/VASP proteins share a conserved domain structure, consisting of an Ena-VASP Homology 1 (EVH1) domain at their amino termini and a carboxy-terminal EVH2 domain. Ena/VASP proteins all contain a more variable central region between the EVH1 and EVH2 domains rich in polyproline clusters. The EVH1 domain has been crystallized and adopts a structure related to PH and PTB domains (Fedorov et al., 1999; Prehoda et al., 1999). The EVH1 domain binds directly to a consensus motif, (D/E)-FPPPP-X(D/E)(D/E) (Niebuhr et al., 1997), and plays an essential role in focal adhesion targeting of Ena/VASP proteins by binding to proteins containing the EVH1 binding motif (Gertler et al., 1996).

Much less is known about the cellular function of the proline-rich region and the EVH2 domain. Various lines of evidence suggest that the EVH2 domain can promote oligomerization of Ena/VASP proteins and can bind directly to F-actin in vitro (Bachmann et al., 1999; Huttelmaier et al., 1999; Harbeck et al., 2000). The proline-rich region can bind to profilins, small actin monomer-binding proteins, as well as proteins containing SH3 and WW domains - protein modules that bind to specific proline-rich motifs (Reinhard et al., 1995; Gertler et al., 1996; Ermekova et al., 1997). Adjacent to the proline-rich region, the three vertebrate Ena/VASP proteins also contain one or more sites for phosphorylation by the cyclic-nucleotide dependent kinases, PKA and PKG (Butt et al., 1994; Gertler et al., 1996; Lambrechts et al., 2000). Phosphorylation of Ena/VASP proteins at the single PKA site found in all three proteins induces changes in electrophoretic mobility and protein:protein interactions (Lambrechts et al., 2000). Although the in vivo functional significance of this phosphorylation is unknown, VASP knockouts exhibit platelet aggregation defects associated with misregulation of PKA-mediated intracellular signaling, suggesting that VASP may be the major physiological substrate for PKA in that cell type (Aszodi et al., 1999).

To gain insight into the function of Ena/VASP proteins, we have performed a systematic mutagenesis of conserved motifs within this protein family. We have previously isolated from *mena;vasp*-null embryos a clonal fibroblastic cell line (MV<sup>D7</sup>) that lacks detectable EVL protein and therefore is deficient in all known Ena/VASP proteins (Bear et al., 2000). MV<sup>D7</sup> cells move more rapidly than do MV<sup>D7</sup> cells expressing physiological levels of Mena (Bear et al., 2000), and a companion study finds that they do not support normal *Listeria* intracellular movement (Geese et al., accepted). We used complementation of the hypermotile phenotype of MV<sup>D7</sup> cells to conduct a structure-function analysis of Ena/VASP-mediated regulation of whole cell motility. The average speed of motile cell populations was quantitated in a videomicroscopy-based long-term cell tracking assay. This allows us to directly measure the effect of Ena/VASP function on cell motility. Our analysis of cells expressing mutant forms of Mena indicates that the proline-rich region is dispensable for function in random cell motility, but identifies two key features for function of this protein family: an F-actin binding motif and Ser/Thr phosphorylation.

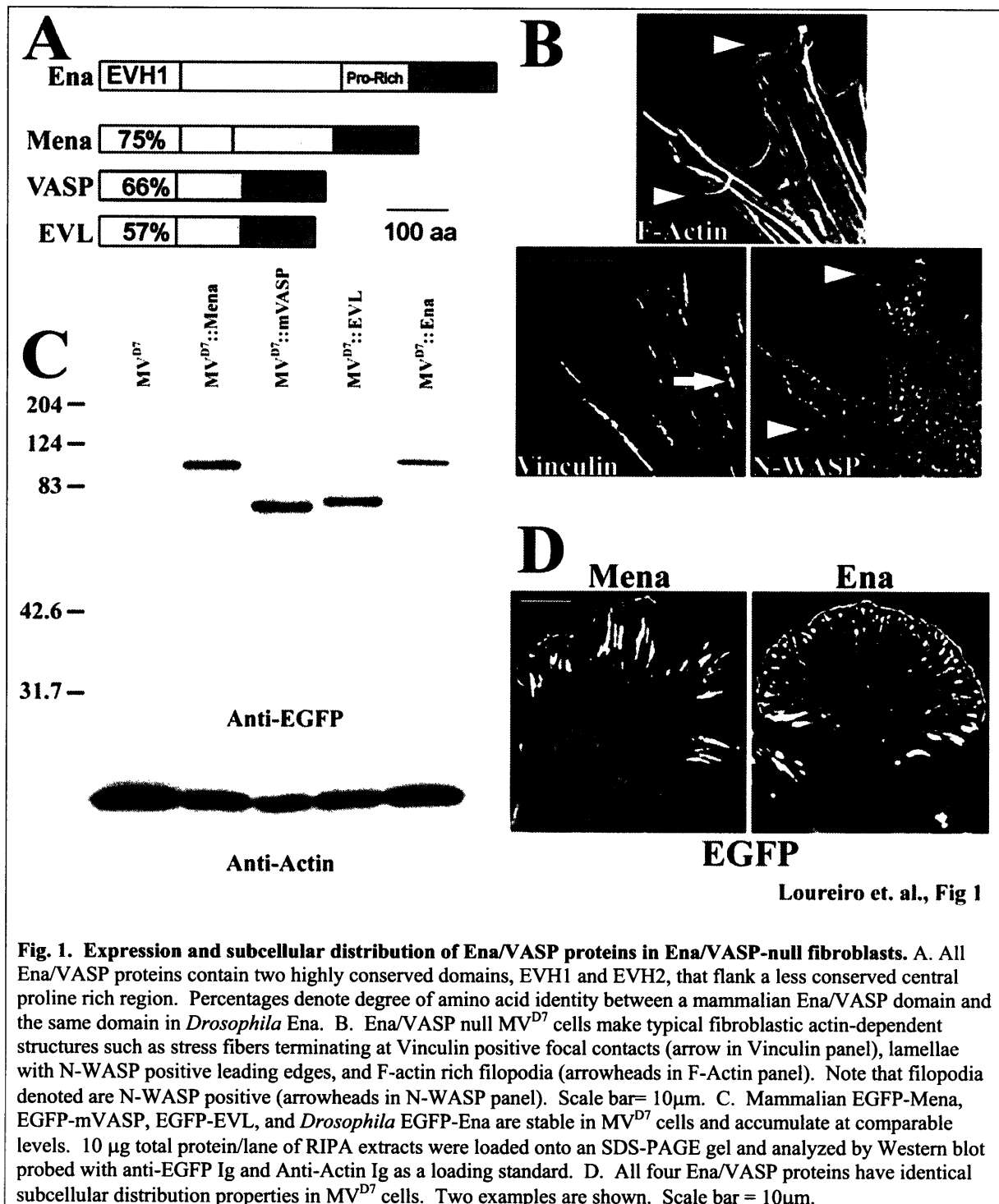
## Results

### **All three murine Ena/VASP proteins rescue Ena/VASP-dependent cell motility defects.**

Ena/VASP proteins have a broad, overlapping expression pattern in developing and adult mice, and a variety of genetic and biochemical experiments indicate they likely share overlapping functions within most cells. We tested the ability of different Ena/VASP proteins to complement MV<sup>D7</sup> cells in a random motility assay. At subconfluent densities, MV<sup>D7</sup> cells have morphological attributes typical to mammalian fibroblasts including filopodia, focal contacts, and protrusive lamellae (Fig 1B). Previously, we demonstrated that stable expression of EGFP-Mena in MV<sup>D7</sup> cells complements loss of Ena/VASP function by decreasing their average speed (Bear et al., 2000). We have extended that analysis to include EGFP-mVASP (murine VASP) and EGFP-EVL. We also tested *Drosophila* EGFP-Ena because it is structurally similar to mammalian Ena/VASP proteins (Fig. 1A), and because mammalian Ena/VASP transgenes complement the loss-of-function phenotype of mutations in *Drosophila Ena* (Ahern-Djamali et al., 1998, and F. Gertler, unpublished).

Each transgene was stably inserted by retroviral infection into MV<sup>D7</sup> cells and sorted by FACS for uniform EGFP signal levels. This approach minimizes genetic drift of the novel transgenic line from the parental cell line and facilitates a direct comparison of activities of different protein variants. All four Ena/VASP proteins were detected by Western blotting at comparable levels (Fig.1C). Therefore, this approach generated cell populations that stably express equivalent levels of EGFP-tagged proteins on a per cell basis within each population, and comparable expression levels of the EGFP-tagged proteins among the different populations.

We previously demonstrated that Ena/VASP activity in cell motility depends on the function of Ena/VASP proteins at the cellular leading edge (Bear et al., 2000). All four family members



