Selectivity in Subunit Composition of Ena/VASP Tetramers
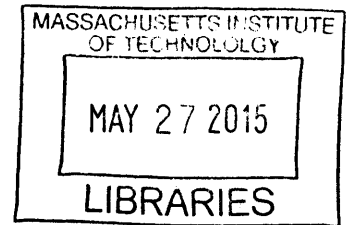
by

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B.A. Biology
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THESIS ABSTRACT

Ena/VASP proteins have an established role in regulating actin dynamics in diverse cell types. The mammalian Ena/VASP family members, Mena, VASP, and EVL have many overlapping activities. However, the three family members are not equivalent and each possess a number of features that are absent in the others. Unique modes of regulation and paralog-specific interacting partners point to potential differences in the activity and function of Ena/VASP proteins.

The function of this family of proteins relies on their ability to form tetramers via a highly conserved tetramerization domain located at the C-terminus of all Ena/VASP proteins. The potential formation of mixed tetramers may combine the unique aspects of each paralog into one molecule.

Here, I describe a series of immunoprecipitation experiments to evaluate hetero-oligomerization of Ena/VASP proteins in a controlled setting. My data demonstrate that VASP can form hetero-oligomers with itself, Mena, and EVL without bias. However, the assembly of Mena and EVL hetero-tetramers is disfavored. In addition, I find that the tetramerization domain mediates the observed selectivity in complex formation. My findings suggest that hetero-tetramerization serve as an additional method to regulate the activity of Ena/VASP proteins.

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Chapter 1:

Introduction

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1.1. Actin Polymerization drives cell migration

A cell's ability to move is critical in many diverse biological contexts, including embryogenesis, axon guidance, immunological responses, and wound healing. In diseases, such as cancer, cell migration is a critical component in the establishment of metastases (Lauffenburger & Horwitz, 1996). Cell motility is generally described in a series of four steps involving: 1) the formation of a protrusive structure at the leading edge of a cell; 2) the establishment of new adhesions between the cell and its the underlying substrata; 3) the release of older adhesions at the rear of the cell; and 4) the detachment of the rear of the cell allowing for the translocation of the cell body (Pollard & Borisy, 2003). The migration of cells within tissues requires the coordinate regulation of polarity, membrane protrusions, adhesion, and contraction (Lauffenburger & Horwitz, 1996).

Dynamic coordination of actin filament networks is a crucial aspect of controlling cell shape and movement (Pollard & Borisy, 2003). Alterations in the organization of actin allow cells to migrate, contract, adhere, or extend protrusions in response to a variety of extracellular and intracellular signaling cues. The actin cytoskeleton is regulated by a large number of proteins that influence its organization through a number of different methods, such as the modulation of polymerization, branching, bundling, or severing of filaments (Ridley, 2011). Membrane protrusions at the cell front are largely generated by actin polymerization (Krause & Gautreau, 2014), but actin is also important in the formation of other types of protrusions, including filopodia and invadopodia

(Ridley, 2011). Proteins associated with the actin cytoskeleton determine the types of structures actin forms.

Our understanding of actin-based protrusion has been aided significantly by the pathogen *Listeria monocytogenes*. This bacterium uses mimicry to exploit a host cell's own actin-machinery and assemble actin-based "comet tails". *Listeria* express the protein ActA, which recruits actin binding proteins to the bacterial surface, where they assemble actin into the comet tails. The actin polymerization at the surface of the bacteria provides the force to push the bacteria through the cell's cytoplasm and to infect neighboring cells. Studies of *Listeria* and other pathogens that also hijack a cell's actin-polymerization machinery have led to the discovery, and the elucidation of the function, of a number of actin associated factors (Lambrechts et al., 2008).

1.2. Actin Filaments

Actin monomers (G-actin) are assembled into a helical, filamentous structure (F-actin) with asymmetric polarity. The two ends of F-actin are structurally differentiable and are identified as the "barbed-end" and the "pointed end," corresponding to the pattern observed by electron microscopy of myosin S1 fragments binding to the sides of actin filaments. The barbed end is more dynamic than the pointed end, with faster rates of actin monomer addition and disassembly. Treadmilling of actin filaments occurs as actin monomers are added to the barbed end and disassembled at the pointed end (Carlier et al., 2003).

Actin filaments form the basis of a variety of cellular substructures. The formation of higher order actin structures occurs through the coordination of multiple actin associated factors that influence actin nucleation or disassembly, filament length, bundling, orientation, or the density of actin structures (Fig. 1-1) (Chhabra & Higgs, 2007). The work of this thesis focuses on the Ena/VASP family, which consists of actin binding proteins capable of influencing the elongation and density of actin filaments. Thus, I will focus on actin-based structures and actin accessory proteins that are most relevant to Ena/VASP function.

1.3. Regulators of actin dynamics

1.3.1. Arp2/3

The Arp2/3 complex consists of seven subunits and functions by binding to the side of a pre-existing actin filament, where it nucleates a new actin filament at a 70° angle, relative to the original filament (Fig. 1-1) (Mullins et al., 1998; Svitkina & Borisy, 1999). Arp2 and Arp3 together are thought to mimic an actin dimer and thus serve as a nucleator of actin filaments (Goley & Welch, 2006). Arp2/3 is responsible for the formation of branched filaments, such as those found in lamellipodia (the leading edge protrusions of migrating cells) and in the actin comet tail of *Listeria*. In reconstitution experiments, Arp2/3 complex was found to be an essential component of *Listeria* movement (Borisy & Svitkina, 2000). For the Arp2/3 complex to nucleate actin filaments, it needs to be activated by nucleation promoting factors (NPFs). There are four families of NPFs that can
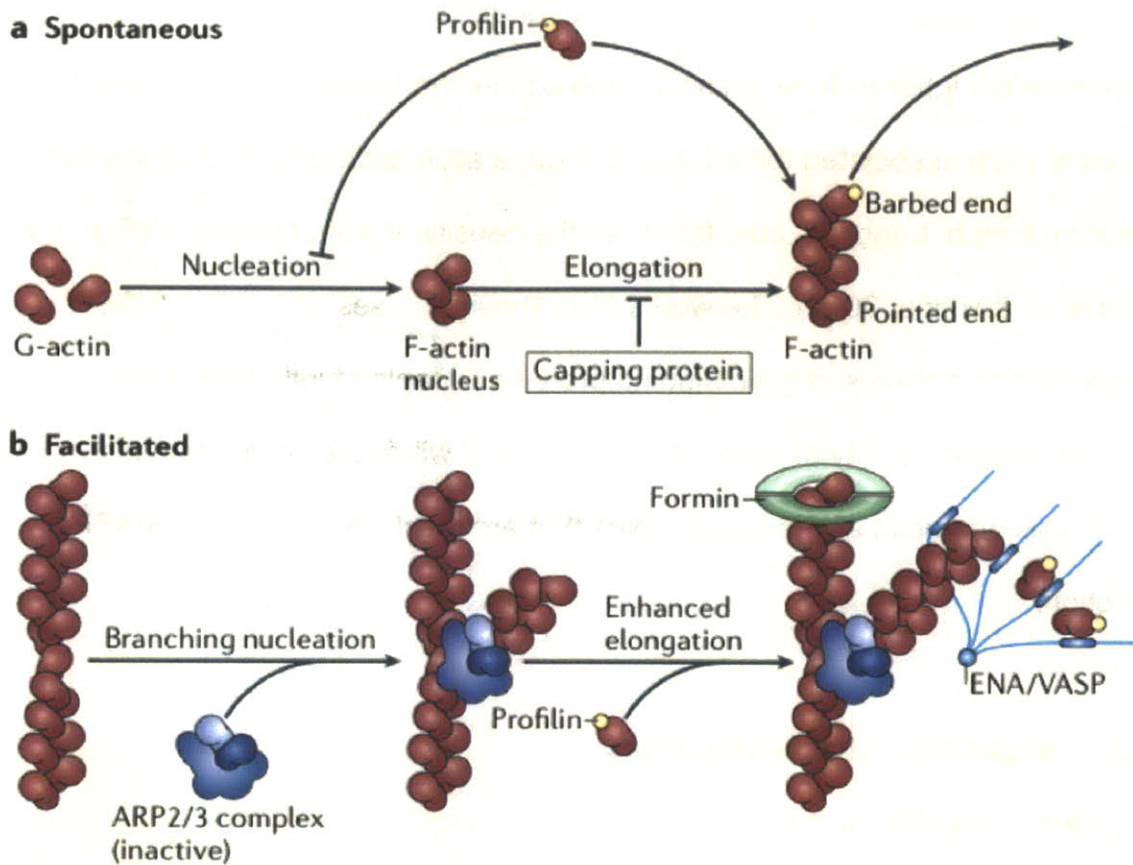
Figure 1-1: Regulation of actin polymerization.
(From Krause and Gautreau, 2014)
(**A**) For the spontaneous formation of actin filaments (F-actin), three actin monomers must come together to create an F-actin nucleus. Once F-actin is nucleated, it is elongated by the addition of actin monomers (G-actin). Elongation can be terminated by the binding of capping protein to the barbed ends of F-actin. Capping proteins bind the barbed ends of F-actin and prevent the addition of new actin monomers, blocking filament elongation. Profilin ensures that the G-actin is competent for polymerization and that elongation of F-actin occurs only at the barbed end. (**B**) The spontaneous nucleation of actin filaments is the rate-limiting step in the formation of F-actin. Actin nucleators, such as Arp 2/3 and formins, have been identified that nucleate actin with greater efficiency. The Arp2/3 complex binds to the side of a pre-existing actin filament to nucleate a new filament at a 70° angle, relative to the parent filament. Arp2/3 remains attached to the pointed end of F-actin. Formins can also nucleate linear actin filaments. They remain processively bound to the barbed ends of F-actin where they increase the rate of actin polymerization. Ena/VASP proteins do no nucleate new actin filaments, but instead enhance the rate of filament elongation. Both formins and Ena/VASP proteins recruit profilin-bound actin monomers to elongate F-actin in a processive manner.

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activate Arp2/3 at different locations within the cell and are linked to various

signaling pathways (Krause & Gautreau, 2014).

Arp2/3 is an essential protein complex and efforts to knock down

components of the Arp2/3 complex proved lethal in yeast and mice, and

negatively affected survival of cell lines (Goley & Welch, 2006). However,

recently, stable depletion of the Arp2/3 complex was obtained in cells derived

from Ink4a/Arf-deficient mice (Wu et al., 2012). These cells are unable to

produce lamellipodia, emphasizing the importance of the Arp2/3 complex in the

formation of lamellipodia.

1.3.2. Capping proteins

Capping proteins (CP) stabilize actin filaments by binding to the growing

barbed ends of actin filaments with high-affinity, and thus preventing the addition

or loss of actin monomers (Fig. 1-1). They indirectly regulate the density of

branched actin networks by limiting filament length, and the formation of

lamellipodial structures is dependent on the presence of CP. By capping barbed

ends, actin assembly is "funneled" to newly nucleated filaments at the cell edge,

keeping branched filaments short and dense (Cooper & Sept, 2008).

CP are expressed in most eukaryotes and consist of a heterodimer of

structurally related subunits (Cooper & Sept, 2008; Edwards et al., 2014). They

are also essential components of the actin machinery found in *Listeria* comet

tails. The dissociation rate of CP and barbed ends is relatively long, but proteins,

such as CARMIL, have been discovered that bind to CP and inhibit its ability to

bind actin barbed ends (Cooper & Sept, 2008). Actin barbed ends can also be

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freed of CP by proteins, such as cofilin, that sever the actin filament. Formins and Ena/VASP proteins antagonize CP by competing for barbed ends, but do not actively remove bound CP from actin filaments (Edwards et al., 2014).

1.3.3. Formins

Formins are a large protein family that directly nucleate the assembly of long, unbranched actin filaments (Fig. 1-1). The structures formed by formins vary by organism. In yeast, which possess two formin family members in *S. Cerevisiae* and three in *S. Pombe*, formins play an essential role in the formation of cytokinetic rings the actin cables necessary for intracellular transport. In mammals, formins are encoded by 15 genes and can promote the assembly of many types of actin-based structures, including filopodia, lamellipodia, and stress fibers. Formins nucleate actin filaments and remain processively bound to growing barbed ends, blocking CP, and accelerating actin filament elongation in the presence of profilin (Breitsprecher & Goode, 2013; Chhabra & Higgs, 2007).

The signature feature of formins is the possession of the formin homology 1 and 2 domains (FH1 and FH2). They function as dimers, with the FH2 domains of two formin molecules assembling into an anti-parallel dimer. The *Diaphanous* related formins, the largest subset of formins, are auto-inhibited by the interactions of two of its domains. Autoinhibition is relieved upon binding of active Rho GTPases (Breitsprecher & Goode, 2013).

1.3.4. Ena/VASP

The Ena/VASP family is a highly conserved group of proteins involved in the regulation of cell morphology, adhesion, and motility (Bear et al., 2000;

Gertler et al., 1996). Vertebrates express three Ena/VASP proteins while invertebrate species possess a single ortholog, such as the *Drosophila* Enabled (Ena*), C. elegans* UNC-34, and *Dictyostelium* VASP (Breitsprecher et al., 2008; Hansen & Mullins, 2010; Krause et al., 2003). The proteins Mena, VASP, and EVL form the vertebrate Ena/VASP family. Mice deficient in all three die *in utero*, displaying neurological and vascular defects (Furman et al., 2007; Kwiatkowski et al., 2007). Deletion of individual Ena/VASP members results in viable mice with only subtle phenotypes, suggesting that Ena/VASP members have overlapping functions and can largely compensate for each other's loss (Aszódi et al., 1999; Hauser et al., 1999; Lanier et al., 1999; Menzies et al., 2004).

Ena/VASP proteins are tetrameric molecules that enhance the rate of actin elongation by effectively competing with capping proteins for barbed ends (delaying the termination of filament growth) and accelerating the rate of filament elongation (Fig. 1-1) (Barzik et al., 2005; Hansen & Mullins, 2010). They alter the architecture of actin networks by decreasing the frequency of branched filaments (Bear et al., 2002). Ena/VASP proteins also support the formation of filopodia by clustering actin filaments and preventing CP from terminating filament elongation (Applewhite et al., 2007; Barzik et al., 2005; Bear et al., 2002). However, unlike formins, they do not nucleate new actin filaments *in vivo* (Trichet et al., 2008). Additionally, Ena/VASP proteins play a role in the pathogenesis of *Listeria monocytogenes. Listeria* recruit Ena/VASP proteins to the bacterial surface via expression of the ActA protein. Here, Ena/VASP proteins are used to promote

polymerization of F-actin "comet tails", which propel the bacterium intracellularly and enable infection to adjacent cells (Auerbuch et al., 2003; Geese, 2002).

Ena/VASP proteins are concentrated at the edges of protruding lamellipodia (Rottner et al., 1999), the tips of filopodia (Applewhite et al., 2007; Lanier et al., 1999), and at sites of cell-cell and cell-matrix adhesions (Gertler et al., 1996; Scott et al., 2006). The family members play important roles in directing cell migration and controlling cell shape in diverse cell types, including fibroblasts (Bear et al., 2002), endothelial cells (Furman et al., 2007), platelets (Aszódi et al., 1999), epithelial cells (Scott et al., 2006), and neurons (Kwiatkowski et al., 2007).

1.3.5. Profilin

Profilins are highly conserved, essential, and abundantly expressed proteins that bind actin monomers, preventing spontaneous actin polymerization and elongation at the pointed ends of actin filaments (Fig. 1-1) (Blanchoin et al., 2014; Witke, 2004). There are multiple profilins expressed in mammals and expression patterns differ between the proteins (Mouneimne et al., 2012; Witke, 2004). Profilin 1 is widely expressed in nearly all tissues and profilin 1 null mice are inviable. Profilin 2 has been found to be expressed in the nervous system and profilin 2 deficiency results in mice with severe neurological defects. Additionally, the affinities of different profilins for various profilin ligands are not uniform (Witke, 2004).

Two well-characterized binding surfaces on profilin mediate interactions with G-actin and proline-rich sequences (Blanchoin et al., 2014; Ferron et al., 2007), such as those found in formins and Ena/VASP proteins. Profilin binding to

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actin monomers prevents the addition of monomers to the pointed end, and thus

limits filament elongation to the barbed ends of F-actin (Ferron et al., 2007).

Recently, profilin has been demonstrated to function as a "gatekeeper",

regulating the balance between branched filaments and unbranched filaments by

preferentially delivering actin monomers to formins and Ena/VASP proteins

(Rotty et al., 2015; Suarez et al., 2015).

1.4.   Actin structures and the role of Ena/VASP proteins

1.4.1.   Lamellipodia

Lamellipodia are thin (<200 nm), actin-rich structures found in migrating

cells. As cells protrude forward, they extend lamellipodia that are supported by a

branched meshwork of actin filaments (Chhabra & Higgs, 2007; Pollard & Borisy,

2003). These filaments are directed such that their barbed ends are facing the

cell edge and actin polymerization pushes the membrane forward. Arp2/3

nucleates actin filaments to form the branched architecture found in these

structures. CP is also required for the assembly of these dendritic actin filament

networks (Edwards et al., 2014). The density of filaments is influenced by the

levels of Ena/VASP proteins, which compete with CP for barbed ends (Bear et

al., 2002).

1.4.2.   Filopodia

Filopodia are finger-like protrusions that consist of various actin-

associated proteins and bundled, parallel actin filaments, with their barbed ends

oriented outwards. Filopodia have demonstrated roles in cell-cell signaling,

adhesion, and cell migration (Gupton & Gertler, 2007). They probe the extracellular environment for various signaling cues and can form attachments to substrata (Schäfer et al., 2010). The formation of filopodia occurs by the reorganization of actin filaments of the lamellipodia and the continued elongation of a sub-population of actin filaments (Svitkina et al., 2003).

Ena/VASP proteins are required for the formation of filopodia in diverse cell types, including fibroblasts (Applewhite et al., 2007) and neuronal growth cones (Lebrand et al., 2004). Members of the Ena/VASP family remain processively bound to the barbed ends of actin filaments (Hansen & Mullins, 2010; Winkelman et al., 2014) and may drive the formation and maintenance of actin bundles by organizing and controlling filopodial length (Barzik et al., 2014). VASP, when bound to IRSp53, can cause cells to switch from forming lamellipodia to filopodia by clustering of actin filaments (Disanza et al., 2013).

1.4.3. Cell-cell contacts

There are several types of cell-cell contacts, including tight junctions and adherens junctions. They function to maintain an interface between neighboring cells to form a barrier, such as occurs with endothelial and epithelial cells. Proteins found at cell-cell contacts form connections with the cytoskeleton. Actin polymerization plays an important role in maintaining the integrity of cell-cell junctions by pushing adjacent membranes together (Leerberg et al., 2014; Trichet et al., 2008). Actin binding proteins are critical regulators of endothelial barrier integrity (García-Ponce et al., 2014).

Ena/VASP proteins localize to cell-cell contacts and are implicated in their maturation (Scott et al., 2006; Trichet et al., 2008; Vasioukhinet al., 2000). Loss of VASP leads to defective sealing of cell-cell contacts, resulting in increased permeability of endothelial cells (Benz et al., 2008). Deletion of the three vertebrate Ena/VASP family members leads to severe vascular defects in mice (Furman et al., 2007).

1.4.4. Cell-matrix contacts

Cell-matrix adhesions link the actin cytoskeleton with integrins, receptors that bind components of the extracellular matrix. They form attachments between cells and the extracellular matrix and are sites of bi-directional signaling (Hynes, 2002). Integrins mediate the rearrangement of the extracellular matrix and transmit extracellular cues that can lead to the reorganization of the cytoskeleton, resulting in changes in cell migration and shape (Hynes, 2002; Petit & Thiery, 2000). In migrating cells, they attach the lamellipodia to the underlying substrate.

Ena/VASP proteins localize to focal adhesions, but not to the more mature fibrillar adhesions (Gupton et al., 2012). They interact with a number of components of focal adhesions, including vinculin, zyxin, Riam, and palladin (Pula & Krause, 2008). VASP regulates $\alpha IIb\beta 3$ integrin activation, but does not bind integrins directly (Aszódi et al., 1999; Hauser et al., 1999), while Mena binds directly to the cytoplasmic tail of $\alpha 5$ integrin. Mena is absent from $\alpha 5$-positive fibrillar adhesions but its expression corresponds to increased formation of fibrillar adhesions (Gupton et al., 2012). The interaction between Mena and $\alpha 5$ in fibroblasts promotes the organization of soluble fibronectin into fibrils. In addition,

outside-in signaling within focal adhesions is enhanced by the presence of Mena. However, the precise mechanistic role performed by Ena/VASP proteins at cell-matrix adhesions remains unclear.

1.4.5. Growth cones

At the growing end of axonal projections are growth cones, fan-shaped, actin-rich structures that integrate environmental cues to direct axon path-finding (Dent et al., 2011; Vitriol & Zheng, 2012). F-actin dynamics are important for a growth cone to explore its environment and respond to guidance cues (Dent et al., 2011). Growth cones are dynamic structures that sense their environment by protruding and retracting filopodia and lamellipodia and adhere to extracellular components to move forward (Vitriol & Zheng, 2012).

Ena/VASP proteins are highly expressed in the developing nervous system. They concentrate to both filopodial tips and to the leading edge of lamellipodia in growth cones (Lanier et al., 1999) and are involved in downstream responses to both attractive and repulsive guidance cues (Dent et al., 2011). Blocking Ena/VASP function blocks the filopodial response of neurons to the chemo-attractive netrin (Lebrand et al., 2004) and Ena is required in *Drosophila* for axon repulsion in response to Slit (Bashaw et al., 2000).

1.5. Domain structure of Ena/VASP family

Ena/VASP proteins possess a modular domain organization comprised of two Ena/VASP Homologous regions (EVH1 and EVH2) and a proline-rich region

(PRR) of varying length (Fig. 1-2) (Krause et al., 2003). The roles of each domain are described in detail below.

1.5.1. EVH1

The EVH1 domain mediates interactions between Ena/VASP members and proteins containing the highly conserved poly-proline core consensus motif "FPPPP" (FP4) (Ball et al., 2002; Fedorov et al., 1999; Niebuhr et al., 1997). It is involved in the subcellular targeting of Ena/VASP proteins to the leading edge (Bear et al., 2000) and to focal adhesions (Ball et al., 2002; Hüttelmaier et al., 1998). Many Ena/VASP binding partners possess an FP4 motif, such as vinculin, but some contain multiple FP4 sequences. The focal adhesion protein zyxin and *Listeria*'s ActA each contain four FP4 sequences, potentially mediating interactions with multiple Ena/VASP tetramers (Ball et al., 2002).