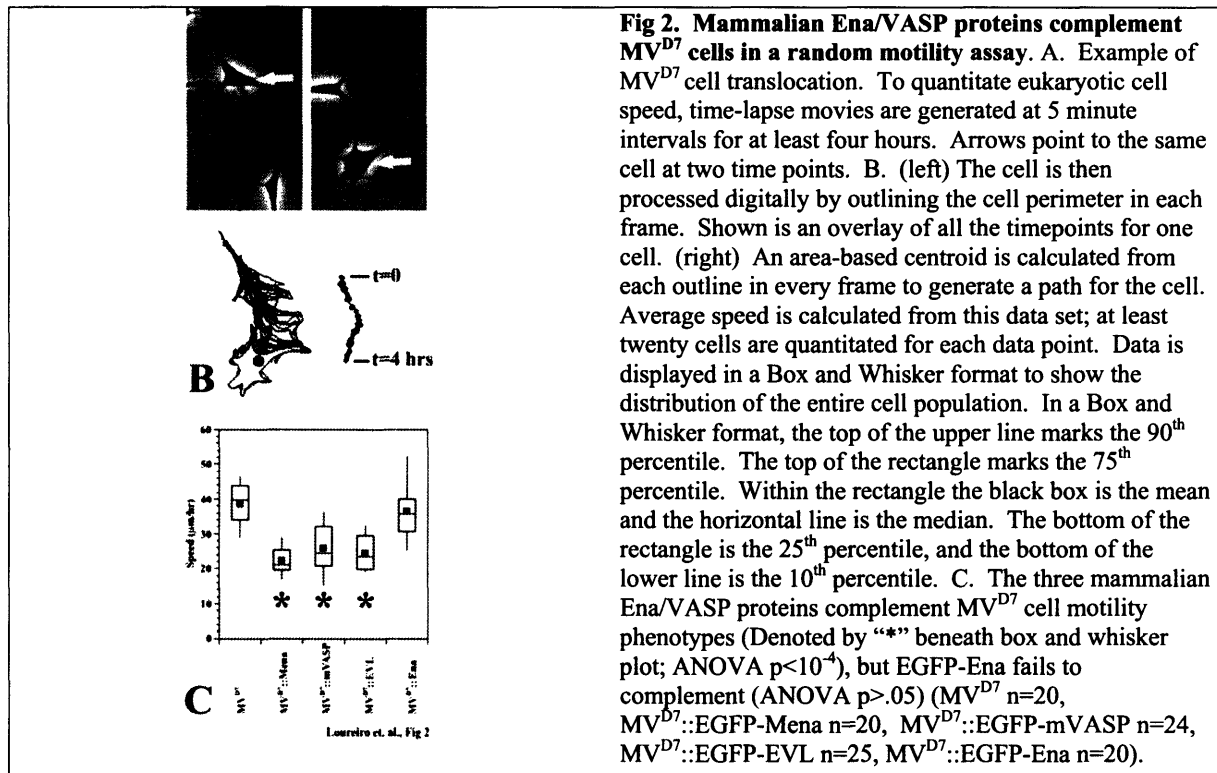
displayed similar subcellular distributions (Fig 1D; Supplemental Fig S1 panels 8, 13, 18, and 23). Colocalization of EGFP signal with Vinculin demonstrated proper localization of all four

Ena/VASP proteins to focal adhesions (Supplemental Fig S1: panels 10, 15, 20, and 25), while colocalization of EGFP signal with N-WASP, a lamellipodial leading edge marker, demonstrated that all four family members localize to the leading edge (Supplemental Fig S1: panels 10, 15, 20, and 25). All four Ena/VASP proteins were also concentrated at the distal tips of filopodia (data not shown). These results indicate that all the Ena/VASP proteins exhibit a subcellular distribution pattern in MV<sup>D7</sup> cells similar to those reported for other fibroblastic cell types (reviewed in Bear et al., 2001).

We used time-lapse video and fluorescence microscopy to analyze the ability of the different Ena/VASP proteins to function in MV<sup>D7</sup> cells. As we previously observed with EGFP-tagged Mena, expression of EGFP-mVASP, EGFP-EVL, or *Drosophila* Ena in MV<sup>D7</sup> cells did not grossly change the F-actin network of MV<sup>D7</sup> cells as judged by phalloidin staining (Supplemental Fig S1: panels 1, 6, 11, 16, and 21). We next analyzed cell behavior. Individual living cells were filmed at high magnification to observe subcellular dynamics and filmed at lower magnification to characterize mean population speeds.

MV<sup>D7</sup> cells complemented with any of the family members were able to form filopodia, focal adhesions and stress fibers that were morphologically indistinguishable from the parental MV<sup>D7</sup> cells as judged by fluorescence, phase-contrast and interference reflection microscopy of fixed and living cells (Fig 1B; Supplemental movies M1 and M2; data not shown). We then quantitated cell speeds by tracking individual cells over four hours, calculating a mean speed for each cell, and comparing the population statistics of the experimental group with the parental MV<sup>D7</sup> control group (Fig 2A and B; Supplemental movie M3). We found, as previously shown,





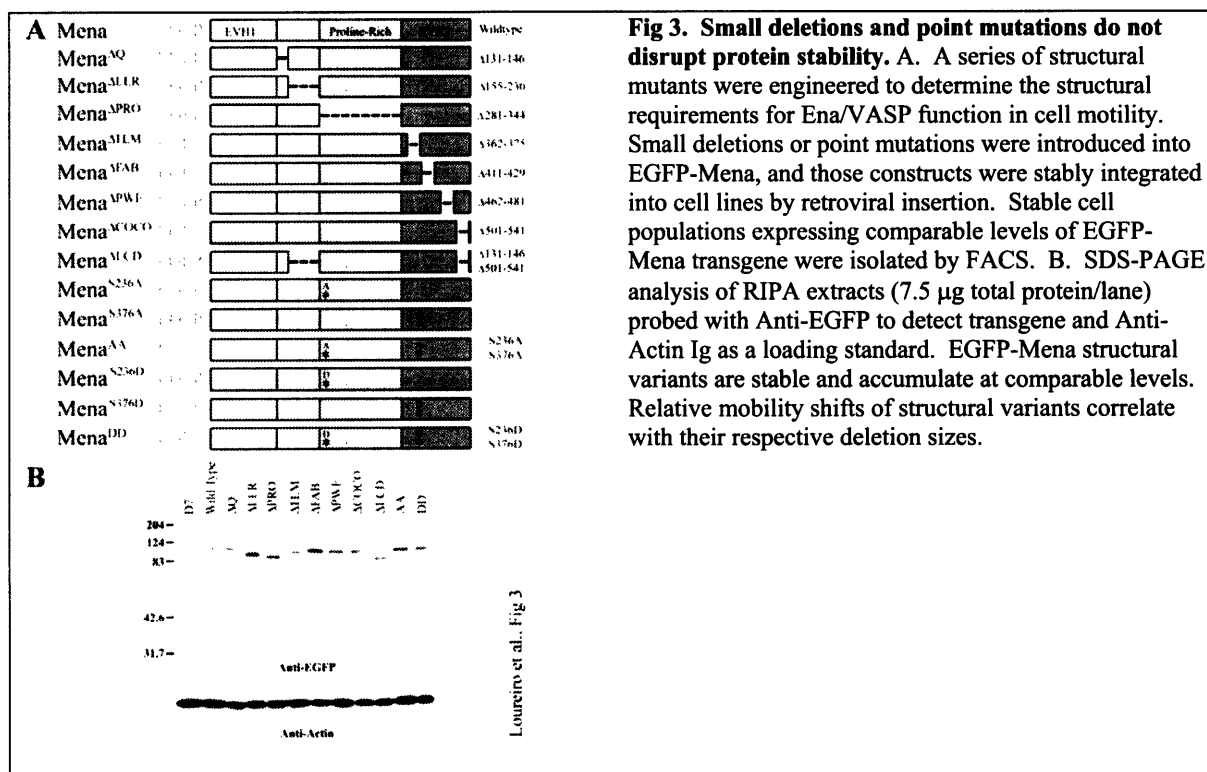
that  $MV^{D7}$  cell speeds were reduced by the expression of EGFP-Mena to physiological levels.

EGFP-EVL and EGFP-mVASP reduced  $MV^{D7}$  cell speed equivalently, indicating that all three murine Ena/VASP proteins function interchangeably in this assay (Fig. 2C). Interestingly, *Drosophila* EGFP-Ena failed to complement the hypermotility phenotype even though its subcellular distribution and expression levels were indistinguishable from mouse Ena/VASP proteins (Fig. 2C), indicating that *Drosophila* Ena lacks some critical feature for function in cell movement present in all murine Ena/VASP proteins.

### EGFP-Mena mutant proteins are stable in $MV^{D7}$ cells.

Since all murine Ena/VASP proteins functioned equivalently in the whole cell motility assay, we chose one member, Mena, to use for a structure-function analysis of Ena/VASP proteins. A series of structural variants of EGFP-Mena were generated to define the regions of EGFP-Mena that regulate cell motility properties of  $MV^{D7}$  cells. As noted above, extensive information on

the structure and function of the EVH1 domain exists. Therefore, we focused our attention on the remainder of the protein. Small internal deletions and point mutations were chosen on the basis of evolutionary conservation and known biochemical properties (Gertler et al., 1996; Bachmann et al., 1999; Fig. 3A). EGFP-Mena mutants were stably expressed in MV<sup>D7</sup> cells and sorted for uniform EGFP signal levels as described above. Western blotting confirmed that the mutant EGFP-Mena proteins accumulated to levels comparable with the wildtype protein in MV<sup>D7</sup> cells and migrated at their predicted sizes (Fig. 3B).



**Fig 3. Small deletions and point mutations do not disrupt protein stability.** A. A series of structural mutants were engineered to determine the structural requirements for Ena/VASP function in cell motility. Small deletions or point mutations were introduced into EGFP-Mena, and those constructs were stably integrated into cell lines by retroviral insertion. Stable cell populations expressing comparable levels of EGFP-Mena transgene were isolated by FACS. B. SDS-PAGE analysis of RIPA extracts (7.5 μg total protein/lane) probed with Anti-EGFP to detect transgene and Anti-Actin Ig as a loading standard. EGFP-Mena structural variants are stable and accumulate at comparable levels. Relative mobility shifts of structural variants correlate with their respective deletion sizes.

**The central proline-rich region is dispensable for Ena/VASP function in random whole cell motility.**

Three structural features are present between the EVH1 and the EVH2 domains of Mena. The first is a conserved block of 16 residues distal to the EVH1 domain that is deleted in EGFP-

Mena<sup>ΔQ</sup>. Although this block is only conserved among vertebrate Ena/VASP proteins, all Ena/VASP proteins have a high incidence of glutamine residues in their primary structure at this region. In VASP and EVL, the “Q” block is twelve residues from the most conserved Ser/Thr phosphorylation site. However, within Mena a repetitive sequence unique to Mena is inserted between the “Q” block and the phosphorylation site. The repeat LERER occurs 6 times in this 77 amino acid stretch, which also contains seven glutamines. These 77 amino acids are deleted in EGFP-Mena<sup>ΔLER</sup>. Finally, all Ena/VASP proteins share a proline-rich region known to bind profilin, Src and Abl SH3 domains, and the WW domain of FE65. This region is deleted in EGFP-Mena<sup>ΔPRO</sup>.

Colocalization with N-WASP and Vinculin indicated that the three mutants all localized normally to the leading edge and focal adhesions, respectively (Fig. 4A, panels 1-3; data not shown). EGFP-Mena<sup>ΔQ</sup>, EGFP-Mena<sup>ΔLER</sup>, and EGFP-Mena<sup>ΔPRO</sup> were each capable of complementing MV<sup>D7</sup> cells in the cell motility assay to the same extent as wildtype EGFP-Mena (Fig. 4B). These results indicate that the LERER repeat unique to Mena and the conserved “Q” motifs are dispensable for subcellular targeting and whole cell movement. Surprisingly, the interactions between the polyproline-cluster of Ena/VASP proteins and proteins such as profilin are not required for proper subcellular localization or for function of Ena/VASP proteins in random cell motility.

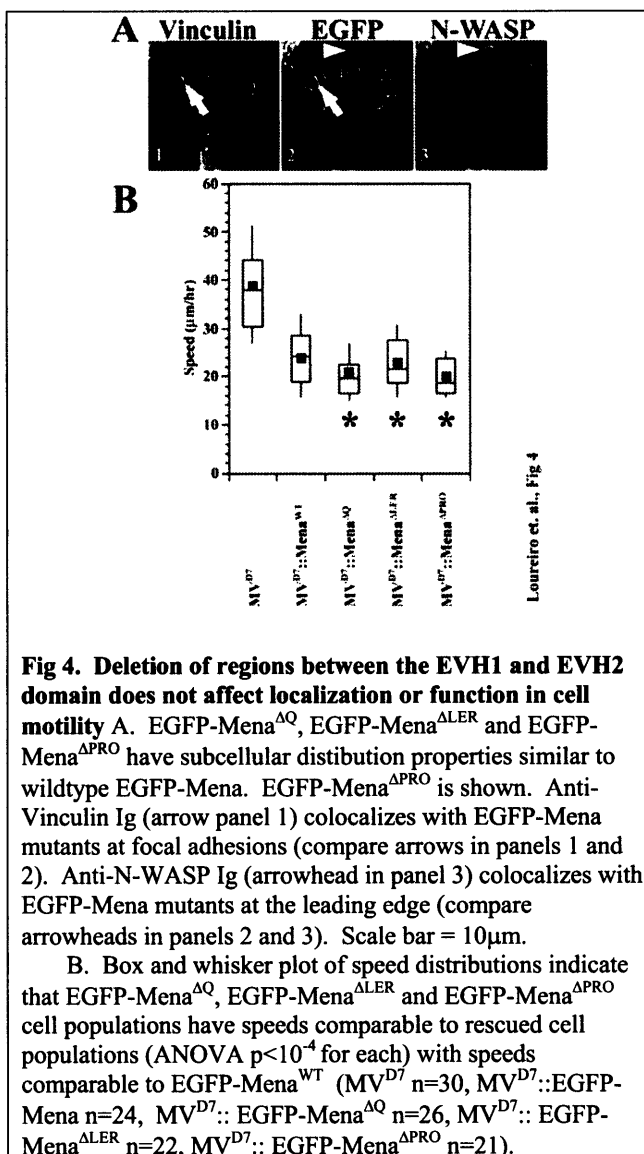
#### **Interaction with the F-actin network is essential for Ena/VASP function in cell motility.**

Our previous study indicated that the EVH1 domain alone is detected in the cytoplasm, at focal adhesions, weakly along the leading edge, and in the nucleus (a property of EGFP alone) (Bear et al., 2000). Since deletion of the proline-rich region resulted in a subcellular distribution equivalent to that of full length Mena, we speculated that the EVH2 domain harbors an activity

that increases the efficiency of leading edge targeting. We deleted four conserved blocks within the EVH2 domain to test this hypothesis.

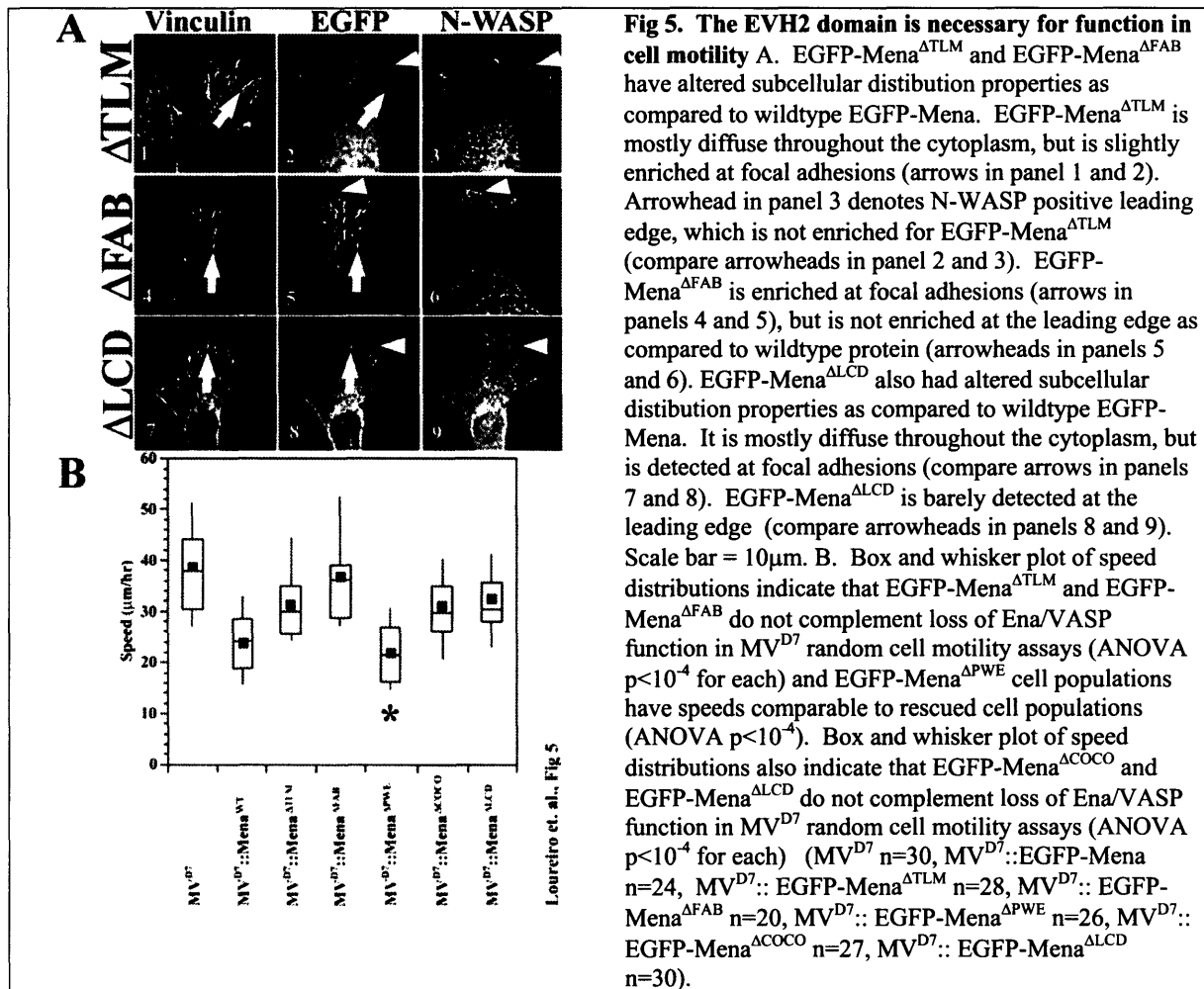
The first conserved region in the EVH2 domain, “TLM” (Thymosin-β4-Like Motif), is a motif related to the actin-monomer binding site in Thymosin-β4 (Van Troys et al., 1996). Although EGFP-Mena<sup>ΔTLM</sup> was excluded from the nucleus, it was diffusely distributed throughout the cytoplasm (Fig 5A panels 1-3), weakly detected at focal adhesions, and barely detectable along the leading edge of lamellipodia. EGFP-Mena<sup>ΔTLM</sup> failed to complement the MV<sup>D7</sup> motility phenotype (Fig 5B).

The next conserved region within the EVH2 domain binds F-actin in vitro (Bachmann et al., 1999; Lambrechts et al., 2000) and was deleted to create EGFP-Mena<sup>ΔFAB</sup> (F-actin binding). EGFP-Mena<sup>ΔFAB</sup> localized robustly to focal adhesions, but was only barely detectable along the leading edge of lamellipodia, comparable to the signal observed with the EVH1 domain alone, suggesting that the F-actin binding motif plays an important role in concentrating Ena/VASP at lamellipodial tips (Fig 5A panels 4-6). EGFP-Mena<sup>ΔFAB</sup> failed to complement the motility



**Fig 4. Deletion of regions between the EVH1 and EVH2 domain does not affect localization or function in cell motility** A. EGFP-Mena<sup>ΔQ</sup>, EGFP-Mena<sup>ΔLER</sup> and EGFP-Mena<sup>ΔPRO</sup> have subcellular distribution properties similar to wildtype EGFP-Mena. EGFP-Mena<sup>ΔPRO</sup> is shown. Anti-Vinculin Ig (arrow panel 1) colocalizes with EGFP-Mena mutants at focal adhesions (compare arrows in panels 1 and 2). Anti-N-WASP Ig (arrowhead in panel 3) colocalizes with EGFP-Mena mutants at the leading edge (compare arrowheads in panels 2 and 3). Scale bar = 10μm.

B. Box and whisker plot of speed distributions indicate that EGFP-Mena<sup>ΔQ</sup>, EGFP-Mena<sup>ΔLER</sup> and EGFP-Mena<sup>ΔPRO</sup> cell populations have speeds comparable to rescued cell populations (ANOVA  $p < 10^{-4}$  for each) with speeds comparable to EGFP-Mena<sup>WT</sup> (MV<sup>D7</sup> n=30, MV<sup>D7</sup>::EGFP-Mena n=24, MV<sup>D7</sup>::EGFP-Mena<sup>ΔQ</sup> n=26, MV<sup>D7</sup>::EGFP-Mena<sup>ΔLER</sup> n=22, MV<sup>D7</sup>::EGFP-Mena<sup>ΔPRO</sup> n=21).