1.5.2. Proline-Rich Region (PRR)

In the center of the protein, the PRR contains binding sites for SH3 and WW-domain containing proteins and profilin (Barzik et al., 2005; Bear et al., 2000; Ferron et al., 2007; Pasic et al., 2008). The PRR of different Ena/VASP proteins differ in their lengths and pattern of profilin-binding sequences (Lambrechts et al., 2000). For Ena/VASP function in whole cell motility, the PRR was dispensable (Loureiro et al., 2002). However, it is necessary for the enhanced efficiency of actin polymerization to propel *Listeria* migration (Auerbuch et al., 2003; Geese, 2002). The PRR preferentially binds profilin-actin over profilin alone and the recruits profilin-actin complexes to growing filaments (Ferron et al., 2007).
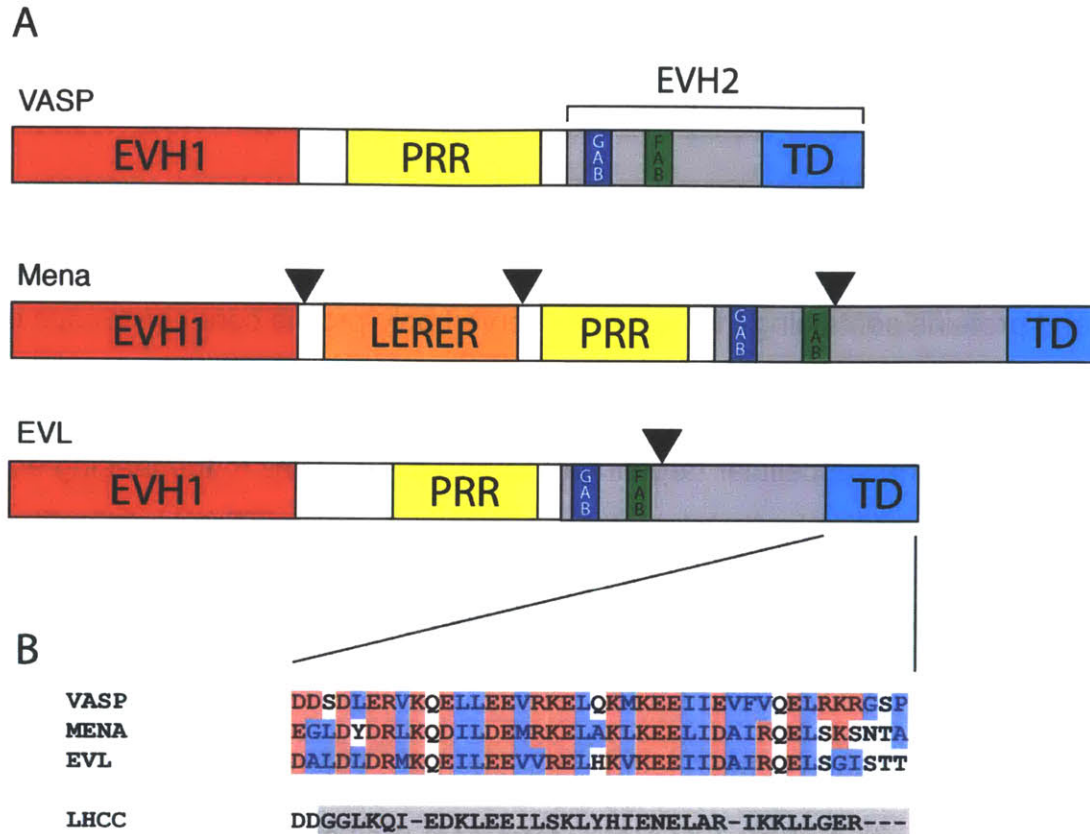
Figure 1-2: Schematic of Ena/VASP family domain structure.
(**A**) The Ena/VASP proteins share a conserved domain structure consisting of the EVH1, Proline-Rich Region (PRR), and the EVH2. Within the EVH2 domain (grey) are the G-actin and F-actin binding motifs (GAB and FAB, respectively) and the tetramerization domain (TD). Arrowheads denote sites of alternative exon inclusion. Mena contains an additional region consisting of a highly repetitive amino acid sequence with a consensus motif of LERER. (**B**) The alignment of the TDs of the mouse Ena/VASP proteins is shown. Sequences are highlighted according to residue properties (red=acidic or basic, blue= hydrophobic). For VASP[LHCC], the TD of VASP was replaced with an unrelated synthetic sequence that forms a left-handed coiled coil, highlighted in grey.

1.5.3. EVH2

The EVH2 domain of Ena/VASP proteins, by itself, is sufficient for anti-capping effects (Barzik et al., 2005) and to enhance filopodia formation in fibroblasts (Applewhite et al., 2007). Conversely, mutations in or deletion of the EVH2 domain hinders filopodia formation (Applewhite et al., 2007). In addition, EVH2 expression influenced cell movement similarly to wild-type Mena in cell-motility assays, and it is involved in the localization of Ena/VASP proteins. The EVH2 domain alone localizes to lamellipodia in fibroblasts, but with a broader distribution than the full length Mena protein (Loureiro et al., 2002).

The C-terminal EVH2 domain consists of G-actin and F-actin binding sites, termed GAB and FAB regions, respectively (Bachmann et al., 1999; Breitsprecher et al., 2011; Hansen & Mullins, 2010; Zimmermann et al., 2002). At the most C-terminal portion of the EVH2 domain, a coiled coil tetramerization domain (TD) mediates the formation of Ena/VASP tetramers (Bachmann et al., 1999; Barzik et al., 2005; Winkelman et al., 2014; Zimmermann et al., 2002).

1.5.3.1. GAB

The sequence of the GAB is related to the actin binding sequence of thymosin $\beta 4$ (Gertler et al., 1996) and the WH2 domains of WASP family proteins (Chereau & Dominguez, 2006). Mammalian Ena/VASP family members possess nearly identical GAB motif sequences (Breitsprecher et al., 2011). Although it can bind monomeric actin, this sequence binds profilin-actin complexes with a higher affinity (Chereau & Dominguez, 2006). It is essential for the anti-capping activity of Ena/VASP proteins (Barzik et al., 2005) and for their role in cell motility and

deletion of the GAB disrupts Ena/VASP localization (Loureiro et al., 2002). The GAB motif is important for the stability of Ena/VAP proteins at the tips of filopodia (Applewhite et al., 2007).

1.5.3.2. FAB

The sequence of the FAB motif, like the GAB, is well conserved between the mammalian Ena/VASP family members. It binds directly to F-actin and likely influences the rate of elongation by affecting the rate of actin monomer transfer to the barbed end (Breitsprecher et al., 2011). This rate depends on the affinity of Ena/VASP for binding of F-actin.

The FAB is dispensable for the role of Ena/VASP proteins in *Listeria* motility (Geese, 2002), but like the GAB, it is necessary for the anti-capping activity of Ena/VASP proteins (Barzik et al., 2005) and for their function in cell motility (Loureiro et al., 2002). Loss of the FAB also disrupts barbed end binding of Ena/VASP proteins (Hansen & Mullins, 2010).

1.5.3.3. TD

The TD of Ena/VASP proteins is highly conserved between family members (Gertler et al., 1996; Zimmermann et al., 2002) (Fig. 1-2). The coiled coil motif of the TD is sufficient for Ena/VASP tetramerization (Bachmann et al., 1999; Zimmermann et al., 2002). The multimerization of Ena/VASP proteins is critical for the ability of Ena/VASP proteins to elongate actin filaments (Breitsprecher et al., 2008; Hansen & Mullins, 2010; Krause et al., 2002).

Previous studies have demonstrated that loss of the TD disrupts multiple functions of Ena/VASP proteins, including anti-capping activity (Barzik et al.,

2005; Krause et al., 2004) and localization (Bear et al., 2000; Loureiro et al., 2002). Experiments with VASP "dimers" have been unable to reproduce actin elongation, demonstrating that higher order oligomers are critical for the function of Ena/VASP proteins (Hansen & Mullins, 2010).

1.6. Model of Ena/VASP function in actin elongation

Ena/VASP family members function as tetramers and an individual tetramer can mediate filament elongation in a processive manner (Fig.1-3) (Breitsprecher et al., 2011; Hansen & Mullins, 2010; Winkelman et al., 2014). The EVH1 domain helps position Ena/VASP proteins via its interactions with FP4 containing proteins (Bear & Gertler, 2009). Profilin-actin complexes are recruited to Ena/VASP via the PRR and the complexes are passed from the PRR to the GAB motif (Ferron et al., 2007). The FAB motif anchors Ena/VASP proteins to the side of filaments, near the barbed end of growing filaments (Hansen & Mullins, 2010). The PRR region binds profilin-actin in a ternary complex and presents them to the GAB, which then transfers the actin monomer to the growing filament (Chereau & Dominguez, 2006; Ferron et al., 2007).

1.7. Ena/VASP binding proteins

1.7.1. EVH1-mediated

The EVH1 domain of Ena/VASP proteins mediates many of their protein-protein interactions. Although exceptions exist, the EVH1 domain binds proteins that possess one or more [FL]PXwP (w is any hydrophobic residue; "FP4")
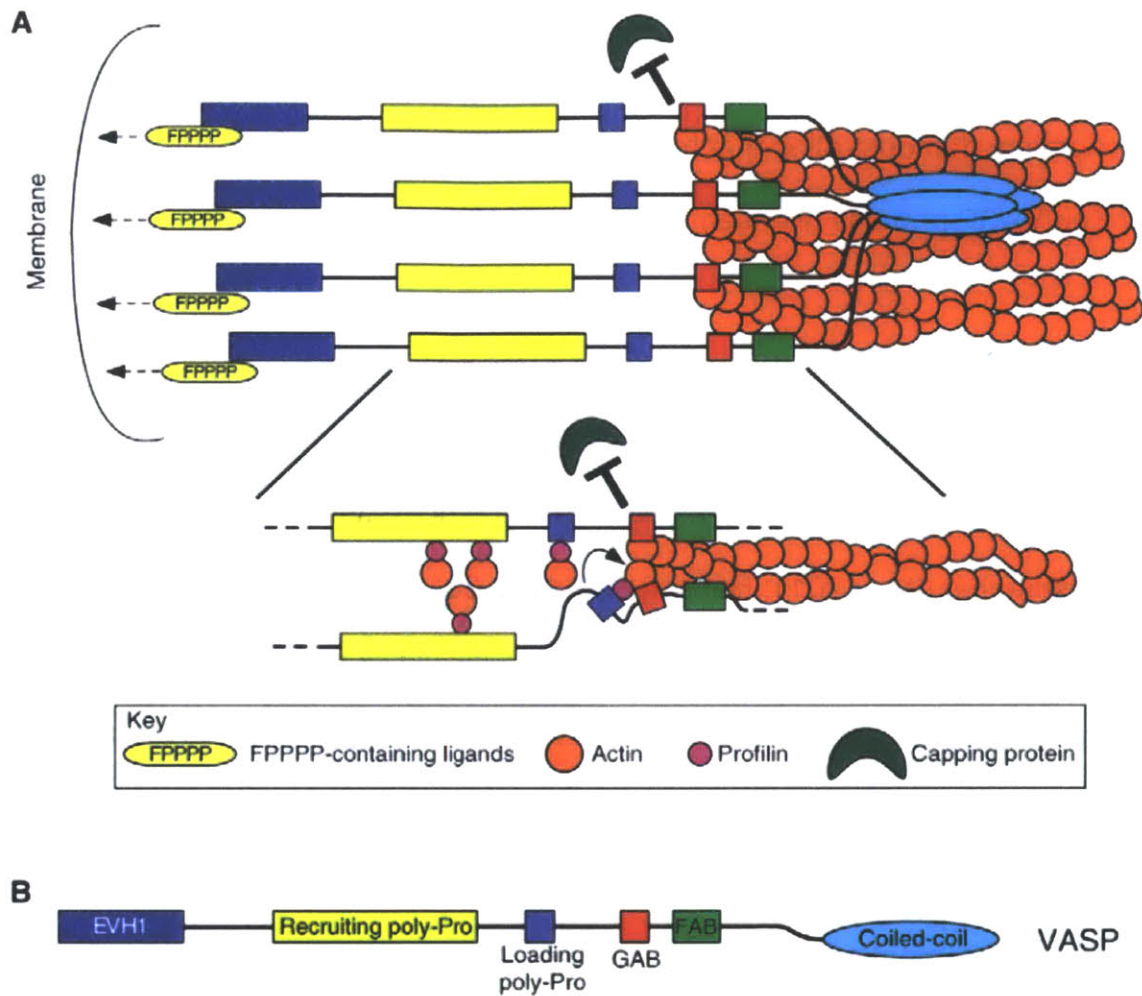
Figure 1-3: Mechanism of anti-capping of Mena and filament elongation.
(Adapted from Bear and Gertler, 2009)
(**A**) The EVH1 domain of Ena/VASP proteins positions Ena/VASP proteins near the plasma membrane via interactions with membrane associated proteins, which likely possess an FP4 motif. The EVH2 domain mediates tetramerization and the localization of Ena/VASP proteins to the barbed ends of uncapped actin filament. Ena/VASP is thought to supply actin monomers to the barbed ends of filaments while antagonizing the ability of CP to terminate filament elongation. Sequences within the PRR recruit profilin-actin, which are then passed onto the GAB domain. Filament elongation results from the addition of the actin subunit at the barbed end and the release of profilin. (**B**) Schematic of VASP domain structure.

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sequences (Ball et al., 2002). *Listeria's* ActA was the first FP4 containing protein described (Niebuhr et al., 1997). This interaction between ActA and Ena/VASP is critical for the recruitment of Ena/VASP proteins to the bacterial surface of *Listeria*. Other interactions are important for the localization of Ena/VASP proteins to their sites of action. Zyxin contains four FP4 repeats and helps direct Ena/VASP proteins to focal adhesions (Drees et al., 2000; Hoffman et al., 2006). Lamellipodin (Lpd) binds to Ras and $PI(3,4)P_2$ and localizes to lamellipodia, where it likely recruits Ena/VASP proteins. Lpd, and the related protein RIAM, contains six FP4 repeats (Krause et al., 2004). Another example of an EVH1 binding protein is the Slit receptor, Robo (Bashaw et al., 2000). The interaction between Ena/VASP proteins and Robo is important in axon guidance in *Drosophila*.

The tumor suppressor Tes does not contain an FP4 sequence, but its LIM3 domain binds to the same region of the EVH1 domain as FP4-containing proteins. It localizes to focal adhesions and binds Mena specifically. Tes interacts with the EVH1 domain of Mena with higher affinity than zyxin (Boëda et al., 2007). The finding that LIM domain containing Tes can interact with Mena opens up the possibility that other LIM containing proteins may also interact with Ena/VASP proteins (Small, 2008).

1.7.2. PRR-mediated

The PRR of Ena/VASP proteins mediates interactions with profilin and the proline-rich SH3 and WW domains (Ahern-Djamali et al., 1999). It was found to preferentially bind profilin-actin in a ternary complex over profilin alone (Ferron et

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al., 2007). The recruitment of profilin-actin by Ena/VASP proteins is important for its role in enhancing the efficiency of *Listeria* actin propulsion (Kwiatkowski et al., 2003). The residues implicated in profilin binding within the PRR are partially overlapping with sequences involved in SH3 binding (Lambrechts et al., 2000).

The PRR of Mena, VASP, and EVL interact with different sets of SH3 or WW domain containing proteins. VASP and Mena bind to the SH3 domains of Abl and Src, while EVL binds to the SH3 domains of Abl, Lyn, and nSrc. Some, but not all, SH3 interactions with Ena/VASP proteins can be regulated by phosphorylation (Lambrechts et al., 2000). The biological relevance of many of these interactions has yet to be identified. One exception is IRSp53, which contains an SH3 domain that mediates its interaction with Mena, VASP, and EVL (Disanza et al., 2013; Krugmann et al., 2001). IRSp53 was shown to cluster VASP to induce filopodia formation in response to Cdc42 activation (Disanza et al., 2013).

1.7.3. EVH2 mediated

As described above, the EVH2 domain includes the FAB and GAB motifs, as well as the TD. Thus, the EVH2 domain is responsible for mediating Ena/VASP binding to actin and for the formation of Ena/VASP tetramers. EVH2-mediated interactions are critical and sufficient for the positioning of Ena/VASP proteins to the barbed ends of actin-filaments and its resulting lamellipodial localization (Applewhite et al., 2007; Bear et al., 2002; Loureiro et al., 2002).

1.8. Unique characteristics of the Mammalian Ena/VASP family members

1.8.1. VASP

VASP was initially identified in platelets as a target of the cyclic nucleotide kinases PKA and PKG (Halbrügge et al., 1990). While all Ena/VASP proteins can be phosphorylated, the sites of phosphorylation are only partially conserved. VASP can be phosphorylated by PKA/PKG at three residues, and phosphorylation at these sites influences its actin polymerization capacities and localization (Benz et al., 2008). Mena retains two of these three residues, while EVL has just one. The activities of the three Ena/VASP families can be regulated differentially by phosphorylation.

VASP, and not Mena nor EVL, has also been shown to be phosphorylated by AMPK at Ser322, altering its binding to F-actin (Thomson et al., 2011). VASP is also phosphorylated at Tyr39 in its EVH1 domain by the tyrosine kinase Abl, which influences its subcellular localization (Maruoka et al., 2012). Another feature of VASP that may be important in its actin regulatory role is it preferential binding to profilin II over profilin I (Hansen & Mullins, 2010; Jonckheere et al., 1999; Mouneimne et al., 2012).

1.8.2. Mena

Between the EVH1 domain and PRR, Mena contains a unique and repetitive sequence termed the "LERER repeat" (Gertler et al., 1996). The LERER region has been shown to mediate a specific interaction between Mena and α5 integrin (Gupton et al., 2012). This interaction influences the formation of fibrillar adhesions and the organization of extracellular matrix through the

formation of fibronectin fibrils. Neither VASP nor EVL possess this repeat or bind α5 integrin. Another Mena-specific interaction is with the focal adhesion protein Tes (Boëda et al., 2007). Mena has not been shown to preferentially interact with a particular profilin family member (Lambrechts et al., 2000).

Alternative splicing of Mena produces five known isoforms (Di Modugno et al., 2007; Gertler et al., 1996; Gertler & Condeelis, 2011). Inclusion of the 19 amino acid INV exon in Mena promotes an enhanced sensitivity of cells to low concentrations of EGF (Philippar et al., 2008). Mena11a results from the inclusion of a 21 amino acid sequence within the EVH2 domain, which has been shown to positively correlate with epithelial-like phenotypes (Di Modugno et al., 2006; Pino et al., 2008) and negatively correlate with invasiveness of breast cancer cells (Goswami, 2004; Roussos et al., 2011).

Mena expression is correlated with increased invasiveness of breast cancer cells and breast cancer grade (Di Modugno et al., 2006; Robinson et al., 2009), and Mena[INV] expression was detected in patients with invasive ductal carcinomas (Roussos et al., 2011). In a subpopulation of highly invasive tumor cells that have migrated towards a chemotactic source, Mena[INV] expression is upregulated (Goswami et al., 2009).

1.8.3. EVL

EVL, like Mena was identified based on its sequence homology to VASP (Gertler et al., 1996). It is not detectable in fibroblasts by western blot, but is highly expressed in cells of the hematopoietic lineage (Lambrechts et al., 2000). Like Mena, EVL can also be alternatively spliced, but only a single alternative

isoform has been identified, EVL-I (Lambrechts et al., 2000). The 21 amino acid

exon is the same length as the 11a exon of Mena and inserted in the same

location, but its sequence is unrelated (Lambrechts et al., 2000). EVL-I contains

an additional phosphorylation site that is targeted by PKD, which may be

involved in fine-tuning lamellipodial dynamics (Janssens et al., 2009). It contains

one PKA phosphorylation site that is conserved in both Mena and VASP.

Similar to VASP, EVL preferentially binds profilin II over profilin I

(Lambrechts et al., 2000; Mouneimne et al., 2012). EVL, but not Mena, was also

found to interact with semaphorin-6A-1, a member of a family of proteins that

function in axon guidance signaling (Klostermann et al., 2000). EVL expression is

negatively correlated with invasiveness and grade of breast cancer cells

(Mouneimne et al., 2012).

The Ena/VASP family plays an important role in regulating actin dynamics

in a variety of cell types. There has been extensive work demonstrating their

overlapping functions, such as in *Listeria* propulsion (Geese, 2002) and

lamellipodial protrusions (Loureiro et al., 2000). However, each member of the

mammalian Ena/VASP family possesses features that are absent in the other

paralogs. The TD of Ena/VASP proteins mediates the oligomerization of Mena,

EVL, and VASP. The formation of homo-tetramers has been demonstrated

biochemically, but the formation of hetero-tetramers has only been assumed to

occur. In this study, I evaluate the composition of mammalian Ena/VASP

tetramers in cells. I confirm that mixed Ena/VASP oligomers form, but,

unexpectedly, find that particular subunit combinations are disfavored.

Furthermore, I demonstrate that the TD controls the selectivity of mixed

Ena/VASP tetramerization.

1.9   References

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Chapter 2:

Selectivity in Subunit Composition of Ena/VASP Tetramers

Table of Contents

2.1   Abstract

The vertebrate members of the Ena/VASP family, VASP, Mena, and EVL, have many overlapping properties and expression patterns, but functional and regulatory differences between paralogs exist. The actin regulatory family of Ena/VASP proteins function as tetramers, and some evidence suggests that Ena/VASP hetero-tetramers can form. The assembly of mixed tetramers may serve a regulatory role to refine the activity of Ena/VASP tetramers. While it has been assumed that family members can form mixed tetramers, this possibility has not been investigated systematically. Using cells expressing controlled combinations of VASP, Mena, and EVL, we evaluated the formation of mixed oligomers and found that VASP forms oligomers with itself, Mena, or EVL without apparent bias. However, Mena and EVL showed weak hetero-oligomerization, suggesting specificity in the association of Ena/VASP family members. Co-expression of VASP increased the interactions of EVL with Mena, suggesting the formation of tetramers containing all three Ena/VASP family members. Additionally, we found that the tetramerization domain at the C-termini of Ena/VASP proteins conferred the observed selectivity. Our results reveal that Ena/VASP proteins form heterotetramers, but in a selective manner predominantly mediated by the tetramerization domain.

2.2. Introduction

Ena/VASP proteins promote actin polymerization by increasing the elongation rate of actin filaments and by protecting barbed ends from capping proteins (CP), thus delaying termination of filament growth (Barzik et al., 2005; Bear et al., 2002; Breitsprecher et al., 2011; Hansen & Mullins, 2010). The importance of this activity has been demonstrated in different cellular contexts. In fibroblasts, for example, loss of Ena/VASP proteins results in the formation of lamellipodia with short, highly branched actin filament networks, while overexpression of Ena/VASP proteins leads to longer, less branched actin networks (Bear et al., 2002). Ena/VASP family members are also key regulators of the formation and elongation of filopodia, structures comprised of long, parallel bundles of F-actin (Applewhite et al., 2007; Dent et al., 2007; Lebrand et al., 2004; Winkelman et al., 2014).

Cells may express more than one Ena/VASP paralog (Bear et al., 2000; Lebrand et al., 2004; Mouneimne et al., 2012), and it has been suggested that Mena, VASP, and EVL can form mixed tetramers. The three paralogs differ in their ability to promote actin polymerization *in vitro*, and additional evidence suggests paralog-specific interactions and modes of regulation (Benz et al., 2008; Gupton et al., 2012; Mouneimne et al., 2012; Philippar et al., 2008). These findings raise the intriguing possibility that Ena/VASP hetero-tetramer formation could provide a means to fine-tune the actin-polymerization activity of Ena/VASP tetramers, as well as to increase the diversity of their interactions and regulation. The ability of Ena/VASP proteins to form oligomers consisting of multiple

Ena/VASP family members has been demonstrated *in vitro* (Ahern-Djamali et al., 1998; Carl et al., 1999), but the extent to which homo- versus hetero-tetramerization occurs *in vivo* has not been addressed systematically.

In this study, I evaluate the composition of mammalian Ena/VASP multimers in cells. I confirm that mixed Ena/VASP oligomers are formed, but unexpectedly find that particular subunit combinations are disfavored. Furthermore, I demonstrate that the C-terminal tetramerization domain (TD) of Ena/VASP proteins controls the selective formation of mixed Ena/VASP tetramers. Hetero-tetramerization may provide a mechanism to fine-tune Ena/VASP function and regulation.

2.3　Results

2.3.1　Mena and VASP associate *in vivo*

We sought to determine the extent to which Ena/VASP paralogs hetero-oligomerize in cells. To confirm that members of the Ena/VASP family form mixed complexes *in vivo*, the association of endogenous Mena and VASP was determined in Rat2 fibroblasts, which express both Mena and VASP at readily detectable levels (Fig. 2-1) (Bear et al., 2000). Endogenous Mena was immunoprecipitated from Rat2 lysates and endogenous VASP was found to co-immunoprecipitate (co-IP) (Fig. 2-1 A). Reciprocally, immunoprecipitation of endogenous VASP confirmed the observed association with Mena (Fig. 2-1 B). Control immunoprecipitation with isotype-matched antibodies confirmed the specificity of the Mena/VASP co-IP. These results demonstrate that Mena and VASP are found in complexes within cells.

2.3.2　Pre-formed homo-tetramers do not reassemble during isolation

I was concerned that the observed associations between VASP and Mena may have resulted from the dissociation and reassembly of oligomers during cell lysis and subsequent immunoprecipitation. To test this, I transfected MV^{D7} cells, a mouse embryonic fibroblast cell line derived from mice genetically null for Mena and VASP and lacking detectable EVL expression (Bear et al., 2000), with either EGFP-Mena or EGFP-VASP alone. Transfected cells were co-cultured at a 1:1 ratio of VASP transfected cells to Mena transfected cells, and Mena was immunoprecipitated from lysates as above. VASP did not co-IP with Mena in this assay (Fig. 2-2), confirming that oligomers assembled within cells are stable
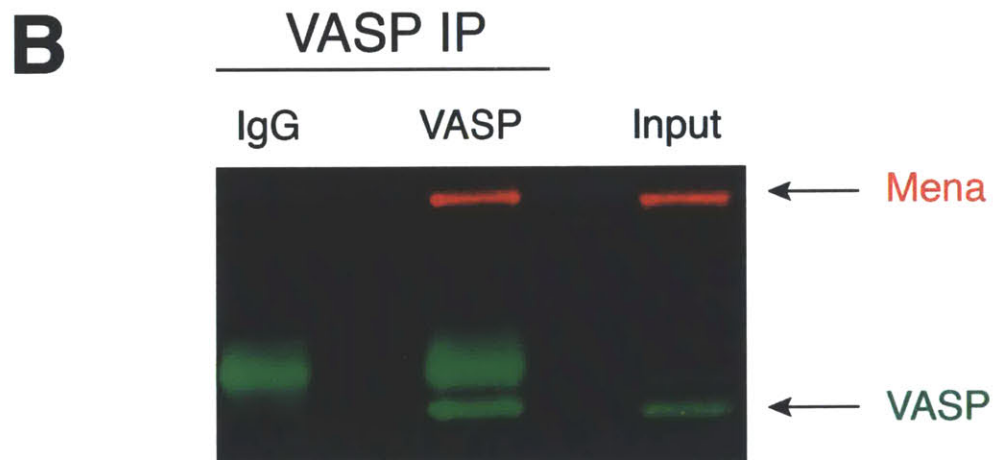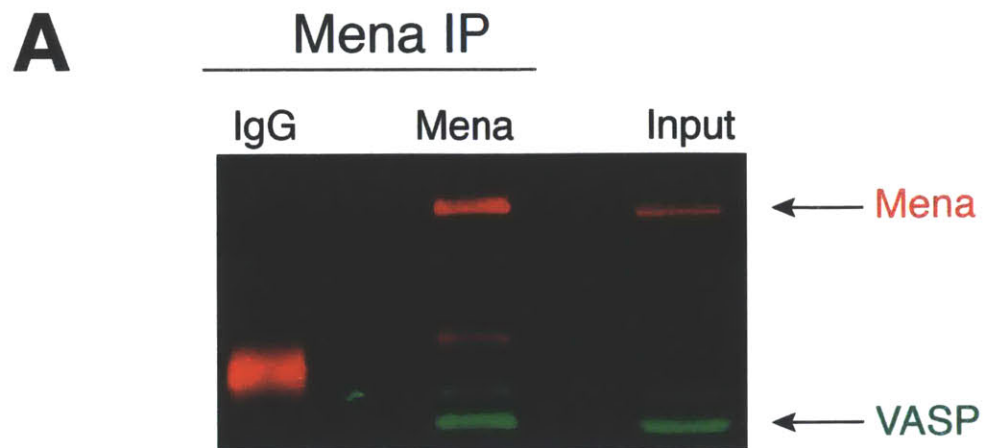
Figure 2-1: Endogenous Mena:VASP protein complexes are detected in vivo.
Mena (**A**) or VASP (**B**) was immunoprecipitated (IP) from Rat2 (fibroblast cell line) lysates using αMena monoclonal antibody or αVASP polyclonal antibody, respectively. Precipitated proteins analyzed by western blots probed with αMena antibody (red) or αVASP antibody (green). Control IPs were performed in parallel using mouse (**A**) or rabbit (**B**) IgG. Input is 10% of IP.
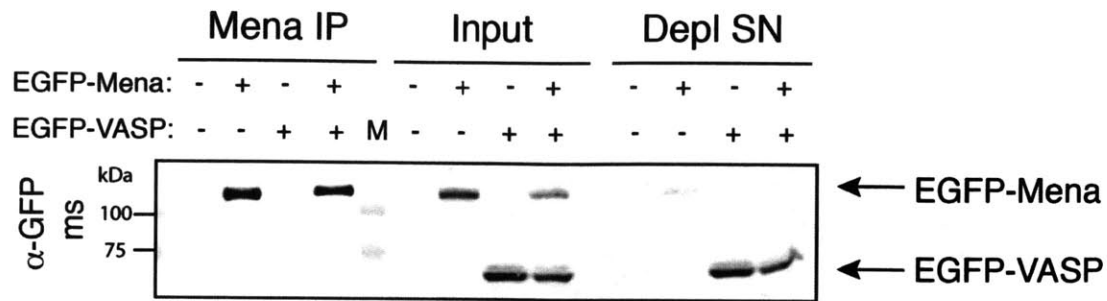
Figure 2-2: Mena and VASP homo-oligomers do not mix in lysate.
MV^{D7} cells were transfected with either EGFP-Mena or EGFP-VASP. Transfected cells were co-cultured at a 1:1 ratio and Mena was immunoprecipitated from lysates. EGFP-tagged proteins were detected with αGFP (rb) antibody and proteins were identified by western blot using αGFP (ms). No co-IP of VASP was observed when VASP and Mena are expressed in separate cells. Depleted supernatant ("Depl SN") indicate near complete depletion of mena from lysates. Input is 10% of IP; M, molecular weight marker.

under the biochemical isolation conditions employed here. This is consistent with structural and biochemical data indicating that VASP homo-tetramers are extremely stable (Barzik et al., 2005; Kühnel et al., 2004; Zimmermann et al., 2002).

2.3.3   Mena:VASP complex assembly is mediated by the TD

Ena/VASP proteins interact with a number of shared binding partners. Some proteins, such as zyxin (Drees et al., 2000) and Lpd (Krause et al., 2004), can bind multiple Ena/VASP proteins simultaneously. These interactions are mediated via the EVH1 domain of Ena/VASP proteins (Ball et al., 2002; Niebuhr et al., 1997). To eliminate the possibility that the association between Mena and VASP observed was a result of potential scaffolding effects through shared EVH1-binding partners (e.g. zyxin), or through interactions mediated by the PRR, we tested whether we could observe isolated EVH2 domains co-IP with EGFP-Mena. We co-transfected EGFP-Mena with the EVH2 domain of Mena or VASP fused to EGFP into MV[D7] cells. Both EVH2 domains associated with immunoprecipitated EGFP-Mena (Fig. 2-3), in agreement with previous studies demonstrating that the EVH2 domain is sufficient for oligomerization (Bachmann et al., 1999; Carl et al., 1999; Kühnel et al., 2004; Zimmermann et al., 2002). These findings imply that the EVH2 domain mediates the observed interactions between Mena and VASP in cells.

The EVH2 domain contains the GAB and FAB regions, which bind to G- and F-actin, respectively (Hüttelmaier et al., 1998), raising the possibility that Ena/VASP complexes may result from scaffolding effects mediated by
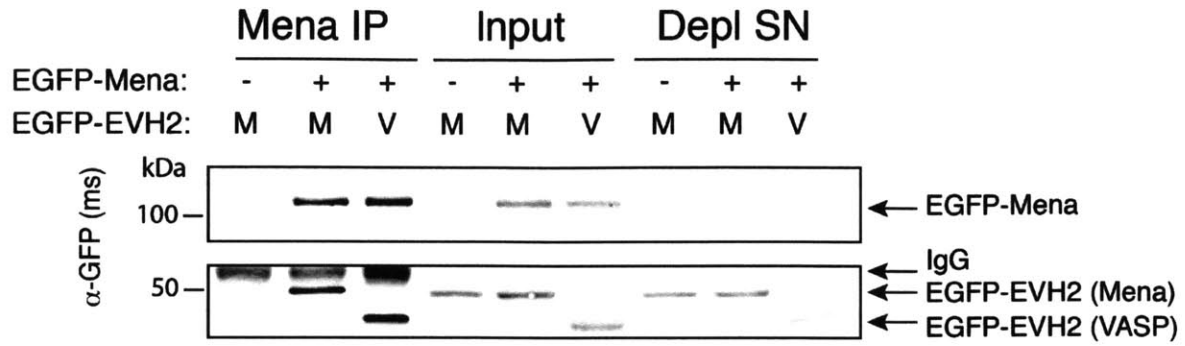
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Figure 2-3: TD mediates oligomerization of Ena/VASP proteins.
MVD7 cells were co-transfected with EGFP-Mena and the EVH2 domains of
Mena ("M") or VASP ("V") fused to EGFP. EGFP-Mena was immunoprecipitated
with αMena antibody and interactions with EVH2 domains were detected with
αGFP antibody. Input is 10% of IP ("Input") and Mena-depleted supernatant
("Depl SN") indicate near complete immuno-depletion of Mena from lysates.
The EVH2 domain of Mena is larger than the EVH2 domain of VASP, and thus
migrates at a higher molecular weight.

interactions with actin. To test if any interactions outside of the TD are sufficient to mediate mixed Ena/VASP complexes, we engineered a version of VASP to force VASP homo-tetramer formation independently of the conserved TD, and assayed for the formation of mixed Ena/VASP complexes by co-IP. The designed synthetic tetramer sequence of GCN4 p-LI was based on the coiled coil oligomerization domain of yeast transcription factor GCN4, a well-studied model of oligomerization (Burkhard et al., 2001; Harbury et al., 1993). GCN4 p-LI d has previously been shown to function modularly in the replacement of the TDs of the pore protein KcsA (Yuchi et al., 2008) and of p53 (Waterman et al., 1996). The crystalized TD of human VASP forms a parallel, right-handed coiled coil (Kühnel et al., 2004), while the synthetic TD forms a left-handed coiled coil. The altered VASP was thus termed VASP^{LHCC} (Fig. 1-2).

We assayed if interactions with actin or other scaffolding proteins were sufficient for VASP^{LHCC} to associate with wild-type VASP (VASP^{WT}). VASP^{LHCC} and VASP^{WT} were expressed at comparable levels (Fig. 2-4 A, input), suggesting that the synthetic coiled-coil did not grossly affect the expression and stability of VASP protein. To test whether scaffolding interactions could lead to complex formation of VASP^{LHCC} with VASP^{WT}, we performed co-IP experiments using lysates of MV^{D7} cells expressing FLAG-VASP^{LHCC} along with either EGFP-VASP^{WT} or EGFP-VASP^{LHCC}. FLAG-VASP^{LHCC} was readily detected to co-IP with EGFP-VASP^{LHCC}, as expected by the interaction of the synthetic oligomerizing sequences. However, FLAG-VASP^{LHCC} did not complex with EGFP-VASP^{WT} (Fig. 2-4 A). Reciprocally, EGFP-VASP^{WT} failed to associate with immunoprecipitated
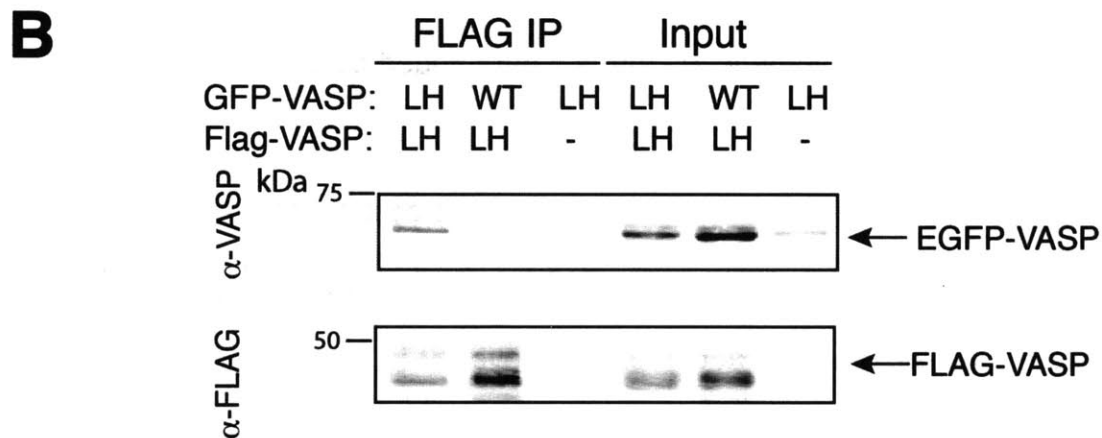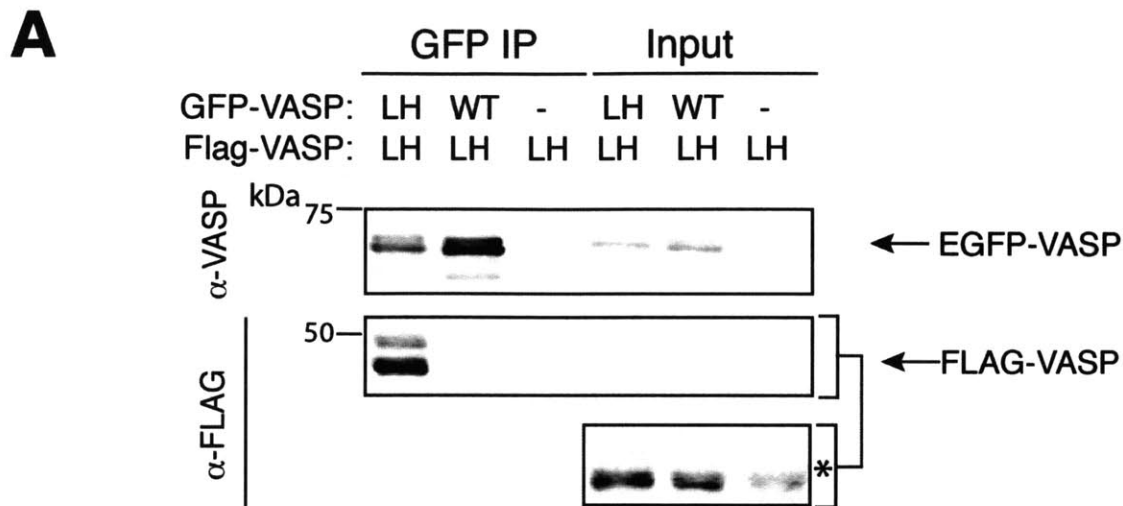
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Figure 2-4: TD mediates oligomerization of Ena/VASP proteins .
MV^{D7} cells were co-transfected with FLAG-VASP^{LHCC} ("LH") and either EGFP-VASP^{WT} ("WT") or EGFP-VASP^{LHCC} ("LH"). αEGFP (**A**) or αFLAG (**B**) antibodies were used to immunoprecipitate tagged proteins. Western blots were probed with αVASP and αFLAG antibodies, as indicated. Contrast-enhanced image of input for (**A**) provided. Input is 5% of IP.

FLAG-VASP^{LHCC}, whereas EGFP-VASP^{LHCC} did co-IP with FLAG-VASP^{LHCC} (Fig.

2-4 B). Thus, the left-handed VASP^{LHCC} forms oligomers only with VASP^{LHCC},

and not with wild-type Ena/VASP proteins. Importantly, these data also confirm

that the observed Ena/VASP co-IPs are dependent upon the TD, and that actin

or other binding partners are not sufficient to mediate Ena/VASP oligomerization.

2.3.4   Mena and VASP association is consistent with random tetramer oligomerization

While co-IP results indicated that endogenous Mena and VASP form

mixed complexes within cells (Fig. 2-1), they did not provide information about

the composition of the observed oligomers. To determine if Ena/VASP family

members are biased towards particular tetramer compositions, such as a

preference to form homo-oligomers over hetero-oligomers, we performed a

series of co-IP experiments to quantify the abundance of Mena and VASP in

mixed oligomers. To control the relative expression of Ena/VASP proteins, we

transfected varying amounts of EGFP-Mena and EGFP-VASP into MV^{D7} cells.

Co-transfected cells were lysed and Mena was immunoprecipitated from the

lysates using an antibody that showed no detectable cross-reactivity with EGFP-

VASP (Fig. 2-5 A).

Our experimental design allowed us to compare the relative levels of

EGFP-VASP and EGFP-Mena expressed in cell lysates and in anti-Mena

immunoprecipitates directly by western blot, using an antibody against the

shared GFP tag (Fig. 2-5 A). The large molecular weight difference between

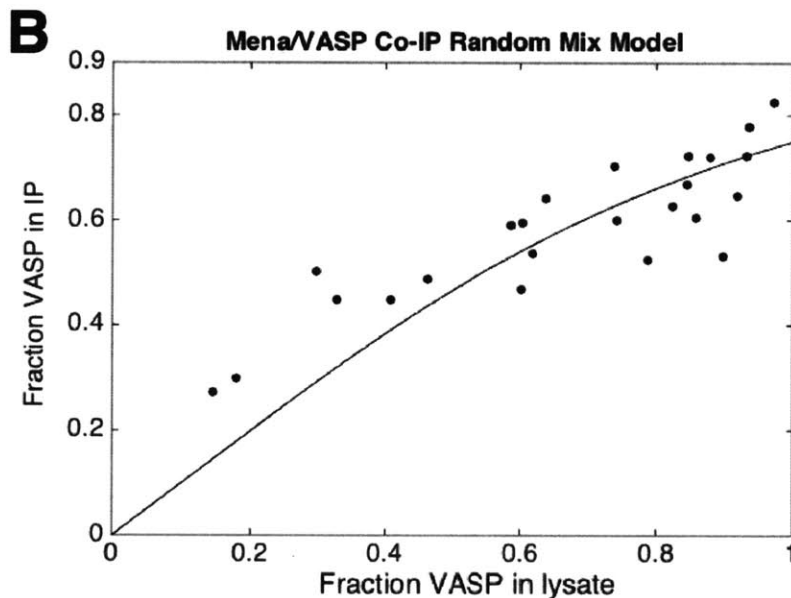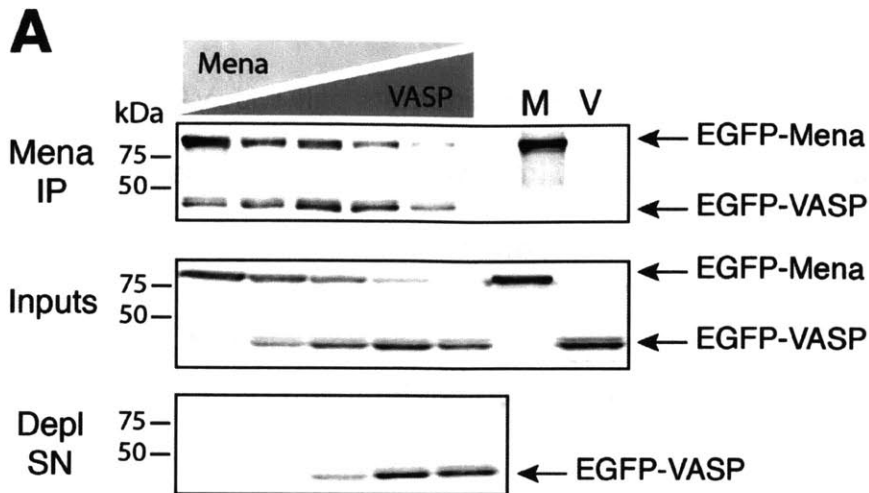EGFP-Mena (~100 kDa) and EGFP-VASP (~80 kDa) allowed unambiguous

Figure 2-5. Mena and VASP oligomerization is consistent with random tetramerization.
(A) EGFP-tagged Mena and VASP were co-expressed in MV^{D7} fibroblasts at varying ratios. For controls, cells were transfected with either Mena ("M") or VASP ("V") alone. Mena was immunoprecipitated from lysates and interacting VASP was detected via the shared EGFP tag using an αGFP antibody (top blot). Protein lysates prior to IP ("Input") and of Mena-depleted supernatant ("Depl SN") (middle and bottom blots, respectively) indicate near complete immuno-depletion of Mena from lysates. Bands were quantified by densitometry and were used to calculate the VASP fraction of total Ena/VASP protein in Mena IPs and in lysates.
(B) The VASP fraction of total Ena/VASP protein in lysates (X-axis) was plotted against the VASP fraction of total Ena/VASP protein in Mena IPs (Y-axis). The curved line indicates expected results based on a model of random tetramer formation. Each point represents one Mena IP experiment.