**Fig 5. The EVH2 domain is necessary for function in cell motility** A. EGFP-Mena <sup>$\Delta$ TLM</sup> and EGFP-Mena <sup>$\Delta$ FAB</sup> have altered subcellular distribution properties as compared to wildtype EGFP-Mena. EGFP-Mena <sup>$\Delta$ TLM</sup> is mostly diffuse throughout the cytoplasm, but is slightly enriched at focal adhesions (arrows in panel 1 and 2). Arrowhead in panel 3 denotes N-WASP positive leading edge, which is not enriched for EGFP-Mena <sup>$\Delta$ TLM</sup> (compare arrowheads in panel 2 and 3). EGFP-Mena <sup>$\Delta$ FAB</sup> is enriched at focal adhesions (arrows in panels 4 and 5), but is not enriched at the leading edge as compared to wildtype protein (arrowheads in panels 5 and 6). EGFP-Mena <sup>$\Delta$ LCD</sup> also had altered subcellular distribution properties as compared to wildtype EGFP-Mena. It is mostly diffuse throughout the cytoplasm, but is detected at focal adhesions (compare arrows in panels 7 and 8). EGFP-Mena <sup>$\Delta$ LCD</sup> is barely detected at the leading edge (compare arrowheads in panels 8 and 9). Scale bar = 10 $\mu\text{m}$ . B. Box and whisker plot of speed distributions indicate that EGFP-Mena <sup>$\Delta$ TLM</sup> and EGFP-Mena <sup>$\Delta$ FAB</sup> do not complement loss of Ena/VASP function in MV<sup>D7</sup> random cell motility assays (ANOVA  $p < 10^{-4}$  for each) and EGFP-Mena <sup>$\Delta$ PWE</sup> cell populations have speeds comparable to rescued cell populations (ANOVA  $p < 10^{-4}$ ). Box and whisker plot of speed distributions also indicate that EGFP-Mena <sup>$\Delta$ COCO</sup> and EGFP-Mena <sup>$\Delta$ LCD</sup> do not complement loss of Ena/VASP function in MV<sup>D7</sup> random cell motility assays (ANOVA  $p < 10^{-4}$  for each) (MV<sup>D7</sup>  $n=30$ , MV<sup>D7</sup>::EGFP-Mena  $n=24$ , MV<sup>D7</sup>::EGFP-Mena <sup>$\Delta$ TLM</sup>  $n=28$ , MV<sup>D7</sup>::EGFP-Mena <sup>$\Delta$ FAB</sup>  $n=20$ , MV<sup>D7</sup>::EGFP-Mena <sup>$\Delta$ PWE</sup>  $n=26$ , MV<sup>D7</sup>::EGFP-Mena <sup>$\Delta$ COCO</sup>  $n=27$ , MV<sup>D7</sup>::EGFP-Mena <sup>$\Delta$ LCD</sup>  $n=30$ ).

defects in MV<sup>D7</sup> cells, indicating that the ability to interact with F-actin is essential for the function of Ena/VASP proteins in whole cell movement (Fig 5B). Interestingly, EGFP-Mena <sup>$\Delta$ FAB</sup> fully supports intracellular *Listeria* motility (Geese et al., accepted), indicating that this mutant retains some type of biological activity.

The third region is a small set of 12 conserved amino acids defined by the EGFP-Mena <sup>$\Delta$ PWE</sup> mutant that is between the FAB and the oligomerization region (COCO, see below). No known function has been ascribed to the PWE region. EGFP-Mena <sup>$\Delta$ PWE</sup> localized in a pattern similar to

the wildtype protein (data not shown), and fully complemented the motility phenotype of MV<sup>D7</sup> cells (Fig. 5B).

#### **Potential oligomerization motifs are required for full Ena/VASP function in cell motility.**

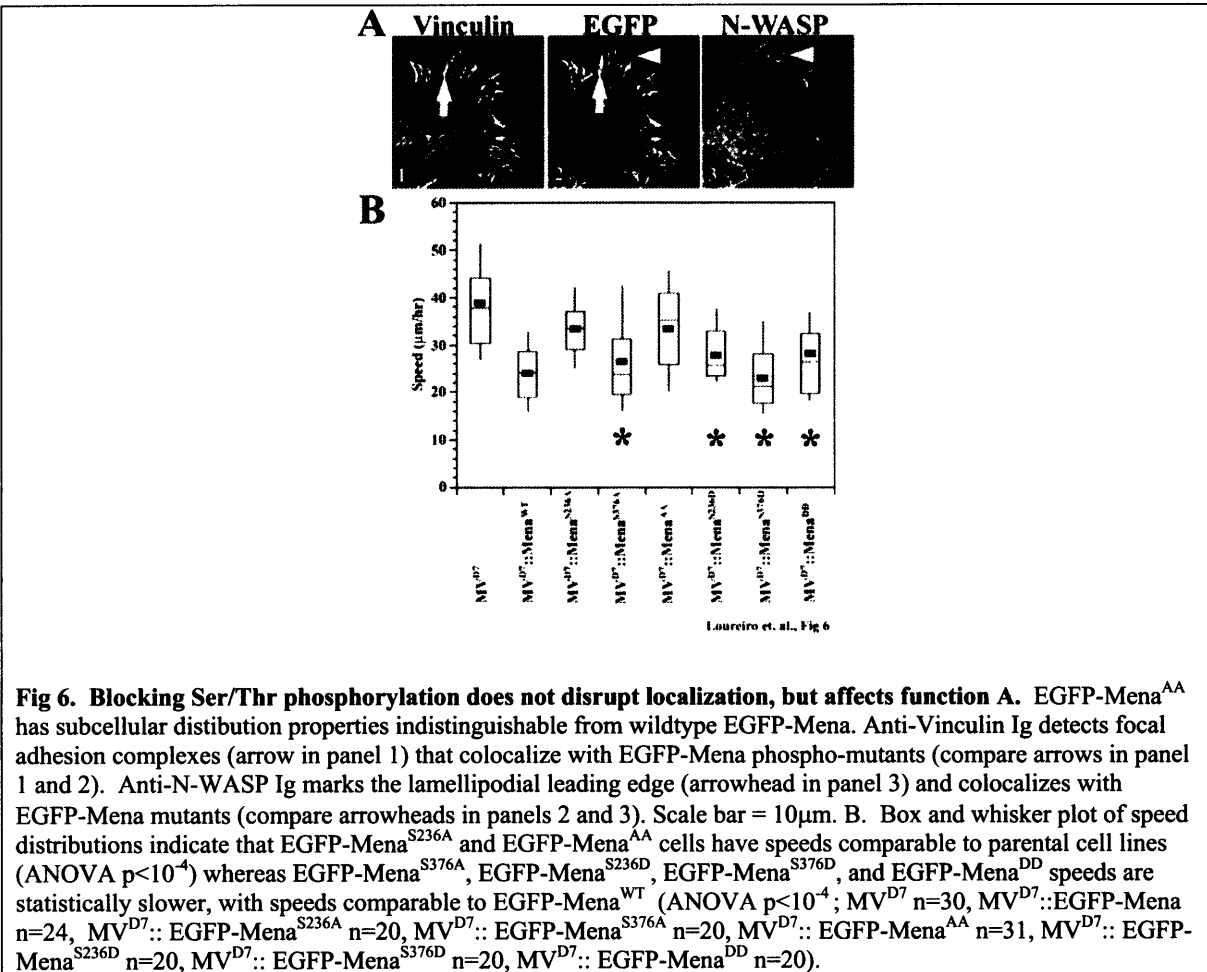
The C-terminus of the EVH2 domain contains a predicted coiled-coil region that can mediate oligomerization of Ena/VASP proteins (Ahern-Djamali et al., 1998; Carl et al., 1999). To assess the requirement for oligomerization in Ena/VASP function, we made two mutants intended to disrupt the formation of EGFP-Mena oligomers. EGFP-Mena<sup>ΔCOCO</sup> harbors an internal deletion that excises the predicted coiled-coil motif in the EVH2 domain. EGFP-Mena<sup>ΔCOCO</sup> localized within the cytosol, was enriched at focal adhesions, but was only weakly detected at lamellipodial leading edges (data not shown). In the motility assay, EGFP-Mena<sup>ΔCOCO</sup> provided only partial function as compared to the wildtype protein, but it did reduce average cell speeds significantly as compared to the parental MV<sup>D7</sup> cell line (ANOVA,  $p < 0.05$ ) (Fig 5B).

We wondered if the Mena-specific LERER region could contribute to function in the absence of the COCO region. The LERER repeat region is predicted to form a potential extended helix with alternating charges, and therefore could serve to form an additional oligomerization motif. As described above, deletion of the LERER region by itself has no effect on localization or function of EGFP-Mena, we deleted both the LERER region and the coiled-coil region to generate EGFP-Mena<sup>ΔLCD</sup> (LER-COCO double-mutant). Although this mutant had similar sub-cellular distribution properties to EGFP-Mena<sup>ΔCOCO</sup>, it was more difficult to detect along the leading edge (Fig. 5A panels 7-9). In the motility assay, EGFP-Mena<sup>ΔLCD</sup> did not alter the hypermotile phenotype of MV<sup>D7</sup> cells (Fig. 5B). These results suggest that oligomerization of Ena/VASP proteins is required for full function of these proteins in cell motility.

#### **Ser/Thr phosphorylation regulates mammalian Ena/VASP protein function.**

The failure of *Drosophila* Ena to replace its murine orthologs in the motility assay prompted us to focus on features conserved within the vertebrate proteins that are missing in Ena. Between one and three cyclic nucleotide-dependent kinase phosphorylation sites flank the proline-rich core in all vertebrate Ena/VASP proteins. *Drosophila* Ena lacks any known PKA/PKG phosphorylation sites. To test whether phosphorylation of the highly conserved PKA/PKG sites is required for Ena/VASP function in mammalian cell motility, six different EGFP-Mena mutants were engineered. Individual phosphorylation sites are mutated from serine to alanine in EGFP-Mena<sup>S236A</sup> and EGFP-Mena<sup>S376A</sup> to block phosphorylation of only one site. Conversely, each phosphorylation site is mutated from serine to aspartic acid in EGFP-Mena<sup>S236D</sup> and EGFP-Mena<sup>S376D</sup> to mimic constitutive phosphorylation at each site. In EGFP-Mena<sup>AA</sup>, the two phosphorylation sites were each mutated to alanine (S236A, S376A), while in EGFP-Mena<sup>DD</sup>, both phosphorylation sites were mutated to aspartic acids (S236D, S376D).

All six phosphomutants localized in a subcellular pattern indistinguishable from wildtype EGFP-Mena, colocalizing with Vinculin at focal adhesions and with N-WASP at the leading edge (Fig. 6A panels 1-3; data not shown). However, EGFP-Mena<sup>AA</sup> failed to complement loss of Ena/VASP function while EGFP-Mena<sup>DD</sup> caused a statistically-significant reduction of cell speed (Fig. 6B). This latter result is consistent with in vitro analysis suggesting that replacing serines with aspartic acid in Ena/VASP proteins mimics phosphorylation (Harbeck et al., 2000). Functional analysis of the single point mutations indicate that Ser236, the only phosphorylation site conserved in all three murine Ena/VASP proteins, is more critical since conversion of that site alone to alanine affects Ena/VASP function. Mutation of Ser376 to alanine alone had no effect on Ena/VASP function. As with EGFP-Mena<sup>DD</sup>, mutation of either phosphorylation site to aspartic acid did not disrupt function in whole cell motility. These results indicate that although

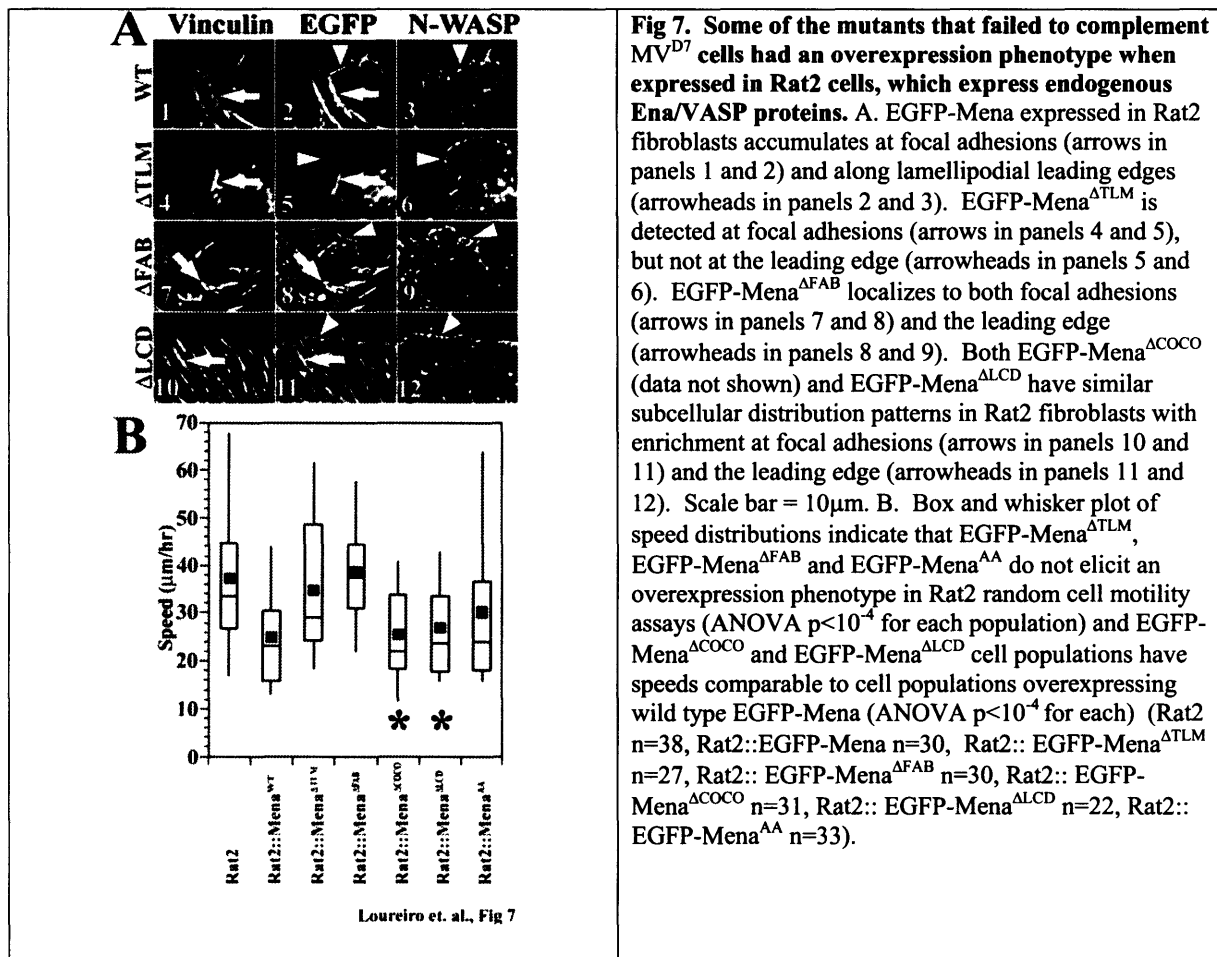


Ena/VASP phosphorylation is essential for function in cell movement, phosphorylation has no obvious role in subcellular targeting of the proteins. Furthermore, blocking phosphorylation at the site common to all three mammalian Ena/VASP proteins is sufficient to block EGFP-Mena function in cell motility.

**Some EGFP-Mena mutants that fail to complement MV<sup>D7</sup> cells induce an overexpression phenotype in Rat2 fibroblasts.**

Previously, we reported that overexpression of Mena to four times normal levels causes a significant reduction in the speed of Rat2 fibroblasts (Bear et al., 2000). We used this overexpression assay as a second way to test whether any of the mutants that failed to rescue





**Fig 7. Some of the mutants that failed to complement  $MV^{D7}$  cells had an overexpression phenotype when expressed in Rat2 cells, which express endogenous Ena/VASP proteins.** A. EGFP-Mena expressed in Rat2 fibroblasts accumulates at focal adhesions (arrows in panels 1 and 2) and along lamellipodial leading edges (arrowheads in panels 2 and 3). EGFP-Mena $^{\Delta TLM}$  is detected at focal adhesions (arrows in panels 4 and 5), but not at the leading edge (arrowheads in panels 5 and 6). EGFP-Mena $^{\Delta FAB}$  localizes to both focal adhesions (arrows in panels 7 and 8) and the leading edge (arrowheads in panels 8 and 9). Both EGFP-Mena $^{\Delta COCO}$  (data not shown) and EGFP-Mena $^{\Delta LCD}$  have similar subcellular distribution patterns in Rat2 fibroblasts with enrichment at focal adhesions (arrows in panels 10 and 11) and the leading edge (arrowheads in panels 11 and 12). Scale bar = 10 $\mu$ m. B. Box and whisker plot of speed distributions indicate that EGFP-Mena $^{\Delta TLM}$ , EGFP-Mena $^{\Delta FAB}$  and EGFP-Mena $^{AA}$  do not elicit an overexpression phenotype in Rat2 random cell motility assays (ANOVA  $p < 10^{-4}$  for each population) and EGFP-Mena $^{\Delta COCO}$  and EGFP-Mena $^{\Delta LCD}$  cell populations have speeds comparable to cell populations overexpressing wild type EGFP-Mena (ANOVA  $p < 10^{-4}$  for each) (Rat2  $n=38$ , Rat2::EGFP-Mena  $n=30$ , Rat2:: EGFP-Mena $^{\Delta TLM}$   $n=27$ , Rat2:: EGFP-Mena $^{\Delta FAB}$   $n=30$ , Rat2:: EGFP-Mena $^{\Delta COCO}$   $n=31$ , Rat2:: EGFP-Mena $^{\Delta LCD}$   $n=22$ , Rat2:: EGFP-Mena $^{AA}$   $n=33$ ).

normal motility of  $MV^{D7}$  retained some function in this overexpression assay. We made stable Rat2 cell lines expressing EGFP-Mena mutants and assayed the subcellular distribution (Fig. 7A) and functional capacity of those mutants as compared to wildtype EGFP-Mena (Fig 7B).

We tested whether overexpression of non-phosphorylatable EGFP-Mena affected cell speed. As in  $MV^{D7}$  cells, EGFP-Mena $^{AA}$  localization was indistinguishable from wildtype EGFP-Mena (data not shown). Analysis of cell speeds indicated that EGFP-Mena $^{AA}$  failed to induce a statistically-significant reduction in the overall speeds of the cell population (Fig. 7B). However, visual inspection of the tracking movies suggested that some cells within the EGFP-Mena $^{AA}$  - expressing population did appear to move more slowly than parental Rat2 cells. In fact, the

median value of EGFP-Mena<sup>AA</sup> speed was nearly identical to that of wildtype EGFP-Mena (indicating that at least half the cells within the population were slowed to the same extent as wildtype EGFP-Mena overexpression).

As in MV<sup>D7</sup> cells, EGFP-Mena<sup>ATLM</sup> again localized diffusely throughout the cytoplasm (Fig 7A panels 4-6). This was surprising since EGFP-Mena<sup>ATLM</sup> should oligomerize with endogenous Ena/VASP proteins, and suggests that deletion of this small conserved region may have broader consequences on the structure of Ena/VASP proteins. Overexpression of EGFP-Mena<sup>ATLM</sup> does not reduce Rat2 cell speed (Fig. 7B).

The subcellular distribution of EGFP-Mena<sup>AFAB</sup> was significantly altered in Rat2 cells as compared to MV<sup>D7</sup> cells. Whereas EGFP-Mena<sup>AFAB</sup> was nearly absent from the leading edge of MV<sup>D7</sup> lamellipodia, it was clearly detected along the leading edge of Rat2 lamellipodia, perhaps due to its ability to oligomerize with endogenous Ena/VASP proteins (Fig. 7A panels 7-9). Although EGFP-Mena<sup>AFAB</sup> is enriched at the leading edge of Rat2 cells, it does not cause an overexpression phenotype (Fig. 7B). This indicates that the F-actin binding motif of the EVH2 domain is essential for the phenotype induced by overexpression of Ena/VASP proteins.