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identification of each species. Band intensities were quantified to calculate the relative composition of Ena/VASP proteins present in immunoprecipitated complexes and the fraction of VASP present in cell lysates [EGFP-VASP/(EGFP-Mena + EGFP-VASP)] across a range of expression levels of the two proteins. The resulting data were plotted and fitted to a model in which tetramer formation between Mena and VASP monomers is assumed to be random (Fig. 2-5 B). The model captured our measured associations ($r^2 = 0.76$), consistent with random association largely determining the amount of Mena:VASP hetero-oligomerization. Additionally, these data are consistent with the assumption that the immunoprecipitated complexes are likely Ena/VASP tetramers. These observations suggest that Mena and VASP can randomly associate into hetero-tetramers.

2.3.5 Mena isoforms oligomerize to form homo- and hetero-tetramers

Mena can be alternatively spliced to create multiple Mena isoforms, including Mena11a (Di Modugno et al., 2007) and Mena^{INV} (Gertler et al., 1996; Philippar et al., 2008). Previous studies have determined that inclusion of either the 11a or INV exon produces Mena variants with functions distinct from Mena lacking these additional sequences (Mena^{classic}) (Goswami et al., 2009; Philippar et al., 2008). Of particular interest, the 11a sequence is included within the EVH2 domain, raising the possibility that it may influence tetramer formation due to its proximity to the TD. Co-IP experiments were performed to determine if 11a or INV inclusion altered the ability of Mena to homo-oligomerize with Mena^{classic}. MV^{D7} cells were co-transfected with FLAG-Mena^{classic} and either EGFP-Mena^{INV},
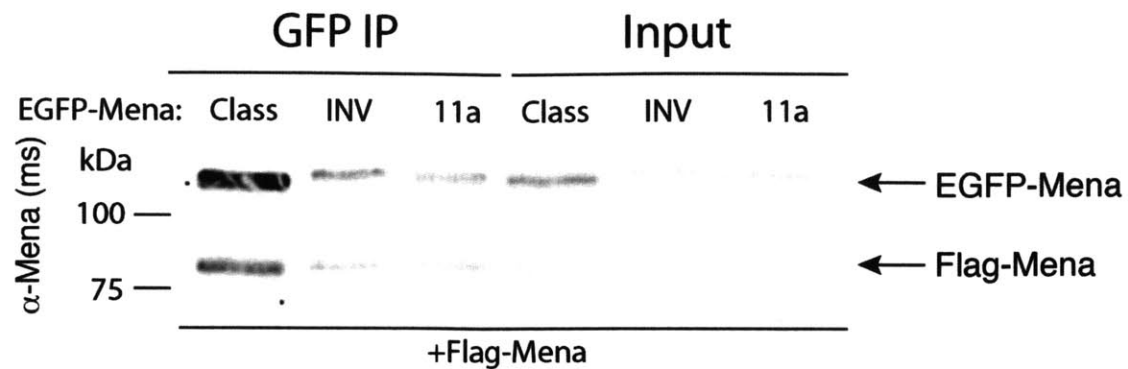
Figure 2-6: Mena isoforms hetero-oligomerize.
MV[D7] cells co-transfected with FLAG-Mena[classic] and either EGFP-Mena[classic]
("class"), EGFP-Mena[INV] ("INV"), or EGFP-Mena11a ("11a"). EGFP-tagged Mena
isoforms were immunoprecipitated from lysates and all expressed Mena proteins
were detected using αMena antibody. Input is 5% of IP.

EGFP-Mena11a, or EGFP-Mena^{classic}. Immunoprecipitation experiments using a GFP antibody indicated that all Mena isoforms bound FLAG-Mena^{classic} to similar extents (Fig. 2-6). This suggests that oligomers consisting of mixed Ena/VASP isoforms can exist in cells expressing multiple Mena isoforms.

2.3.6 VASP forms oligomers with itself, Mena, or EVL in an unbiased manner.

In addition to examining VASP:Mena interactions, we wanted to determine if VASP and EVL also assembled into tetramers without bias. To test this, FLAG-VASP was co-transfected into MV^{D7} cells with EGFP-VASP, EGFP-Mena, or EGFP-EVL. Immunoprecipitation of the EGFP-tagged Ena/VASP proteins using a GFP antibody demonstrated that VASP:VASP complexes occurred to a similar extent as VASP:Mena and VASP:EVL complexes (Fig. 2-7). These results suggest that VASP forms mixed oligomers with all Ena/VASP proteins expressed in a cell with little to no compositional bias.

2.3.7 Mena:EVL hetero-tetramer formation is disfavored

As there appeared to be no bias in oligomer formation between EGFP-Mena and VASP, we tested whether all Ena/VASP proteins can form mixed tetramers by examining the association of Mena, VASP, and EVL in the remaining pairwise combination. To evaluate oligomerization of Mena with EVL, we performed co-IP experiments of EGFP-tagged Ena/VASP proteins (as above) to compare the formation of Mena:EVL complexes with the formation of Mena:VASP complexes. To our surprise, relative to EGFP-VASP, we found only a very weak association of EGFP-EVL with EGFP-Mena (Fig 2-8 A).
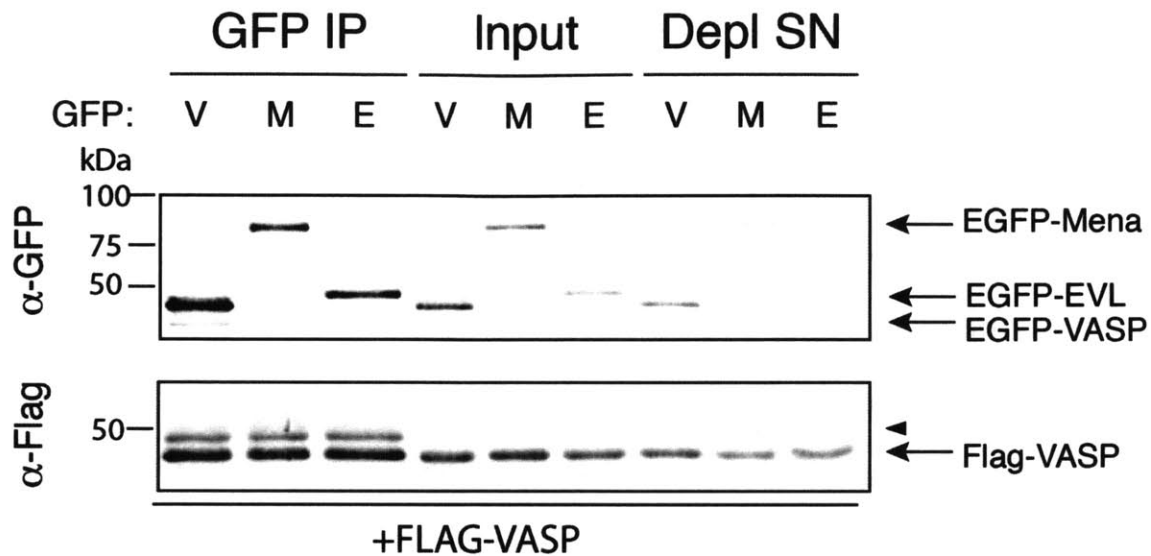
Figure 2-7: VASP oligomerizes with itself, Mena, and EVL without bias.
MV^{D7} cells were co-transfected with FLAG-VASP and either EGFP-VASP ("V"),
EGFP-Mena ("M"), or EGFP-EVL ("E"). EGFP-tagged Ena/VASP proteins were
immunoprecipitated from lysates and interacting FLAG-VASP was detected with
αFLAG antibody. Phosphorylation of VASP induces a well-characterized
electrophoretic mobility shift of VASP and was observed in the IP lanes
(arrowhead). Immuno-depleted supernatants ("Depl SN") illustrate GFP IP
captured a significant fraction of EGFP-tagged Ena/VASP proteins expressed.
Input is 5% of IP.
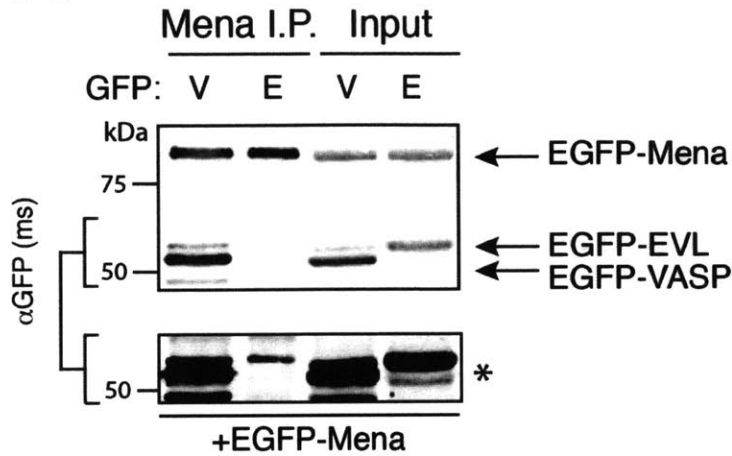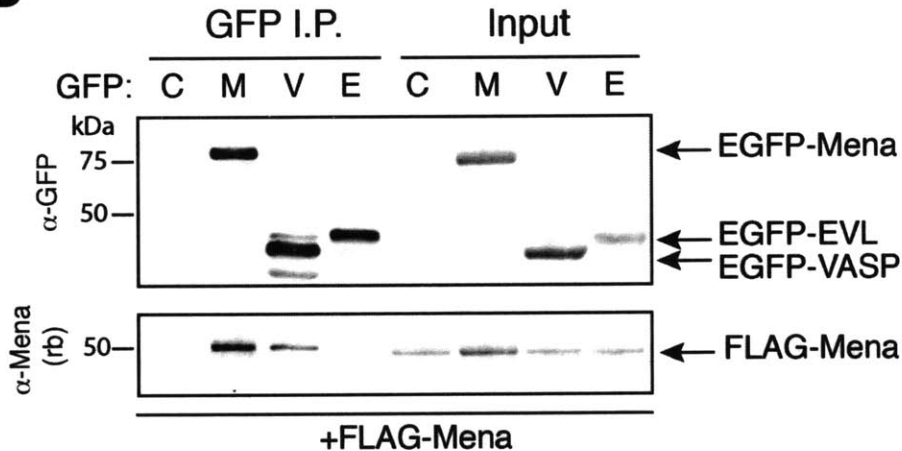
Figure 2-8: Mena and EVL association is disfavored.
(A) EGFP-Mena was immunoprecipitated from lysates of MV^{D7} cells co-transfected with EGFP-Mena and either EGFP-VASP ("V") or EGFP-EVL ("E"). Interacting Ena/VASP proteins were detected by immunoblot with αGFP antibody. A weak co-IP of EGFP-EVL can only be detected by contrast enhancement of the bracketed portion (*, bottom panel). Input is 10% of IP. (B) EGFP-tagged constructs were immunoprecipitated from MV^{D7} fibroblasts co-transfected with FLAG-Mena and control EGFP ("C"), EGFP-Mena ("M"), EGFP-VASP ("V"), or EGFP-EVL ("E"). Interacting FLAG-Mena was detected a rabbit polyclonal αMena antibody. Control EGFP migrates at a molecular weight too small to be visualized on the SDS-PAGE gel used. Input is 10% of IP.

To confirm this result, FLAG-Mena was co-transfected with EGFP, EGFP-Mena, EGFP-VASP, or EGFP-EVL in MV^{D7} fibroblasts. FLAG-Mena associated with immunoprecipitated EGFP-Mena and EGFP-VASP, but not with EGFP-EVL or control EGFP (Fig. 2-8 B). These results suggest that Mena can only weakly complex with EVL and that it oligomerizes much more readily with VASP.

2.3.8   Ena/VASP TD confers the selectivity of hetero-tetramer formation.

Previous experiments demonstrated that interactions between Ena/VASP proteins and shared binding partners were insufficient to mediate oligomerization (Figs. 2-3 & 2-4). I thus hypothesized that the weaker association between Mena and EVL, relative to the association between Mena and VASP, may be due to differences within their EVH2 domain. To confirm that the EVH2 domain is sufficient to mediate the selectivity of Mena's interaction with VASP over EVL, I tested the ability of EVL to bind the EVH2 domains of VASP and Mena. Immunoprecipitated FLAG-VASP interacted with the EVH2 domains of both Mena and VASP, while immunoprecipitated FLAG-EVL bound only to the EVH2 domain of VASP and not of Mena (Fig. 2-9 A). This supports my hypothesis that the EVH2 domain, which contains the TD, dictates the specificity of oligomer formation.

The TD in the EVH2 domain of Ena/VASP proteins has been shown to mediate tetramerization (Ahern-Djamali et al., 1998; Carl et al., 1999; Kühnel et al., 2004), but it is uncertain whether the TD is the sole determinant of specificity in oligomer composition. To test whether the TD directs selectivity in tetramer

composition, we generated a chimeric EGFP-Mena construct (Mena^{TD-EVL}), in

which the TD of Mena was replaced with the homologous sequence of EVL (Fig.

1-2). MV^{D7} cells were co-transfected with EGFP-Mena, EGFP-EVL,or EGFP-

Mena^{TD-EVL} and either FLAG-Mena or FLAG-EVL. EGFP-tagged proteins were

immunoprecipitated and the associated FLAG-tagged proteins were observed by

western blot. In agreement with earlier observations (Fig. 2-8), FLAG-Mena

associated with EGFP-Mena but not EGFP-EVL, while FLAG-EVL associated

with EGFP-EVL but not EGFP-Mena (Fig. 2-9 B & C). However, chimeric

Mena^{TD-EVL} strongly associated with FLAG-EVL, but not with FLAG-Mena. These

observations confirm that the TD is sufficient to mediate the selectivity of

tetramer formation between Ena/VASP proteins.

2.3.9 VASP enhances the association of EVL with Mena

The experiments thus far tested the associations of two Ena/VASP

proteins expressed simultaneously. However, some cell types express all three

Ena/VASP paralogs (Bear et al., 2000; Krause et al., 2003; Lanier et al., 1999;

Lebrand et al., 2004; Mouneimne et al., 2012), raising the possibility that

tetramers containing all three Ena/VASP family members may form. Given the

bias against mixed Mena:EVL oligomers, we wondered whether hetero-tetramers

containing all three Ena/VASP family members could form. In cells expressing all

three paralogs, we hypothesized two potential scenarios for mixed tetramer

formation: (1) oligomers containing all three Ena/VASP proteins form, with VASP

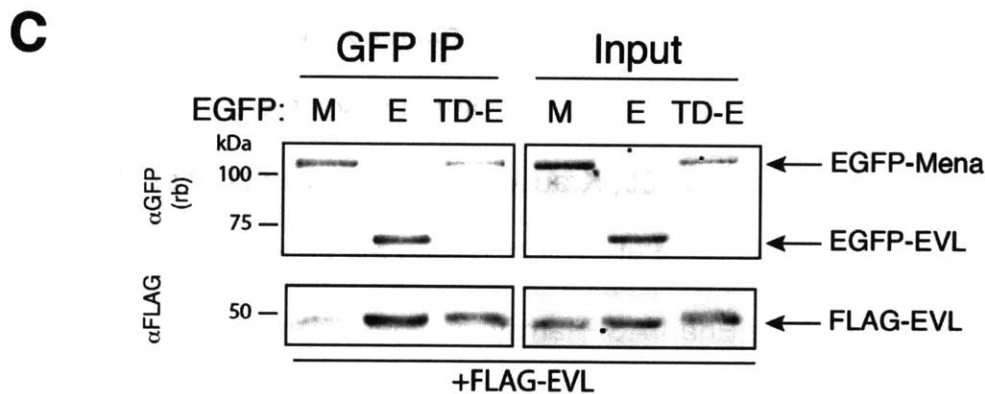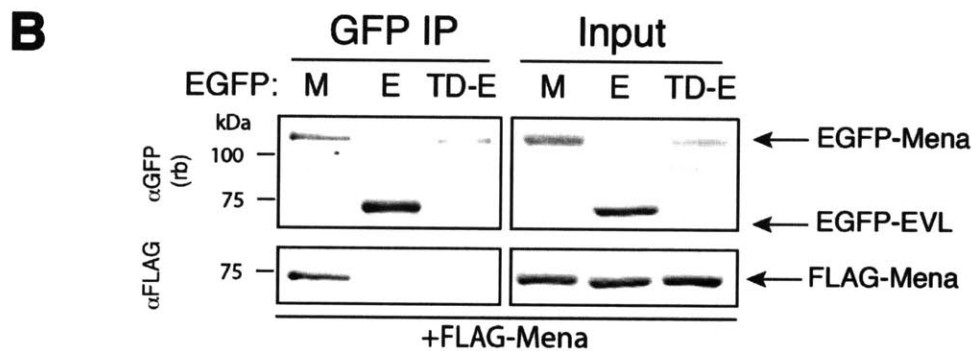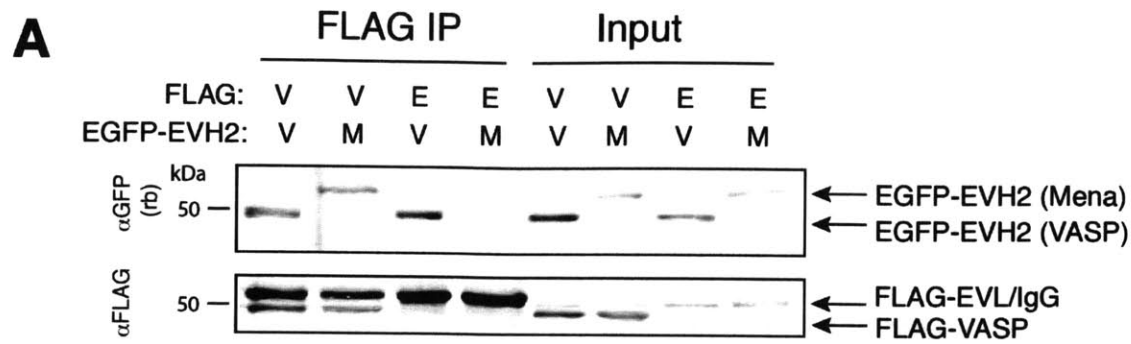supporting inclusion of both Mena and EVL, or (2) assembled oligomers form

Figure 2-9: TD specifies composition of Ena/VASP oligomers.
(**A**) FLAG-tagged proteins were immunoprecipitated from MV^{D7} cells co-transfected with Flag-VASP ("V") or Flag-EVL ("E") and the EGFP-EVH2 domains of VASP ("V") or Mena ("M"). Interactions with EGFP-EVH2 domains were detected using αGFP antibody. FLAG-EVL co-migrates with IgG heavy chain, as indicated. Input is 5% of IP. MV^{D7} fibroblasts were co-transfected with either FLAG-VASP (**B**) or FLAG-EVL (**C**) and either EGFP-Mena ("M"), EGFP-VASP ("V"), or chimeric EGFP-Mena^{TD-EVL} ("TD-E"). EGFP-tagged proteins were immunoprecipitated from lysates and detected with αGFP (rb) antibody and interacting FLAG-tagged proteins were detected with αFLAG-M2 antibody. Input is 5% of IP.

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distinct sets of tetramers consisting only of EVL and VASP, or Mena and VASP, but not all three.

MV^{D7} were co-transfected with EGFP-Mena, EGFP-EVL, and either EGFP or EGFP-VASP. We found that co-expression of EGFP-VASP increased the amount of EVL associated with Mena, compared to EGFP alone (Fig. 2-10). This suggests that Ena/VASP proteins can from oligomers containing all three family members, with VASP acting as a bridge to promote the association of Mena and EVL.

2.3.10 VASP enhances the association of EVL and Mena

The experiments thus far tested the associations of two Ena/VASP proteins expressed simultaneously. However, some cell types express all three Ena/VASP paralogs (Bear et al., 2000; Krause et al., 2003; Lanier et al., 1999; Lebrand et al., 2004; Mouneimne et al., 2012), raising the possibility that tetramers containing all three Ena/VASP family members may form. Given the bias against mixed Mena:EVL oligomers, we wondered whether hetero-tetramers containing all three Ena/VASP family members could form. In cells expressing all three paralogs, we hypothesized two potential scenarios for mixed tetramer formation: (1) oligomers containing all three Ena/VASP proteins form, with VASP supporting inclusion of both Mena and EVL, or (2) assembled oligomers form distinct sets of tetramers consisting only of EVL and VASP, or Mena and VASP, but not all three.

MV^{D7} cells were co-transfected with EGFP-Mena, EGFP-EVL, or EGFP-amount of EVL associated with Mena, compared to EGFP alone (Fig. 2-10). This

suggests that Ena/VASP proteins can form oligomers containing all three family members, with VASP acting as a bridge to promote association of Mena and EVL.
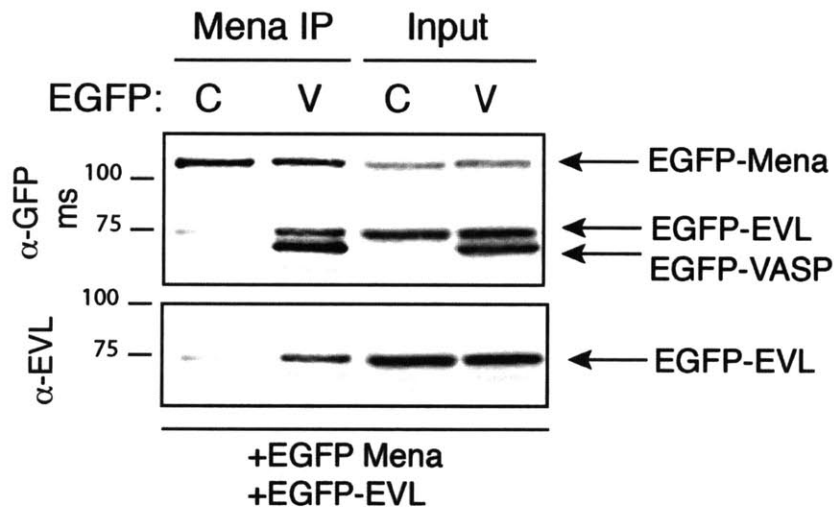

Figure 2-10: VASP enhances Mena:EVL oligomerization.
Mena was immunoprecipitated from MV^{D7} fibroblasts co-transfected with EGFP-Mena, EGFP-EVL, and either control EGFP ("C") or EGFP-VASP ("V"). EGFP-tagged proteins were detected with αGFP antibody and EVL was detected using αEVL polyclonal antibody. Input is 10% of IP.

2.4   Conclusions

In mammals, the Ena/VASP family consists of VASP, Mena, and EVL. VASP was initially discovered as a substrate of cyclic nucleotide-dependent kinases (Halbrügge et al., 1990) while Mena and EVL were identified as orthologs of *Drosophila* Ena (Gertler et al., 1996). Studies have found that the three paralogs can function interchangeably in some, but not all, respects. Mena, VASP, and EVL share substantial sequence similarity and can all rescue the viability of *Drosophila* Ena mutants (Ahern-Djamali et al., 1998) and support propulsion of *Listeria* (Geese, 2002; Laurent et al., 1999). However, these proteins differ biochemically in their effects on actin polymerization. Additional differences in the interacting partners and regulation of Ena/VASP proteins have also been described. Thus, to understand the role of Ena/VASP proteins in cells, it is important to determine how these paralog-specific effects may be combined.