EGFP-Mena<sup>ACOCO</sup> and EGFP-Mena<sup>ALCD</sup> had the same properties in Rat2 cells. Both proteins were found at focal adhesions, but were mostly diffuse throughout the cytoplasm (Fig. 7A panels 10-12; data not shown). However, both EGFP-Mena<sup>ACOCO</sup> and EGFP-Mena<sup>ALCD</sup> expression in Rat2 cells elicited an overexpression phenotype comparable to overexpression of wildtype EGFP-Mena in Rat2 cells (Fig 7B).

**The EVH2 domain alone is sufficient for Ena/VASP function in random whole cell motility in the absence of PKA phosphorylation.**

Our functional assays confirmed the physiological importance of earlier observations indicating that Ena/VASP proteins interact with F-actin and that they can multimerize with each other. Both of these functions map to the conserved EVH2 domain and mutations within that domain disrupt localization and function. We wondered whether expression of the EVH2 domain alone would complement  $MV^{D7}$  cell motility phenotypes.

EGFP-EVH2 localized to the lamellipodia of  $MV^{D7}$  cells, although it exhibited a broader distribution within this structure than EGFP-Mena, which concentrates just at the distal edge of lamellipodia (Fig 8A panels 1-3). EGFP-EVH2 was not enriched at focal adhesions, but did weakly decorate stress fibers. The failure of EGFP-EVH2 to target focal adhesions in  $MV^{D7}$  cells is consistent with earlier work indicating the essential role of EVH1-mediated interactions in focal adhesion targeting of Ena/VASP proteins (Gertler et al., 1996; Bear et al., 2000). When EGFP-EVH2 was expressed in Rat2 cells, the EGFP signal was concentrated at the tips of lamellipodia and in focal adhesions in a pattern identical to the endogenous Ena/VASP proteins, likely due to the ability of the EVH2 domain to oligomerize with endogenous Ena/VASP proteins (Fig. 8A panels 4-6). Together, these results indicate that although the EVH2 domain can target to a broad region of the lamellipodia, other parts of Ena/VASP proteins such as the EVH1 domain are required for targeting to the tip of lamellipodia and to focal adhesions.

We next tested EGFP-EVH2 in the cell motility assays. EGFP-EVH2 complemented loss of Ena/VASP function in  $MV^{D7}$  cells to an extent equivalent to the full-length protein (Fig. 8 C). Similarly, when EGFP-EVH2 was expressed in Rat2 cells, it induced an overexpression phenotype identical to intact Mena (Fig. 8 C). We also tested whether the EVH2 domain of Ena complements  $MV^{D7}$  cells and found that it too reduces average cell speeds (Fig 8 C).



adenylate cyclase thereby increasing the intracellular concentration of cAMP necessary to activate PKA within cells. We found that a mouse monoclonal antibody, 16C2, which was developed for detection of VASP protein that are phosphorylated on Ser238, cross reacts in vitro with PKA phosphorylated Mena and EGFP-EVH2 (Fig 8 B). Surprisingly, we found that forskolin treated Rat2::EGFP-EVH2 cells labeled both VASP and Mena but failed to label EGFP-EVH2. We also found that EGFP-EVH2 is not phosphorylated in forskolin treated MV<sup>D7</sup> cells (data not shown). This suggests that phosphorylation of the EVH2 domain is not required for its function in whole cell motility (see below), and it also suggests that co-recruitment of EGFP-EVH2 to the leading edge by binding to endogenous Ena/VASP proteins in Rat2 cells is not sufficient to phosphorylate the EVH2 domain. Recall that neither full length Ena (Fig 2 C) nor EGFP-Mena<sup>S236A</sup> (Fig 6 B) complements the MV<sup>D7</sup> hypermotility phenotype, suggesting that elements outside the EVH2 domain of full length Ena/VASP protein regulate the physiological activity of the EVH2 domain. Together, these results indicate that the EVH2 domain contains the core mechanistic elements of Ena/VASP proteins required for their function in random whole cell motility, but that the EVH1 and/or central proline-rich region are required to regulate Ena/VASP function.

## Discussion

We have conducted a structure-function analysis to identify features of Ena/VASP proteins required for function in three different assays: subcellular targeting in MV<sup>D7</sup> cells, rescue of the hypermotile phenotype of MV<sup>D7</sup> cells, and overexpression effects on Rat2 fibroblast motility (Table 1). In a companion study, we analyzed several of these mutants for their ability to support intracellular *Listeria* motility (Geese et al., accepted; Table 1).

	Localization in MV <sup>D7</sup>		Functional Assay		
	Focal Adhesions	Leading Edge	Complement Cell motility In MV <sup>D7</sup>	Overexpression Phenotype in Rat2 cells	Complement <i>Listeria</i> motility In MV <sup>D7</sup>
Mena <sup>WT</sup>	+	+	+	+	+
Mena <sup>AQ</sup>	+	+	+	NA	NA
Mena <sup>ALER</sup>	+	+	+	NA	NA
Mena <sup>APRO</sup>	+	+	+	NA	+/-
Mena <sup>ATLM</sup>	+/-	-	-	-	+
Mena <sup>AFAB</sup>	+	-	-	-	++
Mena <sup>APWE</sup>	+	+	+	NA	NA
Mena <sup>ACOCO</sup>	+/-	+/-	+/-	+	+
Mena <sup>ALCD</sup>	+/-	-	-	+	NA
Mena <sup>S236A</sup>	+	+	+/-	NA	NA
Mena <sup>S376A</sup>	+	+	+	NA	NA
Mena <sup>AA</sup>	+	+	-	+/-	+/-
Mena <sup>S236D</sup>	+	+	+	NA	NA
Mena <sup>S376D</sup>	+	+	+	NA	NA
Mena <sup>DD</sup>	+	+	+	NA	++
Mena <sup>EVH2</sup>	-	+	+	+	NA

**Table 1** – Summary of subcellular localization properties and functional capabilities in three distinct assays. For subcellular localization, “+” denotes localization comparable to wildtype EGFP-Mena, “+/-” signifies presence of EGFP signal weaker than wildtype control, and “-“ signifies no or extremely weak EGFP signal. For functional studies, “++” is more active than wildtype, “+” is comparable to wildtype, “+/-” is less than wildtype, and “-“ is no function detected. NA – not analyzed.

One striking result of these studies is that different experimental assays revealed distinct structural requirements for Ena/VASP function. In the case of whole cell motility, the F-actin binding motif within the EVH2 domain is essential for localization within MV<sup>D7</sup> cells and for activity in both MV<sup>D7</sup> and Rat2 cells. In contrast, intracellular *Listeria* motility is unaffected or even enhanced by the absence of the FAB motif. Conversely, the polyproline-rich region of

Mena is dispensable for function in random whole cell movement, but appears to play an important role in intracellular *Listeria* motility (Geese et al., accepted).

We draw three general conclusions from these observations. First, all of the mutants studied displayed partial or complete activity in at least one of the functional assays. This observation combined with the fact that all the mutant proteins were detectable by both FACS analysis and western blotting suggests that failure of a given mutant to function in one of these assays is unlikely to result from a trivial failure in global protein folding. Second, the role of Ena/VASP proteins in *Listeria* motility differs from their function in lamellipodia. We postulate that different sets of features within the same molecules are required differentially for each process. Third, on a more general note, the requirements for various Ena/VASP motifs in these assays suggest that Ena/VASP proteins may be used in distinct ways by different actin-driven processes. As a result, it may not be prudent to assess the function of these molecules in other contexts, such as Jurkat T-cell polarization or axonal growth cone guidance (Lanier et al., 1999; Krause et al., 2000), solely by extrapolation from the results obtained in fibroblast motility or in *Listeria* motility assays. Furthermore, it is possible that other cell types or processes may utilize the three murine Ena/VASP proteins in ways that are not interchangeable.

### **The polyproline-rich region is dispensable for Ena/VASP regulation of random cell motility**

The identification of Ena/VASP proteins as profilin ligands, mediated through the proline-rich region, provided a potential mechanism by which this protein family might regulate actin assembly (Reinhard et al., 1995). Profilin binds G-actin, promotes the exchange of ADP for ATP on G-actin, and permits bound ATP-actin to be added onto the barbed ends of actin

filaments (reviewed in Pollard et al., 2000). The observations that both Ena/VASP proteins and profilin can increase the rate of *Listeria* motility within cell-free systems (Loisel et al., 1999) and that profilin recruitment is proportional to intracellular *Listeria* speed (Geese et al., 2000) supports such a model. In this model, Ena/VASP-profilactin complexes concentrate actin monomer at sites of rapid actin assembly. However, in cell free systems Ena/VASP proteins can increase the rate of *Listeria* motility in the absence of profilin (Loisel et al., 1999). Consistent with this, EGFP-Mena<sup>ΔPRO</sup> can partially restore *Listeria* motility in MV<sup>D7</sup> cells (Geese et al., accepted).

Our results indicate that the central proline-rich region is dispensable for normal subcellular targeting and function in fibroblast motility, therefore interactions with profilin, SH3, and WW domains are all dispensable for the function of Ena/VASP proteins in random cell movement. Previous genetic studies suggest that, in the absence of Mena, the process of neurulation is sensitive to the level of profilin I (Lanier et al., 1999). It is possible that the concentration of profilin in MV<sup>D7</sup> is high enough such that direct interaction with Ena/VASP proteins is not required for their function, even if the molecules do form complexes within cells. Alternatively, profilin recruitment may not be essential to regulate whole cell motility, but it may be important for other functions of this protein family. Additionally, recent reports have postulated a role for WW-mediated binding of Fe65 to Mena in the regulation of cell motility (Sabo et al., 2001), and a requirement for SH3-mediated binding of IRSp53 to Mena in the promotion of filopodial outgrowth (Krugmann et al., 2001). As noted, Ena/VASP-null cells possess morphologically normal filopodia, indicating that they are not strictly required for filopodial formation. Since expression of EGFP-Mena<sup>ΔPRO</sup> is sufficient to complement cell motility phenotypes, binding of ligands to the Pro-rich region is not required for Ena/VASP function in cell motility. Although



this discrepancy may reflect cell-type differences, it does prompt a re-evaluation of models in which profilin or SH3/WW-domain recruitment are critical for the function of Ena/VASP proteins in cell motility.

### **The role of F-actin binding activity in Ena/VASP localization and function**

The F-actin binding motif within the EVH2 domain plays an important role in Ena/VASP targeting to the leading edge within MV<sup>D7</sup> cells. Interestingly, the EGFP-Mena<sup>ΔFAB</sup> mutant displayed a normal subcellular distribution in Rat2 cells, presumably due to oligomerization with endogenous Ena/VASP proteins. Similarly, subcellular targeting by the isolated EVH2 domain was affected by the presence of endogenous Ena/VASP proteins, a factor not controlled for in two recent studies that proposed a role for the EVH2 domain in subcellular targeting (Price and Brindle, 2000; Nakagawa et al., 2001).

Previously we reported that the EVH1 domain could direct GFP to the leading edge and focal adhesions, although the targeting was weak and accompanied by a background nuclear signal. Despite its ability to target GFP, the EVH1 domain alone fails to mediate robust leading edge targeting of full length Ena/VASP proteins. Deletion of the 18 residue F-actin binding motif within the EVH2 domain resulted in a mutant protein that could target appropriately to focal adhesions, but at best weakly to the leading edge. Consistent with these results, we have recently shown that Ena/VASP proteins are recruited to the leading edge by a direct interaction with the barbed ends of elongating actin filaments (Bear et al., submitted).

The EVH2 domain by itself can target GFP to the lamellipodia by a mechanism that depends on the FAB motif (data not shown). By itself, EVH2 does not decorate focal adhesions. Close examination of the distribution of EGFP-EVH2 revealed that EVH2 alone targets a broader region of lamellipodia than full-length Ena/VASP proteins, which decorate only the tips of

protruding lamellipodia. We speculate that the EVH1 domain refines the EVH2-mediated actin dependent targeting of Ena/VASP proteins, thereby restricting them to the very tips of lamellipodia by interacting with either unknown protein ligands or perhaps phosphatidylinositol-containing phospholipids.

### **The EVH2 domain is sufficient to regulate random motility**

Although the EVH2 domain alone does not fully recapitulate wildtype Ena/VASP localization within lamellipodia, it functions equivalently to full length Mena protein in fibroblast motility assays. Within MV<sup>D7</sup> cells, EVH2 localizes to lamellipodia but not to focal adhesions, confirming our previous observations that the hypermotile phenotypes observed by genetic deletion or neutralization approaches within fibroblasts are a consequence of effects on lamellipodia and do not involve loss of Ena/VASP function at focal adhesions.

The EVH2 domain contains two motifs, FAB and COCO, with established biochemical properties. Whereas EGFP-Mena<sup>ΔFAB</sup> localizes predominantly to focal adhesions in MV<sup>D7</sup> cells, in Rat2 cells it is also detected at the leading edge. Since EGFP-Mena<sup>ΔFAB</sup> fails to elicit an overexpression phenotype in Rat2 cells we conclude that F-actin binding is essential for the function of Ena/VASP proteins within lamellipodia. The capacity of EGFP-Mena<sup>ΔFAB</sup> to support normal, or even enhanced rates of intracellular *Listeria* motility (Geese, et al., accepted) provides conclusive evidence that Ena/VASP proteins are utilized by this pathogen by a mechanism that is distinct from the ways in which these same molecules function in lamellipodia during whole cell motility.

In Rat2 cells, as in MV<sup>D7</sup> cells, oligomerization mutants localize to focal adhesions, but are generally diffuse throughout the cytoplasm, suggesting that oligomerization plays a role in targeting to lamellipodia. Since the EVH2 domain alone is sufficient to support normal motility,

and EGFP-Mena<sup>ΔCOCO</sup> is not, we conclude that oligomerization is necessary for full function of the EVH2 domain.

EGFP-Mena<sup>ΔTLM</sup> still contains the coiled-coil region that appears functional in other mutants. TLM is similar to a motif that mediates G-actin binding within molecules such as Thymosin-β4 and Villin (Gertler et al., 1996). The failure of EGFP-Mena<sup>ΔTLM</sup> to localize properly in Rat2 cells suggests that deletion of this 14 amino acid residue region may have a broader impact on Mena structure, though the capacity of EGFP-Mena<sup>ΔTLM</sup> to localize to focal adhesions and to partially support *Listeria* motility suggests that it retains some function. It will be important to determine whether the TLM region actually binds G-actin and to establish what role this motif plays in the overall function of the EVH2 domain.

#### **Regulation of Ena/VASP proteins by phosphorylation.**

PKA/PKG phosphorylation of Ena/VASP proteins has been correlated with a number of physiological processes that involve cytoskeletal remodeling (e.g. - Walter et al., 1993). Furthermore, inhibition of platelet aggregation by cyclic-nucleotide kinase agonists is dramatically attenuated in VASP-deficient mice (Aszodi et al., 1999; Hauser et al., 1999). There are three PKA/PKG sites in VASP, two are present in Mena, and only the amino terminal site is contained within EVL. We analyzed the functional requirements for the two sites found in Mena. Phosphorylation of the first, highly conserved site is essential for function, while we observed no obvious role for the second site in our assays. Since phosphorylation of this first site also induces a shift in the electrophoretic mobility the Mena, EVL and VASP, we propose that it is the major site for regulation of this protein family in vertebrates.

Surprisingly, EGFP-Ena failed to complement the MV<sup>D7</sup> random cell motility phenotype although it is structurally similar to murine Ena/VASP proteins and localized appropriately. This

result is especially striking in light of the ability of vertebrate Ena/VASP proteins to replace *Ena* function in *Drosophila*. While *Drosophila* Ena is phosphorylated on serine as well as tyrosine (Gertler et al., 1995), it lacks the highly conserved PKA/PKG site found in vertebrates (Gertler et al., 1996). The ability of the isolated Ena EVH2 domain to function in mammalian cells indicates that it contains all of the key properties required for function of the domain. It appears likely that the reason why intact Ena fails to function in mammalian cells is that regulation by PKA/PKG is a feature that has been incorporated into Ena/VASP proteins after the divergence between invertebrates and vertebrates.

The isolated Mena EVH2 domain, which complements MV<sup>D7</sup> cells, lacks the key site contained in all the vertebrate proteins. Interestingly, the site within the Mena EVH2 domain is phosphorylated in the intact protein, but not when the EVH2 domain is expressed by itself. It is likely that interactions with the EVH1 or proline-rich region are important for recruiting protein complexes that contain PKA/PKG. One candidate class of proteins may be A-kinase anchoring proteins (AKAPs) which localize PKA to specific regions within cells (reviewed in Diviani and Scott, 2001).

How does phosphorylation regulate Ena/VASP function? Phosphorylation plays no obvious role in subcellular targeting, suggesting that the Ena/VASP proteins are regulated at their sites of function. In addition to causing shifts in electrophoretic mobility, phosphorylation of Ena/VASP proteins is known to alter their affinities for some, but not all, of their binding partners in vitro (Halbrugge et al., 1990; Gertler et al., 1996; Lambrechts et al., 2000). The most conserved phosphorylation site within vertebrate Ena/VASP proteins lies between the EVH1 and EVH2 domains. Substitution of a phospho-mimetic aspartic acid for the serine residue at this site permitted Mena function in MV<sup>D7</sup> cells, providing further evidence that the phosphorylated form

of the protein is active and suggesting that cycling between phospho and dephospho-forms may not be required for Ena/VASP function. Phosphorylation also appears to increase the ability of Ena/VASP proteins to support *Listeria* motility. Therefore, phosphorylation likely activates Ena/VASP proteins in the context of a variety of cellular functions. Together, our results lead us to propose that phosphorylation relieves inhibitory interactions that somehow block the activity of the EVH2 domain.

## Methods and materials

**Subcloning of EGFP-Ena/VASP proteins and engineering of EGFP-Mena structural variants.** EGFP-Ena/VASP family members were subcloned into pMSCV-EGFP retroviral plasmid using standard techniques. EGFP-Mena structural variants were generated using mutagenic PCR primers. For small deletions, two rounds of PCR were required. First, mutagenic primers were used to amplify regions upstream and downstream of the intended deletions. Those PCR products were then purified and combined at equimolar ratios to serve as template for a second PCR reaction to amplify an altered *mena* ORF. For point mutations, mutagenic primers were used to amplify the entire plasmid (pBSII) containing the *mena* ORF. Double-mutant ORFs were generated by subcloning. Mutations were confirmed by sequencing and RFLP analysis. Mutagenic primers were designed in complementary pairs; only the sense orientation is listed below. Q:

5'GACAAAATTCACAGCTACCTGCTCAACTGCAAGAACAGCAGCGACAGAAGGAAC3

'. LER:

5'GAAGGCAACTGCAAGAACAGCAGCGACAGGAGCGCAGAATGTCCAATGCTGCTG  
CCCC3'. PRO: 5'GGGCCTTGTCTTGGGAGCATCTGGAATTTTCTCTGG3'. TLM:

5'GACAATCGCCCTTTAACTTCCCGGGTGGAGGATG3'. FAB:

5'CTGGGCGTGGGAATGGACCTTTCCTCTAGCTGAGAAGGGATCAACAATAGAAAC  
AGAAC3'. PWE:

5'CTGCTAAGGCCCCATCAACAAGTACACCTATGAACGGCAGTAAGTCACCTGTCAT  
CTCC3'. COCO: 5'GGCTGAAGCAGGACATTAGCAAGTCGAACACTGC3'. S236A:

5'GAGCGCAGAATGGCCAATGCTGCTGCC3'. S326D:

GAGCGCAGAATGGACAATGCTGCTGCC. S376A:

5'GCAAAACTTAGGAAAGTGGCCCGGGTGGATGG3'. S376D: 5'  
GCAAAACTTAGGAAAGTGGACCGGGTGGATGG3'.