This is the first study to systematically assess hetero-oligomerization of Mena, VASP, and EVL in cells. Prior to this analysis, it was unknown whether Ena/VASP proteins preferentially formed homo- or hetero-tetramers *in vivo*. My data demonstrate that hetero-oligomeric complexes between Ena/VASP family members form in cells and that oligomerization of Mena and VASP is consistent with random tetramerization. While I observed that VASP can self-associate or oligomerize with either Mena or EVL, we found that there is a bias against the formation of mixed oligomers containing only EVL and Mena. However, the presence of VASP can lead to VASP:Mena:EVL tetramers. I also demonstrated that tetramers consisting of multiple Mena isoforms assemble. Thus indicating

that in cells expressing more than one Mena isoform, the activities of the isoforms may be modulated by mixed tetramerization.

Co-IP experiments of EVL with the EVH2 domains of VASP and Mena confirmed that the EVH2 domain mediates multimerization (Bachmann et al., 1999; Zimmermann et al., 2002) and also demonstrated that the EVH2 domain contains sequences that confer selectivity of oligomer formation. I found that substitution of the TD of Mena with the TD of EVL inverted Mena's bias against binding EVL. Chimeric Mena^{TD-EVL} preferentially bound EVL but did not associate with wild-type Mena. Therefore, I conclude that residues within the TDs of Mena, VASP, and EVL specify their interactions to form select mixed oligomers.

2.5 Materials and Methods

Antibodies

The following antibodies were used in this study and diluted 1:5000 for detection in western blots: monoclonal mouse αMena (Lebrand et al., 2004), polyclonal rabbit αMena (Lanier et al., 1999), polyclonal rabbit αVASP (2010) (Lanier et al., 1999), polyclonal rabbit αEVL (1404) (Lambrechts et al., 2000), monoclonal mouse αGFP (JL-8, Clontech), and polyclonal rabbit αGFP (Invitrogen, Cat. A11122). αFLAG (Clone M2, Sigma, Cat. F1804) was used at 1:2000 for western blots. For immunofluorescence, affinity-purified rabbit anti-Lpd (Krause et al., 2004) was used at 1:400 and chicken αGFP (Ames Laboratory) and was used at 1:500 dilution. Mouse αMena antibody was used for immunoprecipitation experiments of Mena and the rabbit αGFP antibody was used for the immunoprecipitation of EGFP-tagged proteins. Control whole molecule mouse and rabbit IgG was purchased from Jackson ImmunoResearch. Secondaries for western blots were purchased from Licor. 647-donkey αrabbit (Jackson ImmunoResearch) and AlexaFluor 488 goat αchicken (Invitrogen) were used as secondaries for immunofluorescence. 647-Phalloidin was purchased from Invitrogen and phalloidin CF405 conjugate was purchased from Biotium.

Plasmids

Sub-cloning and PCR using Phusion polymerase (NEB) were performed using standard techniques. pMSCV EGFP-VASP^{LHCC} was generated by replacing the TD of mouse VASP (residues 337-370) with residues 250-281 of synthetic pLI-GCN4 using PCR and restriction digests (Harbury et al., 1993). The following pCAX vectors were generated by subcloning: EGFP, EGFP-VASP^{WT}, EGFP-VASP^{LHCC}, EGFP-Mena, and EGFP-EVL (Bear et al., 2000). pCAX EGFP-EVH2(VASP) contains residues 221-375 of mouse VASP and pCAX EGFP-EVH2(Mena) contains residues 345-541 of mouse Mena.

pCAX FLAG-EGFP was created by fusing EGFP in-frame into the pCDNA3-FLAG backbone, and then subcloned into pCAX vector to be used as the backbone for FLAG-tagged constructs. The following plasmids were cloned using the Gibson Assembly Master Mix (NEB, E2611): FLAG-VASP^{LHCC}, FLAG-VASP^{WT}, FLAG EVL, and FLAG-Mena. The TD of mouse Mena (residues 501-541) was replaced with the TD of mouse EVL (residues 380-393) to create the chimeric EGFP Mena^{TD-EVL} construct using Gibson assembly. All PCR primers for Gibson cloning were generated using NEBuilder (http://nebuilder.neb.com/) and were purchased from IDT.

Cell culture and transfections

Rat2, MV^{D7}, and derived cell lines were cultured as previously described (Bear et al., 2000). MV^{D7} cells expressing EGFP-VASP^{WT} cells have been described (Loureiro et al., 2002), and MV^{D7} EGFP-VASP^{LHCC} cells were generated using the same methodology. EGFP positive cells were sorted using FACS and were selected for matching EGFP expression levels.

MV[D7] cells were transiently transfected with the indicated plasmids in the pCAX vector using Amaxa nucleofector technology with cell nucleofection solution MEF-2 and program A-23, according to the manufacturer's recommendations (Lonza). Transfected cells were incubated at 32°C for 16-24 hours prior to cells lysis and immunoprecipitation.

Immunoprecipitation

10 cm dishes of cells were lysed in 250 µl IP lysis buffer [10% glycerol, 1% IGEPAL CA-630, 15 mM sodium pyrophosphate, 50mM sodium fluoride, 50mM TRIS (pH 7.5), 40mM beta-glycerophosphate, 200mM sodium chloride, 1mM sodium vanadate, 2mM magnesium chloride, and protease inhibitor tablet without EDTA (Roche)]. Lysates were incubated on ice for 10 minutes, and then centrifuged at 14,000 g for 10 minutes at 4°C. For EGFP, Mena, and VASP IPs, lysates were precleared with Dynabeads Protein G (Life Technologies) for 1 hour, then incubated with indicated antibody for 1 hour at 4°C, and finally captured with BSA-blocked Dynabeads for 1 hour. For FLAG IPs, clarified lysates were incubated with BSA-blocked Anti-DYKDDDK Magnetic Beads (Clontech, Cat. 635695) for 2 hours. Beads were washed three times in lysis buffer, and proteins were eluted in 4X Laemmli sample buffer [3% SDS, 1% Glycerol, 1.5% β-mercaptoethanol, 50mM Tris (pH 6.8), 100mM DTT and 0.1% Bromophenol Blue

Western blot analysis and quantification

Lysates and immunoprecipitated proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with Odyssey Blocking Buffer (LiCor) and probed with the indicated antibodies. Membranes were washed with PBST and membrane-bound proteins were detected by infrared (LiCor) imaging. Images were recorded as TIFF files for quantification. Band intensities were measured using FIJI (Bear et al., 2002; Schindelin et al., 2012). For presentation, original TIFF files were inverted using Adobe Photoshop. Color images were pseudocolored and merged using Adobe Photoshop.

Random Mixing Model

Assuming random mixing of Mena/VASP members within a cell that expresses a particular ratio of two members, the distribution of tetramers should follow a binomial distribution such that:

$$F_k = \binom{4}{k} p^k (1-p)^{4-k}$$

where F_k is the fraction of tetramers with k members of VASP, and p is the fraction of Mena/VASP protein that is VASP. Co-immunoprecipitation experiments were therefore simulated by removal of tetramers lacking Mena, and comparison to the overall population.

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Chapter 3:

Handedness of TD does not influence VASP localization

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Table of Contents

3.1. Abstract

The tetramerization domain of VASP has been crystalized and shown to form an uncommon right-handed coiled-coil. The TD lies adjacent to the F-actin binding portion of Ena/VASP proteins. Actin also forms a right-handed coiled-coil, raising the possibility that the handedness of the TD may facilitate the interaction of Ena/VASP family members with actin filaments. We sought to evaluate the role that handedness plays in the localization of Ena/VASP proteins. Substitution of the TD of VASP with a synthetic tetramerizing sequence that forms a left-handed coiled-coil supports VASP localization to focal adhesions, lammellopodial edges, and to the tips of filopodia. Additionally, VASP's barbed-end dependent localization to cell edges is unaffected by the handedness of the TD. These findings suggest that the handedness of the coiled-coil is not critical for F-actin barbed-end dependent Ena/VASP localization.

3.2   Introduction

At the most C-terminal portion of Ena/VASP protein, a coiled-coil

tetramerization domain (TD) mediates the formation of Ena/VASP tetramers

(Bachmann, et al., 1999; Barzik et al., 2005; Winkelman, et al. 2014;

Zimmermann et al., 2002). Many Ena/VASP functions are critically dependent

upon their ability to tetramerize (Barzik et al., 2005; Loureiro et al., 2002). For

example, deletion of the TD ablates Ena/VASP-dependent filopodia formation

and elongation (Applewhite et al., 2007). In experiments where the TD of VASP

was replaced with a dimerization sequence, artificial VASP dimers were

compromised in their ability to promote actin elongation, supporting a model in

which higher order oligomers are critical for Ena/VASP protein function (Hansen

& Mullins, 2010).

The crystalized TD of human VASP forms an uncommon, right-handed

coiled-coil (Kühnel et al., 2004). The unusual right-handed coil of VASP's TD led

us to question the biological relevance of the direction of the helix. In light of the

fact that actin also forms a right-handed helix and the close proximity of the TD to

the actin binding portions of VASP, I hypothesized that the direction of the helix

may be important in stabilizing the interaction of VASP with actin-barbed ends.

The coiled-coil oligomerization domain of yeast transcription factor GCN4

is a well-studied model of oligomerization (Burkhard et al., 2001; Harbury et al.

1993). The oligomerization sequence of GCN4 has been mutated to form dimers,

trimers, and tetramers (Harbury et al., 1993). The tetrameric version GCN4

(GCN4 p-LI) forms via a left-handed coiled-coil. The synthetic tetramerizing

sequence of GCN4 p-LI has previously been used to form artificial tetramers with the pore protein KcsA (Yuchi et al., 2008) and with p53 (Waterman, et al., 1996). To test the role of handedness, the α-helix of VASP's TD was replaced with the synthetic left-handed coiled-coil tetramerizing sequence of GCN4 p-LI to form VASP^{LHCC} (Fig. 1-2).

We examined the localization of EGFP-VASP^{LHCC} and found that the synthetic tetramer can support subcellular targeting of VASP similar to that of the wild-type TD. Additionally, we measured the dependence of barbed-end interactions for leading edge localization to be grossly unaffected by the altered TD. These findings suggest that the direction of the coil in VASP does not play a major role in stabilizing the interaction of VASP with barbed ends.

3.3   Results

3.3.1   Altered direction of TD does not affect VASP localization

In fibroblasts, wild-type Ena/VASP proteins localize predominantly to the lamellipodial edges, focal adhesions, and to the tips of filopodia. As some aspects of Ena/VASP localization depend upon their interactions with F-actin barbed ends (Bear et al., 2002; Krause et al., 2004; Loureiro et al., 2002; Scott et al., 2006), we evaluated the effect of switching the handedness of VASP's TD in mediating Ena/VASP localization.

Using MV^{D7} cells stably expressing EGFP-VASP^{WT} or EGFP-VASP^{LHCC}, we found that EGFP-VASP^{LHCC} localized to focal adhesions (Fig. 3-1 A) and to the edges of lamellipodia (Fig. 3-1 A, inset), similarly to EGFP-VASP^{WT}. A subpopulation of MV^{D7} cells extend filopodia while spreading on fibronectin (Applewhite et al., 2007). To determine if VASP^{LHCC} localizes to the tips of filopodia, spreading MV^{D7} cells stably expressing either EGFP-VASP^{WT} or EGFP-VASP^{LHCC} were plated on fibronectin-coated coverslips. In cells spreading in the characteristic filopodial mode, both VASP^{WT} and VASP^{LHCC} were concentrated at the tips of filopodial protrusions (Fig. 3-1 B). These observations suggest that reversing the direction of the TD's coiled-coil does not disrupt VASP localization
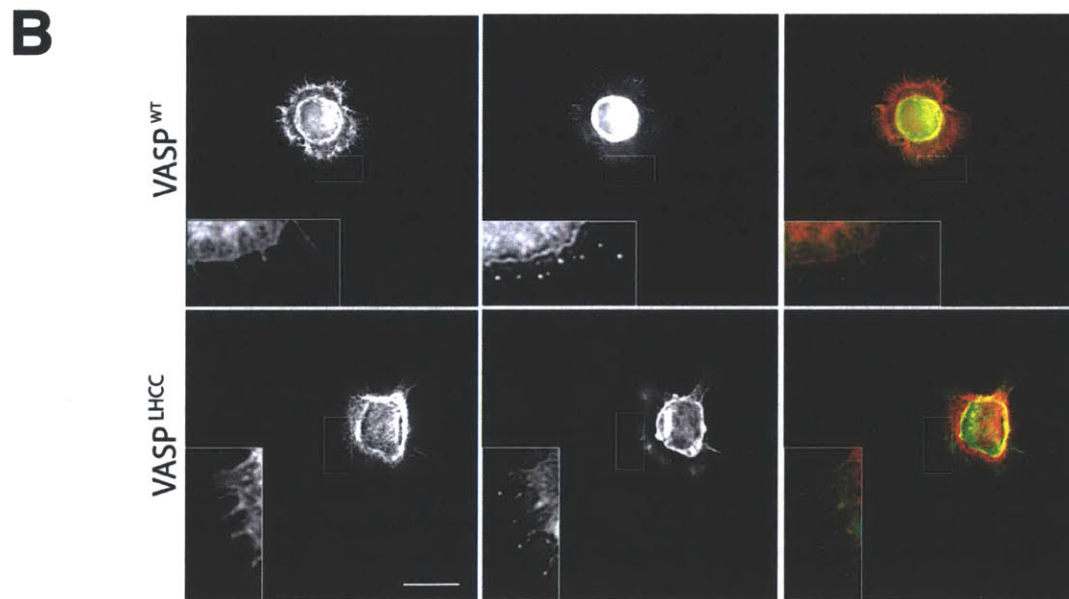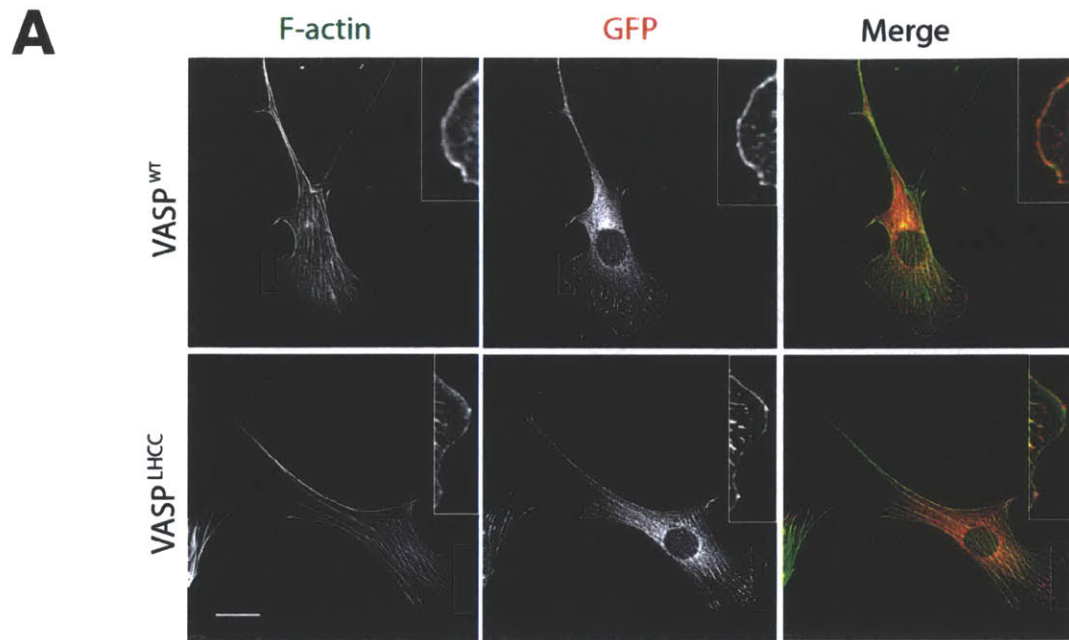
Figure 3-1: VASP^LHCC localization is similar to VASP^WT.
MV^D7 fibroblasts stably expressing EGFP-VASP^WT or EGFP-VASP^LHCC were
fixed and imaged by immunofluorescence. F-actin was visualized with
fluorescent phalloidin. (**A**) EGFP-VASP^WT and EGFP-VASP^LHCC both localize to
focal adhesions and to the leading edge (inset). Scale bar, 20 μm. (**B**) EGFP-
VASP^WT and EGFP-VASP^LHCC both localize to filopodial tips extending from
spreading cells (inset). Scale bar, 20 μm.

3.3.2 Localization dependent on barbed end interactions is not grossly affected by TD replacement

In fibroblasts, accumulation of wild-type Ena/VASP proteins at the leading edge of protruding lamellipodia depends, in part, upon binding to F-actin barbed ends (Bear et al., 2002; Krause et al., 2004; Loureiro et al., 2002; Scott et al., 2006). Treatment of cells with a low dose (25-150nM) of Cytochalasin D (CD) displaces Ena/VASP proteins from the leading edge by binding actin barbed ends, making them unavailable for Ena/VASP proteins (Bear et al., 2002; Krause et al., 2004; Scott et al., 2006). Low-dose CD treatment does not grossly disrupt the actin cytoskeleton (Bear et al., 2002) (Fig. 3-2 A) or displace the lamellipodial protein lamellipodin (Lpd) (Krause et al., 2004).

To test the ability of VASP^{LHCC} to bind free barbed ends, the fluorescence intensity of EGFP-VASP^{LHCC} and VASP^{WT} at protrusions were compared at increasing concentrations (50-100nM) of CD in stably expressing MV^{D7} cells. The fluorescence intensity of VASP at the leading edge of protrusions was measured and normalized to total VASP fluorescence intensity. At each concentration of CD used, both VASP variants were gradually displaced from the cell edge to similar degrees. No statistically significant differences were observed between the localization of VASP^{WT} and VASP^{LHCC} at the leading edge (Fig. 3-2 B). These observations suggest that the handedness of VASP's TD is not crucial for its association with free barbed ends.
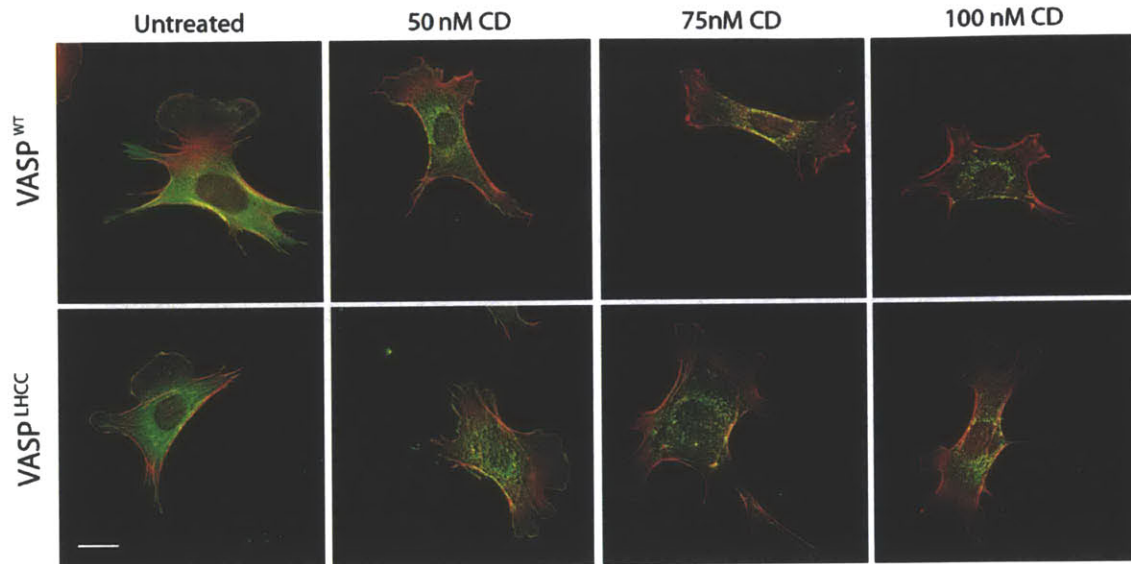
A

| | Untreated | 50 nM CD | 75nM CD | 100 nM CD |

VASP^WT

VASP^LHCC

B

VASP localization at the leading edge

Legend:
- VASP (untr)
- VASP (50nM CD)
- VASP (75 nM CD)
- VASP (100 nM CD)
- LHCC (untr)
- LHCC (50nM CD)
- LHCC (75nM CD)
- LHCC (100nM CD)

Y-axis: Normalized GFP intensity (a.u.)
X-axis: Distance (µm)
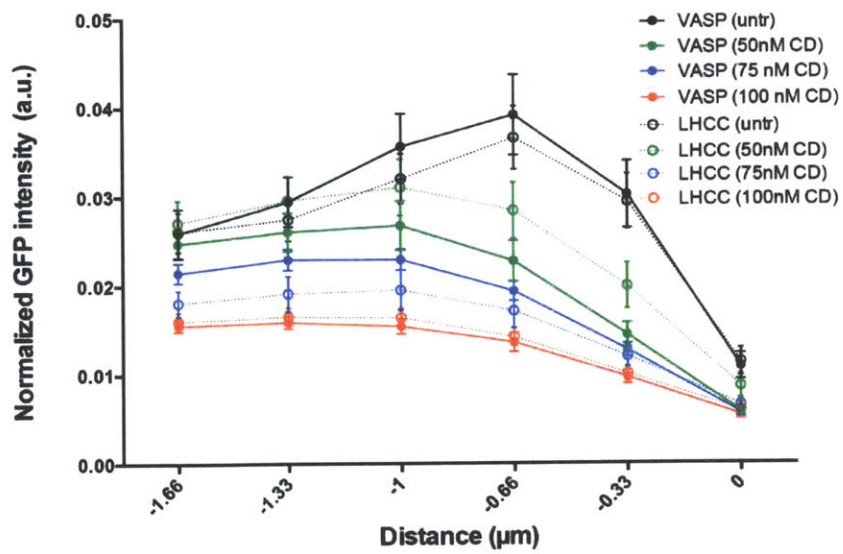
Figure 3-2: Low-dose CD treatment displaces VASPWT and VASPLHCC from cell edge but does not alter cell morphology.

(A) Representative images of MV^{D7} cell stably expressing EGFP-VASP^{WT} or EGFP-VASP^{LHCC}. Merged images of F-actin probed with fluorescent phalloidin (red) and with αGFP antibody to enhance detection of EGFP-VASP (green). Cells were treated for 30 minutes with cytochalasin D (CD) at the indicated concentrations. Scale bar, 20μm. (B) MV^{D7} cells expressing EGFP-VASP^{WT} or EGFP-VASP^{LHCC} plated on fibronectin-coated coverslips were fixed and stained after incubation with the indicated concentration of CD. GFP intensities at cell edges, and at fixed distances away from cell edge, were measured using and ImageJ Plug-in. Measurements were normalized to the total GFP intensity of the cell. The mean (+/- S.E.M.) of the normalized measurements of at least 20 cells were plotted. The edge of the cell is represented by distance 0 on the x-axis and negative values indicate intracellular distances.

3.4 Conclusions

The ability of Ena/VASP proteins to interact with actin filaments depends on sequences within its EVH2 domain. The FAB motif is essential for Ena/VASP binding to actin filaments (Bachmann et al., 1999). The GAB binds actin monomers (Walders-Harbeck et al., 2002) and the presence of actin monomers enhances barbed end binding of Ena/VASP proteins (Hansen & Mullins, 2010). Deletion of the GAB and FAB motifs disrupt the localization of Ena/VASP to the leading edge (Loureiro et al., 2002). The TD is also an essential component of Ena/VASP localization as its deletion disrupts leading edge localization as well (Loureiro et al., 2002). Ena/VASP tetramerization enhances F-actin binding (Bachmann et al., 1999), is required for anti-capping, and was found to be a crucial feature for the function of Ena/VASP proteins in whole cell motility (Loureiro et al., 2002). While the importance of oligomerization has been demonstrated, we sought to determine if the TD has a physical role in positioning Ena/VASP proteins.

My experiments showed that replacement of the right-handed α-helix in VASP's TD with a synthetic left-handed tetramerizing helix did not grossly affect the ability of VASP to localize to the leading edge of lamellipodia or tips of filopodia. Additionally, CD treatment of cells did not indicate that a right-handed helix enhances barbed end dependent localization any more than the synthetic left-handed helix. These findings suggest that the handedness of the coiled-coil is not critical for the barbed-end dependency of Ena/VASP localization. My

results indicated that the direction of the α-helix of VASP's TD is a not a critical

feature of VASP function.

3.5 Materials and Methods

Cell culture
Cell lines derived from MV^{D7} cells were cultured as previously described (Bear et al., 2000). MV^{D7} cells expressing EGFP-VASP^{WT} cells have been described (Loureiro et al., 2002), and MV^{D7} EGFP-VASP^{LHCC} cells were generated using the same methodology. EGFP positive cells were sorted using FACS and were selected for matching EGFP expression levels.

Cell spreading assay and CD treatment
MV^{D7} cells stably expressing EGFP-VASP constructs were trypsinized, resuspended in media, and re-plated on fibronectin-coated coverslips (10 µg/ml). Cells were allowed to spread for 20 min followed by fixation with 4% PFA in PHEM buffer for 15 min at room temperature. Coverslips were then prepared for imaging as above.

Cytochalasin D (CD) (Sigma-Aldrich) was dissolved in DMSO for stock solutions of 5 mM and stored at -20°C. MV^{D7} cells were plated on fibronectin coated (10µg/ml) coverslips for four hours, followed by incubation with media containing diluted CD for 30 minutes, then fixed and prepared for imaging as described above.

Reagents
Sub-cloning and PCR using Phusion polymerase (NEB) were performed using standard techniques. pMSCV EGFP-VASP^{LHCC} was generated by replacing the TD of mouse VASP (residues 337-370) with residues 250-281 of synthetic pLI-GCN4 using PCR and restriction digests (Harbury et al., 1993).

For immunofluorescence, affinity-purified rabbit anti-Lpd (Krause et al., 2004) was used at 1:400 and chicken αGFP (Ames Laboratory) and was used at 1:500 dilutions. Secondaries antibodies used were 647-donkey αrabbit (Jackson Immunoresearch) and AlexaFluor 488 goat αchicken (Invitrogen). 647-Phalloidin was purchased from Invitrogen and phalloidin CF405 conjugate was purchased from Biotium.

Immunofluorescence
MV^{D7} cells stably expressing constructs were plated on acid washed coverslips coated with fibronectin (10 µg/ml). Cells were fixed with 4% paraformaldehyde (PFA) in PHEM buffer [60 mM PIPES (pH 7.0), 25 mM HEPES (pH 7.0), 10 mM EGTA, 2 mM $MgCl_2$, 0.12 M sucrose] for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Coverslips were rinsed with PBS, then incubated with 10% donkey serum for 1 hour, followed by incubation with Alexa 647-Phalloidin (Invitrogen), diluted (1:400) in 1% donkey serum. Coverslips were washed three times with PBS and mounted on glass slides using Fluoroshield with DABCO (Sigma).

Imaging

Cells were imaged on a DeltaVision microscope (Applied Precision) using either a 60× 1.3 NA Plan-Apochromat or a 40x 1.3NA UPlan-FL N objective lens (Olympus). Z stacks were acquired with a CoolSNAP HQ camera (Photometrics) and SoftWoRx acquisition software (Applied Precision). Images were deconvolved using Deltavision SoftWoRx software and objective specific point spread function. Projections of three z-stacks were made using FIJI (Schindelin et al., 2012).

Quantification of VASP leading edge localization

Masks of the cell area were created by thresholding phalloidin images and total GFP intensity was determined by measuring the intensity of the appropriate channel in the masked area. The ImageJ Edge ratio plugin (Cai, Makhov, & Bear, 2007) (http://www.unc.edu/~cail/code/EdgeRatio.txt) was used to quantify the GFP intensity at fixed distances from the cell edge of Lpd-positive regions. The value of the intensity was normalized to the total cell intensity to compensate for variations in GFP expression. The values of the normalized intensity were plotted with error bar representing S.E.M. The edge of the cell is represented by distance 0 on the x-axis and negative values indicate intracellular distances.

Statistical Analysis of VASP at cell edge

Two-way ANOVA was performed with Tukey Multiple Comparison post test using GraphPad Prism version 6.04 for Mac, GraphPad Software (La Jolla, CA). The mean values +/- S.E.M. are plotted.

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Chapter 4:

Conclusions and Future Directions

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4.1. The TD of Ena/VASP family members mediates the selective formation of mixed Ena/VASP tetramers

It had been postulated that Ena/VASP family members assemble randomly into oligomers (Ahern-Djamali et al., 1998; Gertler & Condeelis, 2011). My data demonstrate that hetero-oligomeric complexes between Ena/VASP family members can form within cells and that VASP oligomerization with Mena is consistent with random tetramerization. However, there is a bias against the formation of Mena:EVL hetero-tetramers. Additionally, we demonstrated that the TD of Ena/VASP proteins govern this selectivity. Scaffolding via actin or other binding partners does not play a dominant role in Ena/VASP oligomerization. This is supported by the results observed when the right-handed TD in VASP was substituted with a left-handed TD. This allowed for homo-oligomerization, but did not support complex formation between VASP^{LHCC} and VASP^{WT}.

Although I mapped the role of selectivity to the TD, future studies are necessary to shed light on the critical residues involved in specifying the composition of hetero-tetramers. Despite the high degree of similarity between the TDs of Ena/VASP proteins (Bachmann et al., 1999; Chereau & Dominguez, 2006; Gertler et al., 1996) (Fig. 1-2), potential side-chain interactions between residues of Mena and EVL may inhibit their association. Currently, only the TD of VASP homo-tetramers has been crystalized (Kühnel et al., 2004). Structural and mutational analysis of the TDs of Mena and EVL homo-tetramers and of Ena/VASP hetero-tetramers are necessary to determine which residues may inhibit the association between the TDs of Mena and EVL.

To confirm that VASP can form tetramers in a random fashion with Mena in cells expressing endogenous Mena and VASP, size exclusion chromatography should be performed. The various compositions that Mena:VASP tetramers can be distinguished by changes in the molecular weight of the tetramers. Fractions collected can be compared to standards of known molecular weights and the composition of tetramers in each fraction identified by size. If tetramerization were in fact random, we would expect to find homo-tetramers and all the various hetero-tetramers (1:3, 2:2, and 3:1 ratios of Mena:VASP) in accordance with a random probability distribution.

4.2. Potential effects of Ena/VASP hetero-tetramerization on actin dynamics

Tetramerization is critical to Ena/VASP-mediated elongation of actin filaments (Barzik et al., 2005; Breitsprecher et al., 2008; Hansen & Mullins, 2010). Ena/VASP tetramers are processively attached to the barbed ends of actin filaments (Barzik et al., 2005; Hansen & Mullins, 2010), mediated by the FAB motif within the EVH2 domain (Ferron et al., 2007; Winkelman et al., 2014). The PRR region binds profilin-actin in a ternary complex and presents them to the GAB, which then transfers the actin monomer to the growing filament (Chereau & Dominguez, 2006; Ferron et al., 2007).

Recent data determined that changes in the relative levels of profilin paralogs influences the architecture of the actin cytoskeleton and alters the behavior of both normal and cancer cells (Mouneimne et al., 2012; Rotty et al.,

2015). There are multiple profilin isoforms in mammalian cells and differences in the function and biochemical properties have been observed between isoforms (Mouneimne et al., 2012; Witke, 2004). Previously, it was shown that profilin enhanced Ena/VASP mediated actin polymerization rates (Barzik et al., 2005; Winkelman et al., 2014). VASP and EVL have an enhanced affinity for profilin-2 over profilin-1, whereas Mena shows no preference (Lambrechts et al., 2000; Mouneimne et al., 2012). Additional differences between Ena/VASP members can be found within the PRR, which consists of profilin recruiting regions as well as profilin "loading sites", which have a stronger capacity to bind profilins (Ferron et al., 2007). Mena possesses four G-actin:profilin "loading sites", whereas EVL and VASP each possess only one (Ferron et al., 2007; Gertler & Condeelis, 2011). It has been shown that EVL increases actin elongation to a rate of ~20 subunits/second only in the presence of profilin-2 while VASP increases actin polymerization to ~30 subunits/second with either profilin-1 or 2 (Hansen & Mullins, 2010; Mouneimne et al., 2012). Actin elongation rates correlate with the affinity of the GAB domain for actin monomers, which vary among Ena/VASP orthologs (Breitsprecher et al., 2011), and the GAB domain also preferentially binds profilin-actin complexes over free actin monomers (Chereau & Dominguez, 2006). Tetramer mixing, therefore, may fine-tune the activity of Ena/VASP molecules by adjusting interactions with profilin and actin.

In a recent study (Winkelman et al., 2014), *Drosophila* Ena was shown to elongate actin filaments in a "tunable" manner to promote the formation of filopodia. It was also shown that a single Ena tetramer could simultaneously

elongate two filaments, although at a slower rate than when bound to the barbed end of a single filament. In the context of our results, these findings suggest that the kinetics of filopodia formation and dynamics could be influenced by the composition of Ena/VASP hetero-tetramers.

4.3. Implications of mixed tetramer formation on the regulation of Ena/VASP function

Although Mena, VASP, and EVL possess some overlapping functions, differences in their regulation and protein-protein interactions have been demonstrated. For example, VASP possesses multiple phosphorylation sites regulating its localization and activity that are absent in other family members. Additionally, Mena contains a unique sequence, termed the LERER region (Gertler et al., 1996), which mediates its interaction with $\alpha5$ integrin (Gupton et al., 2012). The interaction of Mena and $\alpha5$ integrin influences the formation of fibrillar adhesions and the organization of extracellular matrix through the formation of fibronectin fibrils (Gupton et al., 2012). Mixed Mena:VASP tetramers have the potential to both bind $\alpha5$ integrin and have their activity and localization regulated by phosphorylation (described in detail below).

VASP activity can be regulated by phosphorylation of residues that are not found in Mena or EVL. For instance, VASP phosphorylated Threonine 278 alters F-actin accumulation in cells (Benz et al., 2009), while Abl phosphorylation of VASP at Tyrosine 39, another phosphorylation site absent in Mena and EVL, diminishes its localization at focal adhesions (Maruoka et al., 2012). These

additional sites allow for unique modes of regulation not found in other Ena/VASP family members. However, how phosphorylation of VASP in mixed tetramers potentially alters the function and localization of these molecular complexes has not yet been evaluated.

Additional complexities in tetramer formation arise when one considers alternatively spliced isoforms of Ena/VASP proteins. EVL possesses a single alternatively included exon to produce the EVL-I isoform (Lambrechts et al., 2000) and alternative splicing of Mena produces five distinct isoforms (Di Modugno et al., 2007; Gertler et al., 1996). It has been demonstrated that alternative splicing of Mena alters the behavior of cells. For example, cells expressing Mena^{INV} possess an enhanced sensitivity to low concentrations of EGF relative to cells expressing Mena^{classsic} (Philippar et al., 2008). Another Mena isoform, Mena11a, has been shown to positively correlate with epithelial-like phenotypes (Di Modugno et al., 2006; Pino et al., 2008) and negatively correlate with invasiveness (Goswami, 2004; Roussos et al., 2011). How the function of Mena^{INV}:Mena^{classic} or Mena11a:Mena^{classic} hetero-tetramers compare to Mena^{INV} or Mena11a homo-tetramers has not yet been evaluated.

Intriguingly, the role of Ena/VASP proteins in cancer progression and/or prognosis is not the same for all family members. For instance, increased expression of Mena is positively correlated with increased invasiveness of breast cancer cells and breast cancer grade (Philippar et al., 2008), while EVL expression is negatively correlated with invasiveness and grade (Mouneimne et al., 2012). Our findings, in combination with the preferences of different

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Ena/VASP proteins for different profilin paralogs (Ferron et al., 2007; Lambrechts et al., 2000; Mouneimne et al., 2012), suggest that distinct pools of Mena and EVL tetramers can exist within a single cell, and may therefore function independently of one another.

4.4. Synthetic tetramerization domain allows for analysis of Ena/VASP homo-tetramers

Most studies examining Ena/VASP proteins have focused on one family member at a time. However, studies performed in cells expressing endogenous Ena/VASP proteins are complicated by the potential formation of mixed oligomers, which may modify the activities of the protein under investigation. Replacement of the native TD with a synthetic TD enables formation of pure oligomers containing only exogenously expressed Ena/VASP proteins. Thus, VASP^{LHCC} may provide a method to study the function of Ena/VASP homo-tetramers without concern for hetero-tetramerization with endogenous proteins.

While I found that the handedness of the TD is not critical for its localization, experiments comparing the function of VASP^{LHCC} to VASP^{WT}, both at a cellular and molecular level, should be performed to definitively conclude that the handedness of the TD is not important for VASP function. The EVH2 domain is sufficient to support its lamellipodial localization and to recapitulate the function of full length Ena/VASP proteins in whole cell motility assays, while Mena lacking the TD is not (Loureiro et al., 2002). As it appears that the TD plays a critical role in the function of Ena/VASP proteins during whole cell translocation, whole cell

motility assays should be performed to test if the ability of the EVH2 domain containing a left-handed coiled coil is comparable to wild-type EVH2 domains.

Single molecule experiments using purified VASP^{WT} and VASP^{LHCC} should be performed to evaluate the strength of each version of VASP to associate with F-actin barbed ends (Hansen & Mullins, 2010). The affinity of the VASP^{LHCC} for barbed ends can be measured and compared to that of VASP^{WT}. The rate of actin elongation and dwell-time on barbed ends (Winkelman et al., 2014) should also be compared. If no significant differences between the two forms of VASP can be detected, it would suggest that the sole function of the TD of Ena/VASP proteins is to mediate the selective formation of Ena/VASP homo- and hetero-tetramers.

Ena/VASP proteins are convergence points for regulation of actin polymerization by upstream signaling pathways (Gertler & Condeelis, 2011). Our findings strongly suggest that mixed tetramers provide another layer of regulation to this landscape. By blending or segregating Ena/VASP family members, the control of tetramer composition provides a mechanism to fine-tune actin polymerization in response to diverse signaling events.

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Appendix A:

Mena is phosphorylated at Ser125 upon activation of the p38(MAPK)/MK2 pathway

Table of contents

A.1. Abstract

In a large number of quantitative phosphoproteomics studies, Mena was found to be phosphorylated in a variety of cell types and contexts at an uncharacterized phosphorylation site. Here, I confirm that Mena is phosphorylated at Ser125 through the use of a customized, phospho-specific antibody. I demonstrate that Mena(Ser125) phosphorylation is induced upon treatment of cells with doxorubicin and upon stimulation with TNFα. Additionally, I show that phosphorylation of Mena is dependent on the p38MAPK/MK2 signaling pathway and provide evidence suggesting that Mena is a direct target of MK2.

A.2.   Introduction

Mena contains multiple residues that have been demonstrated to be targets of phosphorylation (Krause et al., 2003). Mena contains two sites targeted by the cyclic nucleotide-dependent proteins kinases PKA and PKG (Benz et al., 2009) and Abl phosphorylates Mena at tyrosine 296 (Tani et al., 2003). In a large number of high-throughput phosphorylation studies performed in a variety of cell types and conditions, a novel and uncharacterized serine residue has been identified as phosphorylated. This residue, S125, is located between the EVH1 domain and the LERER region and is immediately downstream of the INV inclusion site. Its location suggests a potential role influencing EVH1 or LERER mediated interactions.

Mena is subject to the inclusion of alternative exons, two of which lead to the insertion of an additional four (++) or 19 amino acids (+++/INV) between the EVH1 domain and LERER region (Gertler et al., 1996). The effect of ++ inclusion has not been determined, but inclusion of the INV exon to produce Mena^{INV} has been shown to sensitizes cell to low levels of EGF (Philippar et al., 2008). Phosphorylation at S125 (S144 in Mena^{INV}) may alter Mena function differentially in the context of INV inclusion. Thus, I sought to investigate the role of Mena phosphorylation at S125 on the activity of Mena in cells.

Coincidentally, Mena(S125) was identified as a substrate of the DNA damage responsive kinase, MK2, in a screen conducted by Dr. Mun Kyung Hwang, a postdoctoral fellow in the Yaffe Lab (M. Yaffe, personal communication). MK2 activation is triggered by doxorubicin, a topoisomerase II

poison and commonly used chemotherapeutic agent. In addition to DNA

damage, MK2 is activated in response to activation of the p38 MAPK pathway by

cytokine stimulation or stresses such as hypoxia or heat-shock (Manke et al.,

2005; Rousseau et al., 2000; Roux & Blenis, 2004). Activated/phosphorylated

p38 MAPK phosphorylates and activates MK2, which is then able to

phosphorylate its targets.

MK2 has been shown to function in the regulation of cytokine biosynthesis

and cell migration by influencing post-transcriptional gene regulation (Kotlyarov &

Gaestel, 2002). MK2 has previously been shown to have effects on actin

dynamics by phosphorylating (and thus modulating the activity) of actin

regulatory proteins, such as LIMK and HSP25/27 (Guay et al., 1997; Kobayashi

et al., 2006). Additionally, it is essential for cell migration *in vitro* as MK2 deficient

cells have defects in migration (Kotlyarov et al., 2002). We hypothesized that

phosphorylation of Mena by MK2 may be an additional mechanism by which

MK2 can regulate cytoskeletal dynamics.

I confirmed that MK2 can phosphorylate Mena and developed a phospho-

specific antibody to study Mena phosphorylation. In addition to doxorubicin

treatment, I found that Mena is phosphorylated upon TNFα stimulation and that

under these conditions is also dependent on activation of the p38/MAPK

pathway.

A.3. Results

A.3.1. Generation of phosphorylation state sensitive antibody to detect Mena(S125) phosphorylation

To aid in the study of S125 phosphorylation, we developed antibody to detect phosphorylation of Mena at this site (α-pS125). To formally test the specificity of the purified antibody, Mena was immunoprecipitated from MV^{D7} cells stably expressing EGFP-tagged wild-type or Mena containing a mutation replacing serine at position 125 with alanine (EGFP-Mena^{WT} or EGFP-Mena^{S125A}, respectively). To induce phosphorylation of Mena, immunoprecipitated proteins were incubated with constitutively activated, purified recombinant MK2. α-pS125 antibody detected Mena only after incubation with MK2, while an αMena antibody detected both EGFP-Mena^{WT} or EGFP-Mena^{S125A} (Fig. A-1). This experiment both confirmed the specificity of the α-pS125 antibody and confirmed that MK2 can lead to S125 phosphorylation. Additionally, it shows that under steady state conditions, Mena phosphorylation at S125 is not readily detectable.
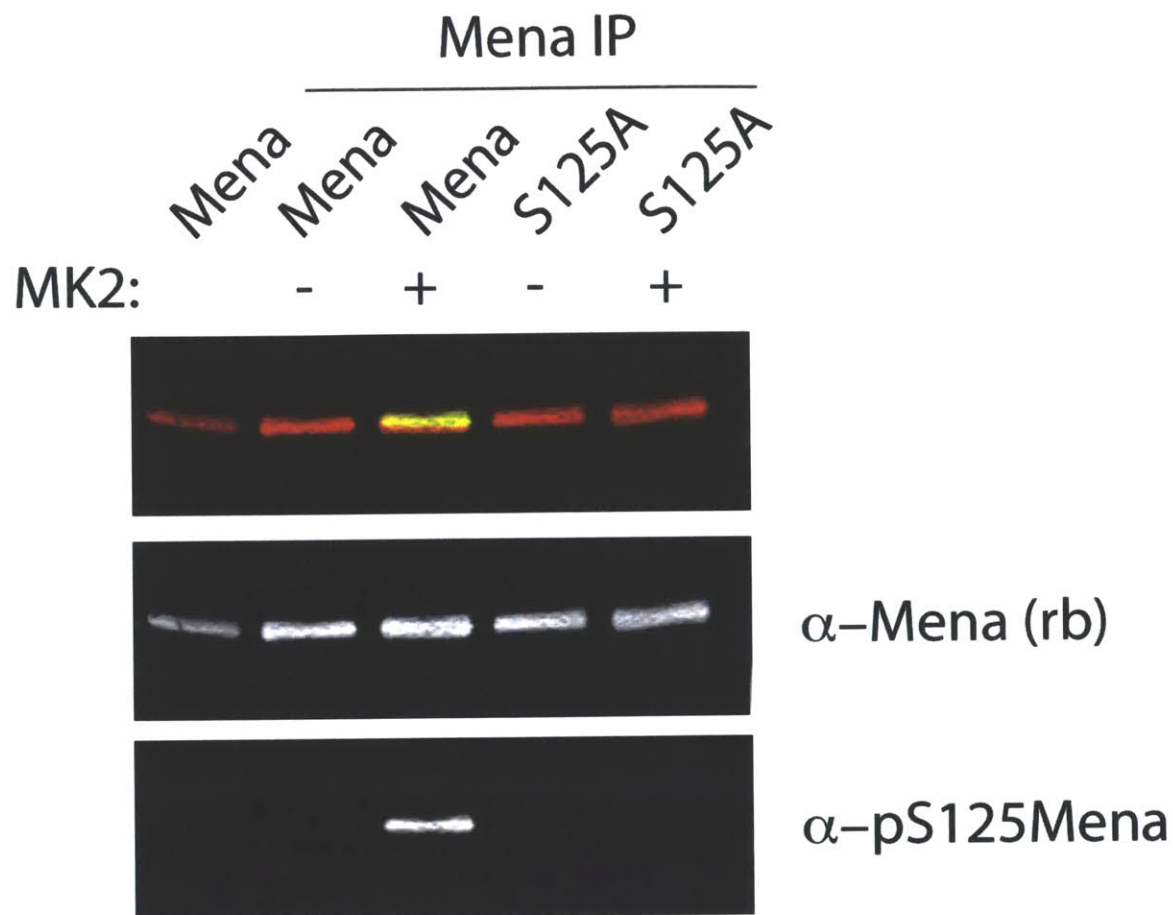
Figure A-1: Ser125 phospho-specific antibody recognizes Mena incubated with MK2.
Mena was immunoprecipitated from MV^{D7} cells expressing EGFP-Mena^{WT} ("Mena") or EGFP-Mena^{S125A} ("S125A"). Isolated protein was split into two tubes and one set was incubated with recombinant, purified constitutively-active MK2. Western blot was probed with αMena (rb) polyclonal antibody and α-pS125 Mena antibody.