### **Retroviral packaging, infection, FACS sorting, and cell culture**

Culture of Rat2 cells is described in Gertler, et al, 1996. Isolation of MV<sup>D7</sup> fibroblastic cells is described in Bear, et al., 2000. The MV<sup>D7</sup> cells were cultured at 32 °C in Immorto media (high glucose DME w/ 15% fetal calf serum, pen/strep, L-glut and 50U/ml recombinant mouse interferon-gamma (Gibco-BRL)). Retroviral plasmids described above were transiently calcium-phosphate transfected into Bosc23 packaging cells (3.3 µg retroviral plasmid and 1.7 µg pCL-Eco helper plasmid), and supernatant was collected after 48 hours. MV<sup>D7</sup> cells or Rat2 cells (ATCC) plated at 50% confluency were exposed to infectious supernatant for 24 hours in the presence of 4µg/ml polybrene. Infected cells were cultured to 90% confluency, trypsinized, and FACS sorted in PBS/5% FCS. EGFP positive cells were harvested and cultured for one passage and then resorted for EGFP signal intensity levels that matched EGFP-Mena wildtype controls. To confirm FACS analysis and assess protein stability, RIPA extracts from each cell line were resolved by SDS-PAGE and probed with Anti-EGFP Ig, using Anti-Actin Ig as a loading control.

### **Immunofluorescence and microscopy**

Cells were plated on acid-washed coverslips coated with 10µg/ml fibronectin (Sigma), and allowed to spread for 6-8 hours. They were fixed and stained as described in Gertler et al., 1996. Anti-Vinculin Ig (hvin-1, Sigma) was used at 1:400. Anti-N-WASP was used at 1:1000 (Gift of R. Rohgati and M. Kirschner). Coumarin-phalloidin (Molecular Probes) was used at 1:20. Images were collected on a Deltavision Zeiss microscope and digitally deconvolved using SGI Softworx graphics processing software.

### **Videomicroscopy, quantitation and statistical analysis of motility movies**

Cells were first adapted overnight in CO<sub>2</sub>-independent video microscopy media (high-glucose DME, 350mg/L NaHCO<sub>3</sub>, 25mM HEPES, L-Glut, Pen/Strep, 15% FCS, 50U/ml interferon-gamma). 4,000 MV<sup>D7</sup> or 10,000 Rat2 adapted cells were plated on a  $\Delta$ T dish (Bioptechs) pretreated with 10 $\mu$ g/ml fibronectin and blocked for 1 hour with 1mg/ml tissue culture grade BSA. MV<sup>D7</sup> cells were plated for 8 hours before filming and Rat2 cells were plated for 2 hours before filming. Time-lapse images were collected every 5 minutes for 4 hours for MV<sup>D7</sup> cells, and every 5 minutes for 2 hours for Rat2 cells. At least two acceptable movies from each genotype were quantitated using DIAS software (Solltech). Individual cells chosen for quantitation a) were not in contact with other cells for more than 15 minutes (i.e. – 3 frames), b) did not undergo mitosis, and c) stayed within the viewing area for the duration of the movie. The cell periphery was outlined in each frame using a Wacom digital tablet. DIAS software then computed an area-based centroid for each cell in each frame that subsequently defined a motility path for each cell. Average speed was calculated from paths for at least twenty cells per genotype. Data sets were analyzed by one-way unstacked ANOVA (n = number of cells). Statistical significance was determined by one-way unstacked analysis of variance. EGFP-Mena structural variants complemented loss of Ena/VASP function if their mean 95% confidence intervals overlapped with that of wildtype EGFP-Mena.

### **Biochemical analysis of Ser/Thr phosphorylation of EVH2 domain**

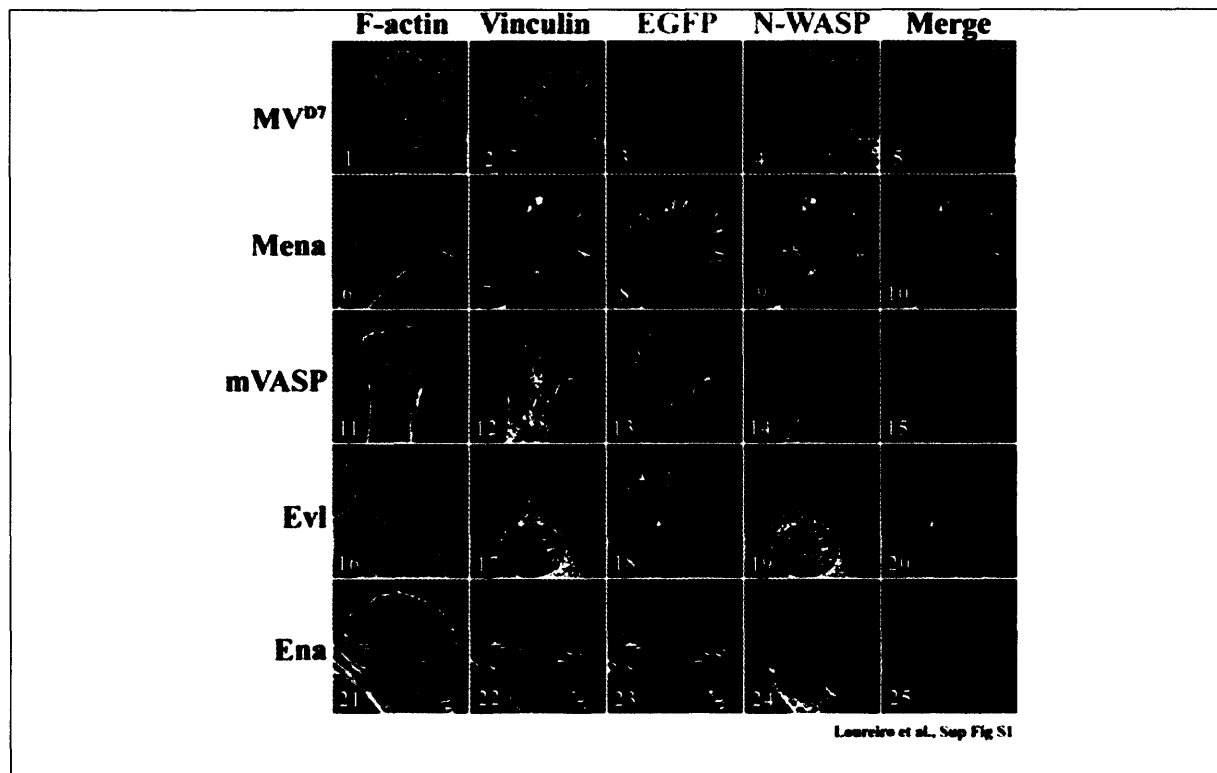
Rat2 cells stably expressing EGFP-EVH2 were grown to 90% confluency and treated with forskolin solubilized in DMSO (10  $\mu$ M or 100  $\mu$ M final forskolin concentration) or DMSO alone for 30 minutes. Cells were then lysed in NP-40 buffer (1% NP-40, 150mM NaCl, 50 mM Tris, pH 8.0) containing protease inhibitors (Complete tablets, Roche Diagnostics) and phosphatase



inhibitors (1 mM sodium vanadate and 1 mM sodium fluoride). Protein extracts were run on 8% SDS-PAGE gels and probed with mouse monoclonal 16C2 antibody (1:100, Vasopharm), rabbit polyclonal anti-EGFP (1:100, Clontech), and anti-Mena rabbit polyclonal 2197 (1:5000). Positive and negative controls for PKA ser/thr phosphorylation of Mena, VASP, and EGFP-EVH2 were generated by harvesting Rat2::EGFP-EVH2 extracts in NP-40 buffer with protease inhibitors and running either in vitro phosphatase reactions (NEB) or PKA kinase reactions (NEB) for 30 min.

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**Supplemental Fig S1. All four Ena/VASP proteins have similar subcellular distribution patterns in MV<sup>D7</sup> cells.** MV<sup>D7</sup> cells stably expressing individual EGFP-Ena/VASP fusion proteins were characterized by immunofluorescence for colocalization with vinculin, a focal adhesion marker, and N-WASP, a leading edge marker. Cells were also labeled with phalloidin to characterize the actin cytoskeleton. For each cell population, each of the four channels is shown in black and white, and a merger of the vinculin (blue), EGFP (green), and N-WASP (red) is shown in color. Note the presence of typical actin structures including stress fibers and enrichment along lamellipodial edges is grossly unchanged in MV<sup>D7</sup> and MV<sup>D7</sup> cells expressing Ena/VASP proteins (compare panel 1 to 6, 11, 16, and 21). All four Ena/VASP proteins colocalize with vinculin at focal adhesions (compare “vinculin” column with “EGFP” column, and note merger of signals in color panels 10, 15, 20, and 25). Colocalization at the leading edge between N-WASP and EGFP fusion proteins is also observed with all four Ena/VASP proteins (compare “NWASP” column with “EGFP” column, and note merger of signals in color panels 10, 15, 20, and 25). Scale bar = 10µm.

### Supplemental Movies available online at the Molecular Biology of the Cell website

**Supplemental Movie M1.** Timelapse movie of an MV<sup>D7</sup> cell recorded at a magnification of 600x using phase-contrast optics. Frames were collected every 15 seconds. MV<sup>D7</sup> cells have features typical to fibroblastic cells including protrusive lamellipodia and dynamic filopodia.

**Supplemental Movie M2.** Timelapse movie of an MV<sup>D7</sup>::EGFP-Mena cell recorded at a magnification of 600x using phase-contrast optics. Frames were collected every 15 seconds. Stable expression of EGFP-Mena does not grossly alter MV<sup>D7</sup> lamellipodial dynamics.

**Supplemental Movie M3.** Side-by-side comparison of MV<sup>D7</sup> and MV<sup>D7</sup>::EGFP-Mena cell populations. Frames were recorded at five minute intervals at 40x magnification using phase-contrast optics.

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