A.3.2. Mena is phosphorylated in cells treated with doxorubicin

Mass spectrometry experiments indicated that Mena is phosphorylated at S125 in the U2OS (human osteosarcoma) cell line following high-dose and prolonged doxorubicin treatment (M. Yaffe, personal communication). To confirm these results, I generated U2OS cells expressing EGFP-Mena^{WT} and EGFP-Mena^{S125A}. I treated wild-type U2OS and derived U2OS cells with 5μM doxorubicin for twelve hours, the same conditions used for the mass spectrometry experiments. I evaluated the phosphorylation status of Mena(S125) in lysates by western blot. In all treated conditions, the α-pS125 antibody detected increased phosphorylation of endogenous Mena and EGFP-Mena^{WT} upon doxorubicin treatment (Fig. A-2).

In U2OS cells expressing a hairpin against MK2 that effectively reduces its expression, doxorubicin treatment failed to induce phosphorylation of Mena at S125 (M. Yaffe, personal communication). These experiments demonstrate that MK2 is required for phosphorylation of Mena at S125.
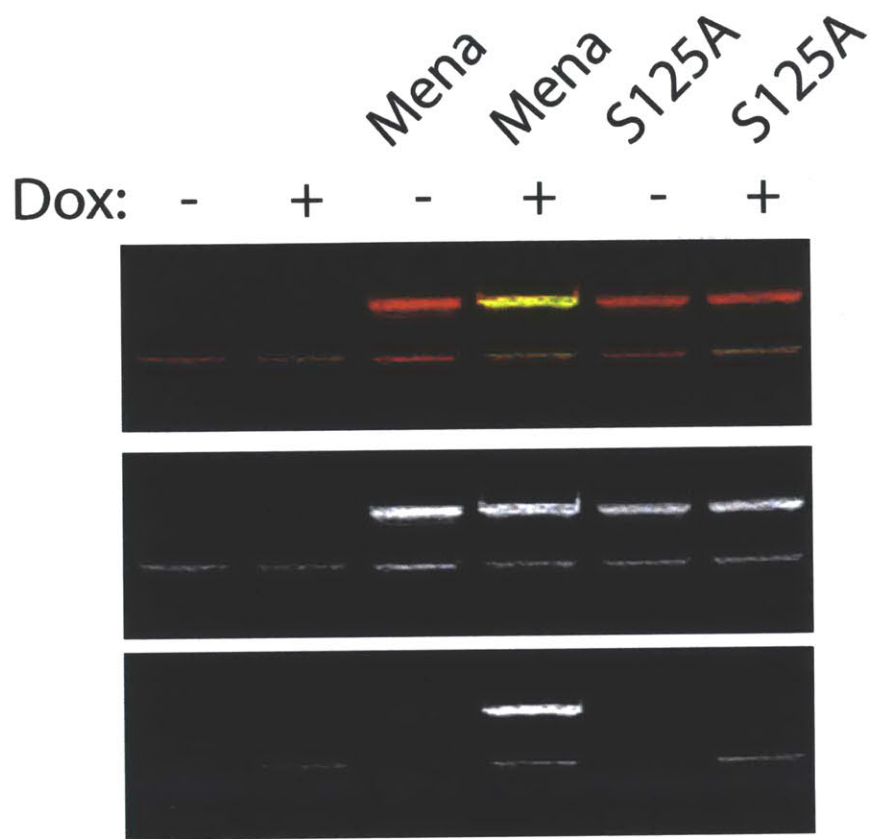
Figure A-2. S125 of Mena is phosphorylated upon doxorubicin treatment.
Wild-type U2OS cells and U2OS cells stably expressing EGFP-Mena^{WT} ("Mena")
or EGFP-Mena^{S125A} ("S125A") were plated in duplicate. One dish of each were
treated with 5µM doxorubicin ("Dox") for 12 hours. Cells were lysed and analyzed
by SDS-PAGE. Phosphorylation of endogenous Mena and EGFP-Mena^{WT} was
detected with α-pS125 antibody in treated conditions.

A.3.3. TNFα treatment leads to short-term phosphorylation of Mena

The MK2/p38 pathway can be activated following stress or stimulation by a variety of signaling molecules. TNFα signaling activates p38α/β isoforms (Cargnello & Roux, 2011) and MK2 (Chang et al., 2011). It has also been shown to have effects on the actin cytoskeleton (Mathew et al., 2009). To determine if Mena phosphorylation at S125 can be induced under physiological conditions, I evaluated the phosphorylation status of Mena upon activation of p38 and MK2 in response to TNFα. In U2OS cells, and in MV[D7] cells expressing EGFP-Mena, TNFα induced phosphorylation of S125 in Mena. However, phosphorylation peaked at approximately twenty minutes post-treatment in both cell types and was no longer detectable after one hour of TNFα treatment (Fig. A-3). MK2 phosphorylation, indicative of activation, can also be detected upon TNFα stimulation. This is similar to the short-term phosphorylation of HSP27 observed in endothelial cells in response to TNFα treatment (Chang et al., 2011).
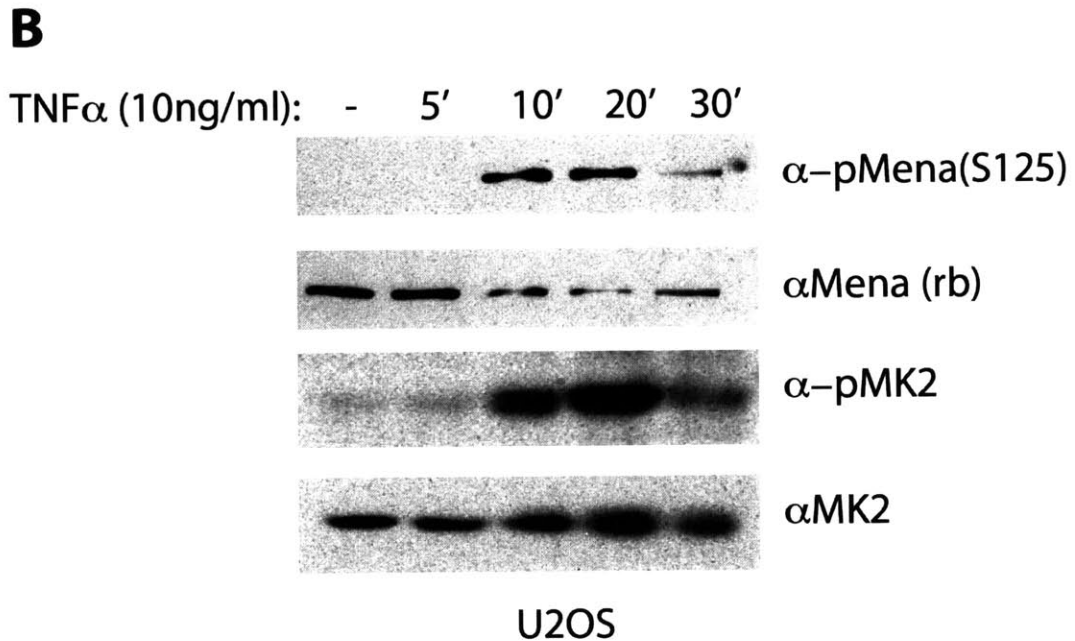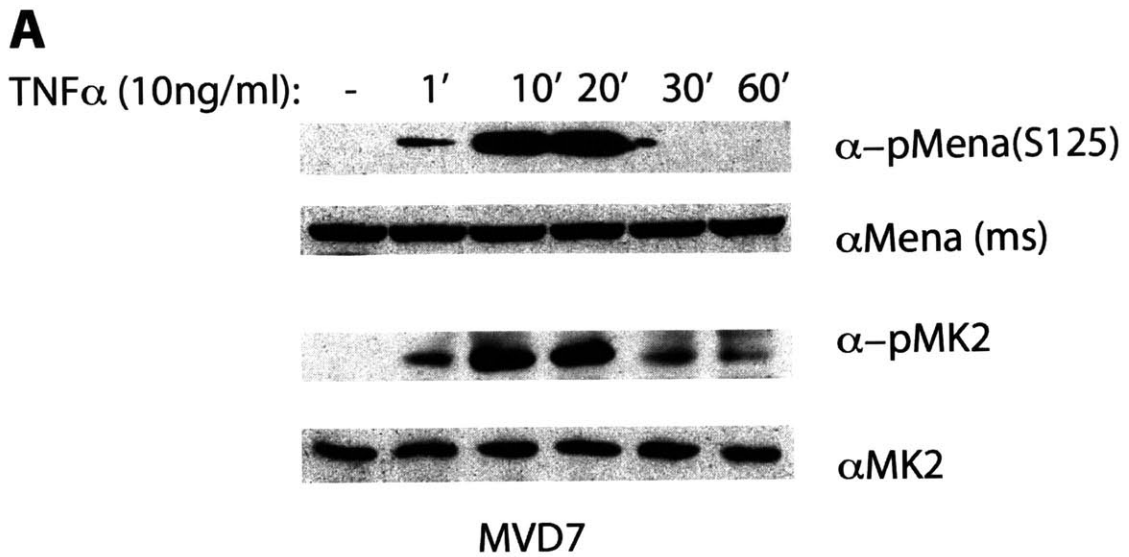
A

TNFα (10ng/ml):   -   1'   10' 20'   30' 60'

α–pMena(S125)

αMena (ms)

α–pMK2

αMK2

MVD7

B

TNFα (10ng/ml):   -   5'   10'   20'   30'

α–pMena(S125)

αMena (rb)

α–pMK2

αMK2

U2OS

Figure A-3. S125 of Mena is phosphorylated upon TNFα stimulation
MV^{D7} cells expressing EGFP-Mena^{WT} (**A**) and wild-type U2OS cells (**B**) were treated with 10ng/ml TNFα for the indicated times (minutes). Cells were lysed and analyzed by SDS-PAGE in duplicate to detect phosphorylated and total protein levels. Blots were probed with indicated antibodies. Phosphorylation of MK2 correlates with Mena phosphorylation.

A.3.4. TNFα phosphorylation of Mena is dependent upon the p38(MAPK) signaling pathway

TNFα signaling activates a number of different kinases and has demonstrated roles in apoptosis, inflammation, and endothelial permeability (Mathew et al., 2009). As it remains possible that kinases outside of the p38α signaling pathway may also phosphorylate Mena, I sought to confirm that TNFα-induced phosphorylation of Mena(S125) is dependent upon p38 signaling. The inhibitor, SB20385, binds to p38 and prevents the kinase from phosphorylating downstream targets. In cells pre-incubated with SB20385 and then stimulated with TNFα, p38 is phosphorylated, but its target MK2 is not (Fig. A-4). Mena phosphorylation is not observed in the presence of the p38 inhibitor, indicating that Mena phosphorylation upon TNFα treatment is dependent on the p38 signaling pathways.
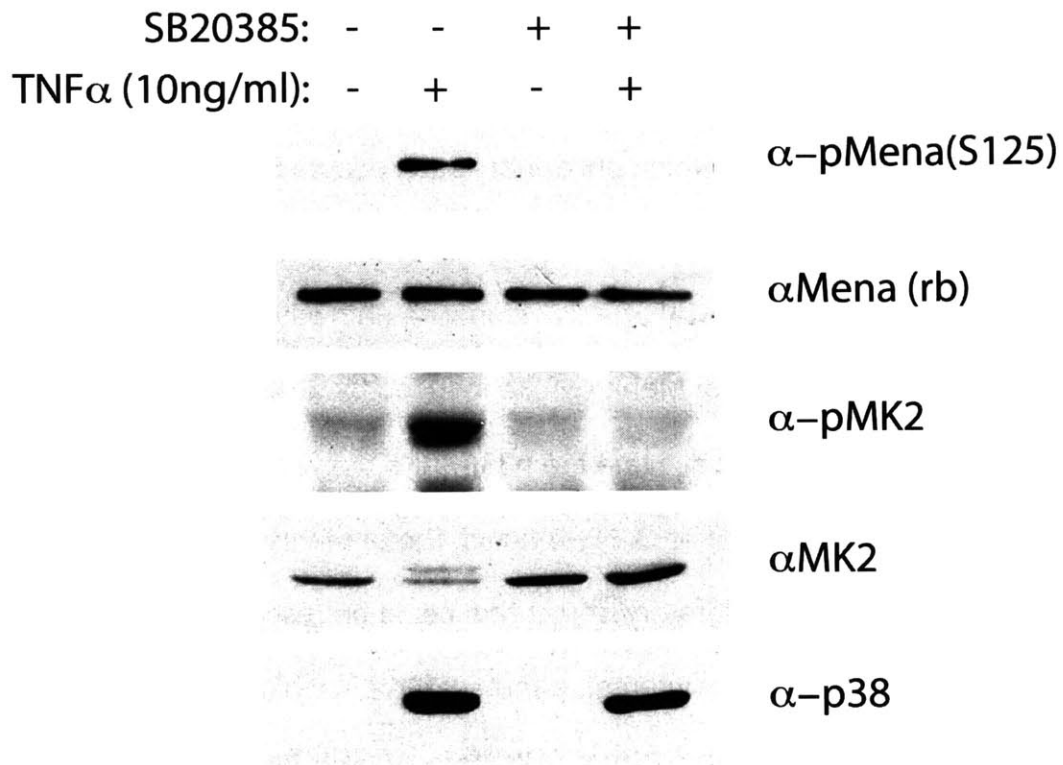
Figure A-4: TNFα induced phosphorylation of S125 is dependent on signaling downstream of p38.
U2OS cells were incubated with DMEM +/- 10μM SB203580, an inhibitor of p38 signaling, for one hour. Cells were treated with 10ng/ml TNFα for 15 minutes prior to lysis and analyzed by SDS-PAGE. Western blots were probed with the indicated antibodies and demonstrated that TNFα induced phosphorylation of MK2, but pre-treatment with the p38 inhibitor blocked phosphorylation, and thus activation, of MK2. Mena phosphorylation is also blocked upon inhibition of the p38 pathway.

A.4. Conclusions and Future Directions

A.4.1. Mena is phosphorylated at S125 upon MK2 activation

Phosphorylation of Mena at S125 had been detected by mass spectrometry, but conditions by which phosphorylation occurs had not been elucidated until now. Mena is likely directly phosphorylated by MK2 under conditions that activate the p38(MAPK)/MK2 signaling pathway. Here, I have confirmed that phosphorylation of Mena can be induced by doxorubicin and TNFα treatment, conditions that activate the p38(MAPK)/MK2 pathway.

MK2 contains a FP4 motif at its N-terminus. Experiments performed using MK2 deficient MEFs found a migratory defect that could be rescued upon re-introduction of MK2. Of particular interest, a mutant MK2 in which the FP4 sequence (residues 13-17) is altered from FPSPP to AASPP fails rescue the migratory defect (Kotlyarov et al., 2002). The presence of the FP4 sequence suggests a potential direct interaction with members of the Ena/VASP family (Niebuhr et al., 1997; Renfranz & Beckerle, 2002). The inability of MK2 with a mutated FP4 motif to rescue the migratory defect of MK2 deficient cells also suggests that the potential interaction between Mena and MK2 is critical for MK2's role in cell migration. In future studies, it should be determined if Mena and MK2 interact directly the incubating purified MK2 with recombinant Mena fragments. Additional binding assays with the EVH1 domains of VASP and EVL should be performed to determine if all Ena/VASP family members can interact with MK2, or if it is specific to Mena. While S125 site is not conserved in VASP or

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EVL, interactions with Ena/VASP proteins may be a general mechanism to target MK2 to potential sites of action.

A.4.2. Function of Mena phosphorylation remains unknown

The p38(MAPK) pathway is considered to be a general stress signaling pathway (Reinhardt & Yaffe, 2009) and can be activated by environmental stresses and inflammatory cytokines (Roux & Blenis, 2004). An important role for MK2 has been established in the DNA damage response, especially in cells deficient in p53 (Reinhardt et al., 2007). A role for Mena in the DNA damage response has not been observed, but the finding that Mena can be phosphorylated after doxorubicin treatment may suggest Mena is involved in helping cells survive chemotherapy. However, how phosphorylation of Mena at S125 alters its function has not yet been determined.

In MK2-deficient macrophages, filopodia formation in response to a variety of extracellular stimuli is reduced (Kotlyarov et al., 2002). Mena localizes to the tips of filopodia and is phosphorylated by MK2. As it is demonstrated that Ena/VASP proteins are involved in filopodia formation (Applewhite et al., 2007; Barzik, McClain, Gupton, & Gertler, 2014), it would be of interest to determine if filopodia induction upon various cytokines is dependent upon MK2 phosphorylation of Mena. Using stimuli that activate filopodia formation and MK2 signaling, the number and lengths of filopodia formed after stimulation in cells expressing EGFP-Mena^{WT} or EGFP-Mena^{S125A} should be compared.

The localization of Mena upon MK2 activation should also be observed. If localization of EGFP-Mena^{WT} is altered under conditions of p38 activation, the

dependence of localization changes on S125 phosphorylation should also be evaluated. In addition, the importance of Mena in MK2 deficient cells can be evaluated by using shRNAs to knockdown Mena. If re-introduction of MK2 does not rescue the migratory defect, it would support the hypothesis that Mena is critical for MK2 dependent migration of MEFs.

A.5. Materials and Methods

Antibody purification
Custom anti-sera was produced in guinea pigs were injected with peptide containing mouse Mena residues 117-133 phosphorylated at S125 and coupled to conjugated to KLH [Ac-CKKGPTLPRQN(pS)QLPAQVQN] and was purchased from Covance. Purification of pS125Mena antibody was performed according to protocol from (Archuleta, Stutzke, Nixon, & Browning, 2011). Peptides used for purification (phosphorylated peptide: Ac-CKKGPTLPRQN(pS)QLPAQVQN; de-phosphorylated: CKKGPTLPRQNSQLPAQVAN) were prepared by Covance and ThermoScientific, respectively. Peptides were coupled to SulfoLink Coupling Resin Column (Thermo Scientific) according to manufacturer's instructions. Sera was sequentially passed over column containing de-phosphorylated peptide, and then over the column containing phosphorylated peptide. Antibodies bound to second column were eluted using IgG Elution buffer (ThermoScientific).

Generation of S125A point mutant
pMSCV-EGFP MenaS125A was generated using QuickChange II Site-Directed Mutagenesis Kit (Stratagene) to mutate the sequence TCA in Mena that coded for a Serine at the amino acid position 125, to GCA resulting in expression of Alanine.

Cell culture, drug treatment, and TNFα stimulation
MV^{D7}, and derived cell lines, were cultured in DMEM with 15%FBS, 1% penicillin/streptomycin and L-Glutamine, and 50 U/ml of mouse interferon-gamma (Gibco) at 32°C in 5% CO_2, as previously described (Bear et al., 2000). MV^{D7} cells expressing EGFP-Mena cells have been described (Loureiro et al., 2002), and MV^{D7} EGFP-Mena^{S125A} cells were generated using retrovirus made in HEK 293 cells with pMSCV-EGFP Mena^{WT} or pMSCV-EGFP Mena^{S125A}. EGFP positive cells were sorted using FACS and were selected for matching EGFP expression levels.

U2OS cells were originally purchased from ATCC by M. Yaffe. Cells were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin and L-Glutamine at 37°C in 5% CO_2. EGFP-Mena^{WT} and EGFP-Mena^{S125A} overexpressing cells were generated as above.

Doxorubicin was purchased from Sigma and SB203580 were purchased from Calbiochem by M. Yaffe. TNFα was purchased from PeproTech.

Immunoprecipitation
10cm dishes of cells were lysed in 120 μl lysis buffer [1% Triton-X, 20mM Tris-HCl (pH 7.5), 150mM sodium chloride, 1mM EDTA, 1mM EGTA, and PhosStop and protease inhibitor tablet without EDTA (Roche)]. Lysates were incubated on ice for 15 minutes, and then centrifuged at 14,000 g for 10 minutes at 4°C. For Mena IPs, lysates were precleared with protein A+ beads (ThermoScientific) for 2

hours, then incubated with Mena monoclonal antibody for 2 hours at 4°C, and finally captured with BSA-blocked protein A+ beads for 2 hours. Beads were washed three times in lysis buffer, and proteins were eluted in 4X Laemmli sample buffer [3% SDS, 1% Glycerol, 1.5% β-mercaptoethanol, 50mM Tris (pH 6.8), 100mM DTT and 0.1% Bromophenol Blue].

Kinase assay

MV^{D7} cells expressing EGFP-Mena^{WT} or EGFP-Mena^{S125A} were lysed in 500 μl IP lysis buffer [10% glycerol, 1% IGEPAL CA-630, 15 mM sodium pyrophosphate, 50mM sodium fluoride, 50mM TRIS (pH 7.5), 40mM beta-glycerophosphate, 200mM sodium chloride, 1mM sodium vanadate, 2mM magnesium chloride, and protease inhibitor tablet without EDTA (Roche)]. Lysates were incubated on ice for 15 minutes, passed through a 23g needle five times, and then centrifuged for centrifuged at 14,000 g for 15 minutes at 4°C. Mena^{WT} and Mena^{S125A} were immunoprecipitated with αGFP antibody (rabbit polyclonal; Invitrogen) for 2 hours, rotating at 4°C, and then captured with BSA-blocked protein A+ beads for 2 hours.

Beads were washed tree times with Kinase Wash Buffer [20mM Tris-Hcl (pH 7.5), 15 mM $MgCl_2$, 5mM β-glycerophosphate, 1mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM DTT] and then two times with Kinase Reaction Buffer [50mM Tris-Hcl (pH 7.5), 150mM NaCl, 10 mM $MgCl_2$, 0.2mM ATP, 1 mM DTT] and . Beads were resuspended in 50μl Kinase Reaction buffer and incubated with 200ng purified MK2 (diluted in 20mM HEPES, 200mM NaCl, 2mM DTT) at 30°C for 40 minutes. Reaction was stopped by the addition of 25μl 4X Laemmli sample buffer. Purified MK2 was provided by M. Hwang of the Yaffe lab.

Western blot and antibodies

Lysates and immunoprecipitated proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad) for detection with Licor Odyssey or onto PVDF membranes (Bio-Rad) for detections by chemiluminescence.

Licor immunoblots: membranes were blocked with Odyssey Blocking Buffer (LiCor) and probed with the indicated antibodies for one hour. Membranes were washed with PBST and membrane-bound proteins were detected by infrared (LiCor) imaging. Images were recorded as TIFF files for quantification. Band intensities were measured using FIJI (Schindelin et al., 2012).

Chemiluminescence: Membranes were blocked in 5% milk in TBST and incubated with the indicated antibodies overnight at 4°C. Membranes were then washed in TBST and subsequently probed with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) diluted at 1:5000 and visualized by enhanced chemiluminescence (Roche).

The following antibodies were used for detection of western blots: α-p38α/β (A-12) from SantaCruz Biotechnology. αphospho-p38MAPK, αMK2, and αphospho-MK2 antibodies was purchased from Cell Signaling Technology.

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Appendix B:

Mena binds α5 integrin directly and modulates α5β1 function

Table of Contents

B.1. Abstract

Mena is an Ena/VASP family actin regulator with roles in cell migration, chemotaxis, cell–cell adhesion, tumor cell invasion, and metastasis. Although enriched in focal adhesions, Mena has no established function within these structures. We find that Mena forms an adhesion-regulated complex with $\alpha5\beta1$ integrin, a fibronectin receptor involved in cell adhesion, motility, fibronectin fibrillogenesis, signaling, and growth factor receptor trafficking. Mena bound directly to the carboxy-terminal portion of the $\alpha5$ cytoplasmic tail via a 91-residue region containing 13 five-residue "LERER" repeats. In fibroblasts, the Mena–$\alpha5$ complex was required for "outside-in" $\alpha5\beta1$ functions, including normal phosphorylation of FAK and paxillin and formation of fibrillar adhesions. It also supported fibrillogenesis and cell spreading and controlled cell migration speed. Thus, fibroblasts require Mena for multiple $\alpha5\beta1$-dependent processes involving bidirectional interactions between the extracellular matrix and cytoplasmic focal adhesion proteins.

B.2. Introduction

Cell migration and differentiation are among many processes controlled by the extracellular matrix (ECM) as it engages adhesion receptors. The ECM protein fibronectin (FN) is a ubiquitous component of the interstitial matrix (Singh et al., 2010). Outside the bloodstream, cells typically assemble soluble FN dimers into complex meshworks of fibrils (Schwarzbauer & DeSimone, 2011), which provide a supporting scaffold to deliver multivalent, spatially organized biochemical and mechanical signals that influence cell behavior (Hynes, 2009; Huttenlocher & Horwitz, 2011; Geiger & Yamada, 2011).

The predominant ECM receptors are integrins, a family of heterodimeric transmembrane proteins composed of α and β subunits that link the ECM to the cytoskeleton and transmit signals and mechanical forces bidirectionally across the plasma membrane (Hynes, 2002). Integrins are regulated by clustering and conformational changes triggered either by binding to ECM ligands or by interaction between the intracellular tails of integrin subunits and cytoplasmic proteins (Margadant et al., 2011).

$\alpha V\beta 3$ and $\alpha 5\beta 1$ are the two major FN receptors (Hynes, 2002). $\alpha 5\beta 1$ is the primary receptor for soluble FN and has a key role in assembling FN into fibrils, though $\alpha V\beta 3$ can assemble fibrils in cells that lack $\alpha 5\beta 1$ (Yang et al., 1999). Typically, however, the two receptors exert distinct effects on cell motility, invasion, signaling, and matrix remodeling (Clark et al., 2005; Caswell et al., 2008, 2009; Wickström et al., 2011).

Integrin-based ECM adhesions are complex structures that turn over

continually and change their composition and morphology (Geiger & Yamada, 2011). New adhesions form as small integrin-rich punctae at the cell periphery; associated cytoplasmic proteins bound to integrin tails recruit additional signaling, adaptor, or actin-binding proteins (Vicente-Manzanares & Horwitz, 2011). Nascent adhesions enlarge into transient focal complexes (FXs) that mature into focal adhesions (FAs), elongated structures of variable size and composition that are connected to the distal ends of F-actin bundles. In some cell types, including fibroblasts, α5β1 exits FAs, moves toward the cell interior along stress fibers (Pankov et al., 2000), and forms stable fibrillar adhesions (FBs) that mediate FN fibrillogenesis. FBs are enriched for FN, α5β1, and tensin (Pankov et al., 2000; Zamir et al., 2000; Zaidel-Bar et al., 2003). Tensin is absent from FXs and is found only weakly in FAs (Zaidel-Bar et al., 2003). FBs lack components found in FAs, including phosphotyrosine (pY)-containing proteins, vinculin, FAK, and zyxin. α5β1 drives fibrillogenesis by translocating bound FN out of FAs to FBs: the movement generates contractile forces on the α5β1 connection between the cytoskeleton and FN, causing conformational changes in both α5β1 and FN; these changes strengthen and prolong binding (Margadant et al., 2011) and expose self-association sites that align nascent FN fibrils with intracellular actin bundles (Schwarzbauer & DeSimone, 2011).

Ena/vasodilator-stimulated phosphoprotein (VASP) actin-regulatory proteins have diverse roles in cell movement and morphogenesis (Drees & Gertler, 2008; Bear & Gertler, 2009; Homem & Peifer, 2009): they promote formation of longer, less-branched F-actin networks and increase F-actin

elongation rates by transferring actin monomer from profilin to free barbed ends while protecting growing filaments from capping proteins that terminate polymerization (Bear & Gertler, 2009; Dominguez, 2009; Hansen & Mullins, 2010). Ena/VASP proteins are concentrated at the tips of lamellipodia and filopodia (sites of rapid actin assembly), and localize prominently to cell–cell and cell–matrix adhesions; they interact with several FA components, including vinculin, zyxin, Rap1-GTP–interacting adaptor molecule (RIAM), and palladin (Pula & Krause, 2008). The function of Ena/VASP in FAs is not well understood, but they regulate integrin activation. For example, VASP negatively regulates $\alpha IIb\beta 3$ activation (Aszódi et al., 1999; Hauser et al., 1999).

The three vertebrate Ena/VASP proteins Mena, VASP, and EVL share conserved domains that consists of the EVH1 and EVH2 domains as well as the proline rich region (PRR) (Gertler et al., 1996) (described in detail in Chapter 1). Each of the three proteins can support many Ena/VASP-dependent cellular functions such as filopodial protrusion (Applewhite et al., 2007; Dent et al., 2007), formation of functional endothelial barriers (Furman et al., 2007), or stimulation of actin-based motility of the intracellular pathogen *Listeria monocytogenes* (Geese, 2002). However, Mena contains the "LERER repeat," a unique region of unknown function, with 13 repeats of a 5-residue motif within a 91-residue span between the EVH1 domain and proline-rich core (Gertler et al., 1996).

We found that the LERER repeat interacts with the cytoplasmic tail of $\alpha 5$ integrin, and mediates a robust adhesion-modulated interaction between Mena and $\alpha 5\beta 1$ that contributes to key $\alpha 5\beta 1$ functions: FN fibrillogenesis, cell

spreading, motility, and activation of adhesion-dependent signaling. We conclude that Mena is involved in both inside-out and outside-in signaling through $\alpha5\beta1$.

Here, I will describe my contributions to this manuscript and emphasize aspects of the story for which I provided data or replicated results. However, I am only including figures that I can claim responsibility for the production of the raw data.

B.3. Results

B.3.1. Relocalization of Mena to mitochondria recruits α5, but not other focal adhesion proteins

While investigating Ena/VASP- and integrin-mediated neuritogenesis (Gupton & Gertler, 2010), it was observed that artificially relocalized Ena/VASP protein influenced α5β1 subcellular distribution. We depleted Ena/VASP from their normal locations and sequestered them on the mitochondrial surface by expressing a construct containing multiple EVH1-binding sites fused to a mitochondrial-targeting motif (FP4-Mito; Bear et al., 2000). FP4-Mito expression phenocopies defects that arise from loss of Ena/VASP function in fibroblasts, endothelial cells, neurons, and in *Drosophila melanogaster*, where transgenic expression of FP4-Mito phenocopies axon guidance and epithelial defects observed in Ena mutants (Bear et al., 2002; Dent et al., 2007; Furman et al., 2007; Gates et al., 2007).

Despite redistribution of Ena/VASP proteins to the mitochondrial surface by FP4-Mito, localization of known Ena/VASP-binding partners such as the FA proteins zyxin and vinculin is unaffected, and no defects are evident when FP4-Mito is expressed in Ena/VASP-deficient cells (Bear et al., 2000). However, in cells expressing Mena, transfection of EGFP-tagged FP4 construct resulted in the redistribution of α5 integrin to mitochondrial surfaces in a Mena dependent manner.

To determine if Ena/VASP could recruit other integrins or FA components to the mitochondrial surface, we used immunostaining of cells expressing FP4-

Mito: Mena redistributed to the mitochondrial surface, as was a fraction of the $\beta 1$ integrin pool (likely by association with $\alpha 5$); however, we saw no significant relocalization of αv- and $\alpha 6$-integrins and zyxin (Fig. B-1). Therefore, Ena/VASP-dependent $\alpha 5\beta 1$ recruitment to mitochondria via FP4-Mito is specific and does not affect other integrins or FA proteins tested.

It is possible that such recruitment to mitochondria occurs by capture of $\alpha 5\beta 1$-containing vesicles by Ena/VASP, in which case the cytoplasmic tails of $\alpha 5\beta 1$ may remain accessible to bind the mitochondrial-tethered Ena/VASP proteins directly or indirectly. To determine whether such vesicle capture occurs during a particular stage of trafficking, FP4-Mito–expressing cells were immunostained for markers of vesicle populations involved in $\alpha 5\beta 1$ trafficking pathways (Caswell et al., 2009; Margadant et al., 2011): EEA1, an early endosomal marker; Rab7, for vesicles containing activated $\beta 1$ integrins (Arjonen et al., 2012); and Rab11, which decorates $\alpha 5\beta 1$-containing vesicles as they pass through the perinuclear recycling compartment (Margadant et al., 2011). None of the markers were enriched on the $\alpha 5\beta 1$-coated mitochondria of FP4-Mito expressing cells (Fig. B-2).
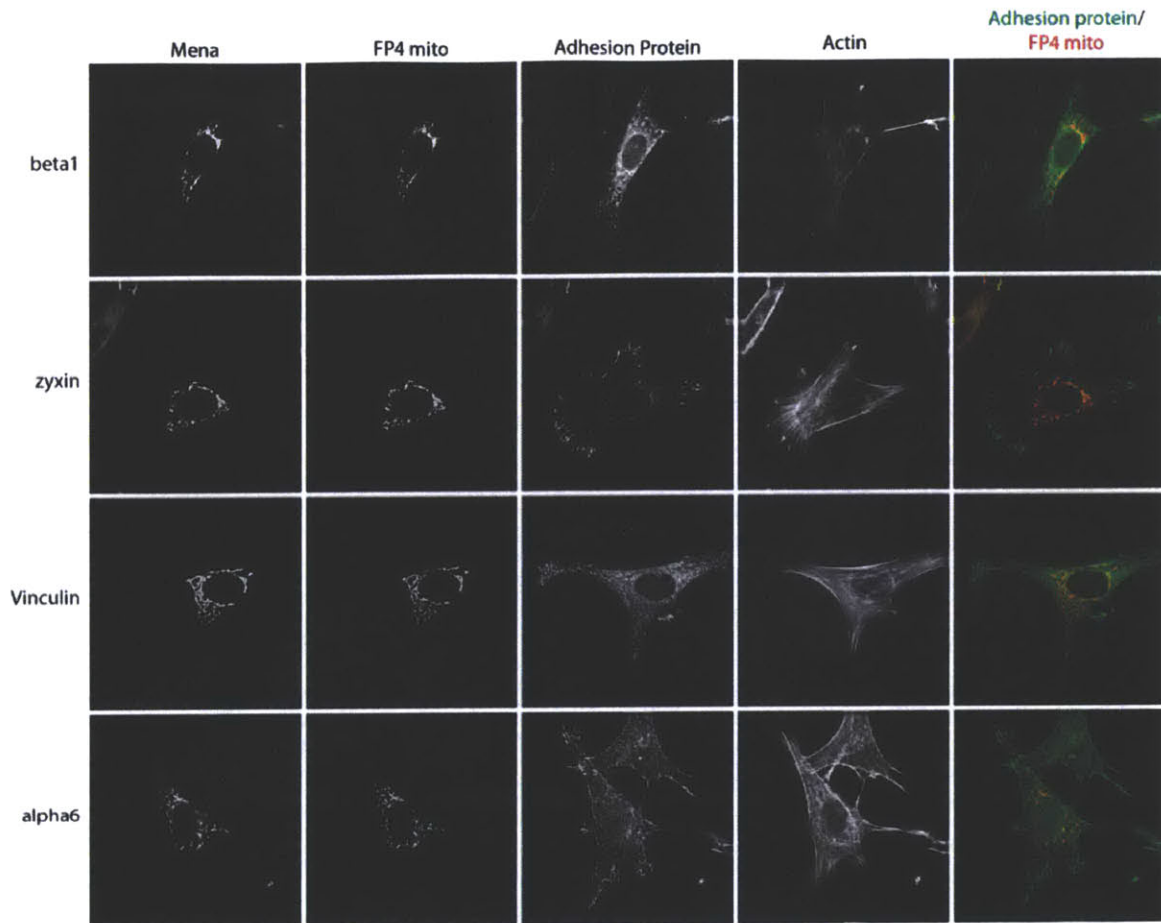
Figure B-1: FP4-Mito does not recruit other adhesion proteins to the mitochondrial surface.
MV^{D7} cells expressing EGFP-Mena were transiently transfected with mCherry-FP4-Mito (red) and stained for indicated adhesion component (green).
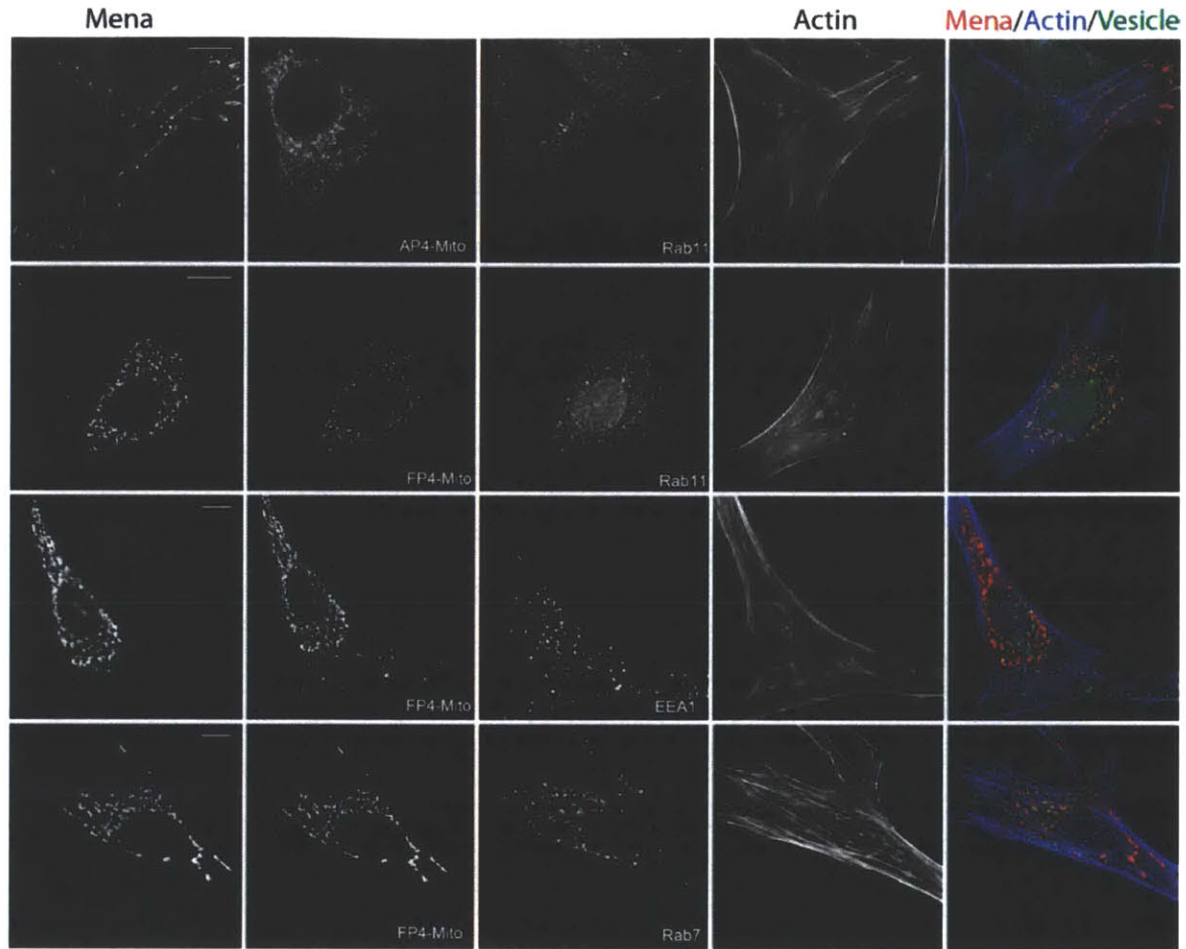
Figure B-2: Recruitment of Mena to mitochondria does not result in co-localization with vesicles.
MV^{D7} cells expressing EGFP-Mena were transfected with mCherry-FP4Mito and stained for EEA1 (early endosomal marker; Pearson's colocalization coefficient between Mena and EE1A = 0.069), Rab7 (late endosomal marker, Pearson's colocalization coefficient between Mena and Rab7 = 0.082), or Rab11 (perinuclear, recycling endosomal marker, Pearson's colocalization coefficient between Mena and Rab11 =0.32). Higher levels of colocalization between Mena and Rab11 are likely because of colocalization of Rab11 with mitochondria, even in parental MV^{D7} cells lacking Mena (not depicted; Pearson colocalization coefficient = 0.48). Scale bar, 15 μm.

B.3.2.   The LERER repeats mediate the Mena:α5 interaction

Using MV^{D7} cells expressing a series of characterized Mena deletion mutants (Loureiro et al., 2002), we transfected FP4-Mito to determine which region of Mena mediates the interaction with α5 integrin. In cells expressing a Mena mutant lacking the LERER repeat, the distribution of α5 in cells expressing FP4-mito, indicating that the interaction with α5 requires the LERER repeat. In western blot analysis of α5 immunoprecipitates from cells expressing full length EGFP-Mena or MenaΔLERER, only EGFP Mena was found in complex with α5 integrin. This led us to conclude that the LERER repeat is necessary for complex formation between Mena and α5 integrin.

B.3.3.   Mena's LERER repeat modulates subcellular distribution of α5

Mena and α5β1 levels vary dynamically within cell–matrix adhesions as they mature during cell spreading and migration (Zaidel-Bar et al., 2003). We explored whether the Mena:α5 interaction influences the distribution of either molecule to the different types of adhesions. In fibroblasts cultured on FN, α5β1 is in nascent FXs, FAs, and FBs. In MV^{D7} cells that express EGFP-Mena, Mena, α5, and paxillin co-localized extensively in peripheral FAs, whereas the cell center displayed robust α5 signal (typical of FBs), but little, if any, EGFP-Mena. When endogenous Mena was localized by immunofluorescence in fibroblasts transiently transfected with EGFP-tensin (a major component of FBs; Zamir et al., 2000), we found only weak overlap of Mena with tensin in central FBs (Fig. B-3).
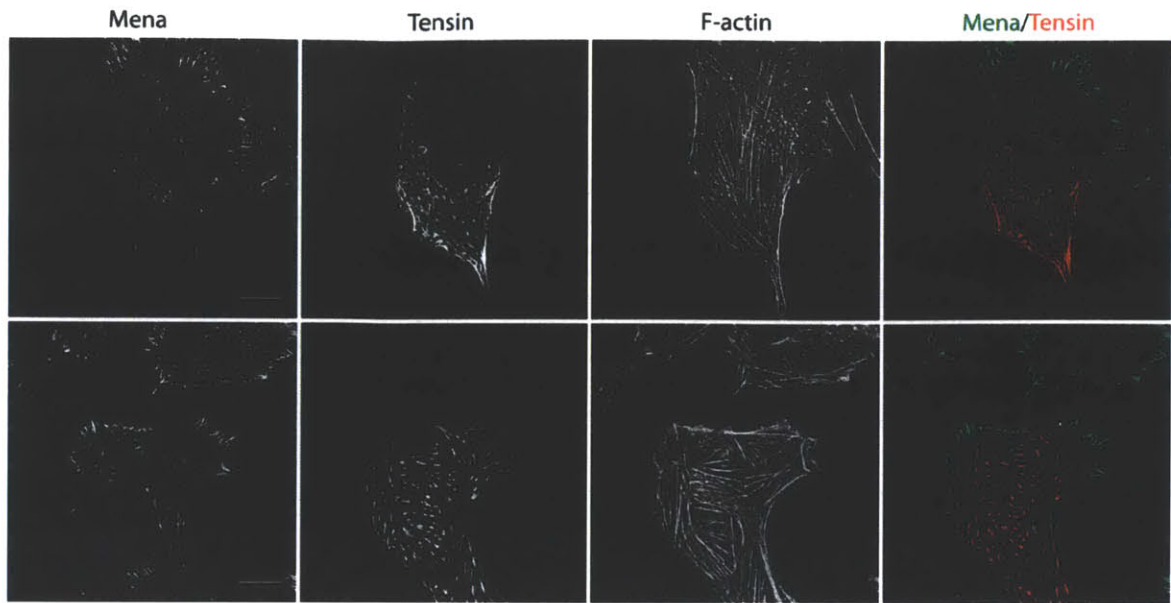
Figure B-3: Mena is excluded from fibrillar adhesions.
Rat2 fibroblasts were transfected with EGFP-tensin, a marker of fibrillar adhesions, and stained for Mena. Bar, 15 μm.

Parental MV^{D7} cells contained peripheral FAs with α5 and paxillin, but lacked prominent FB-like α5 signal. Similarly, MV^{D7} cells expressing EGFP-MenaΔLERER contained α5, paxillin, and EGFP-MenaΔLERER within peripheral FAs, but lacked α5-positive FBs in the cell center. However, in cells expressing EGFP-Mena, there was an increase in the area of α5-positive adhesions that was almost double relative to that of paxillin. To determine if the increase in α5-positive adhesions was a result of altered α5 distribution or an increase in α5 expression, I analyzed the surface levels of α5 using FACS. I found that α5 surface levels were similar in adherent cells of all cell lines (Fig. B-4).
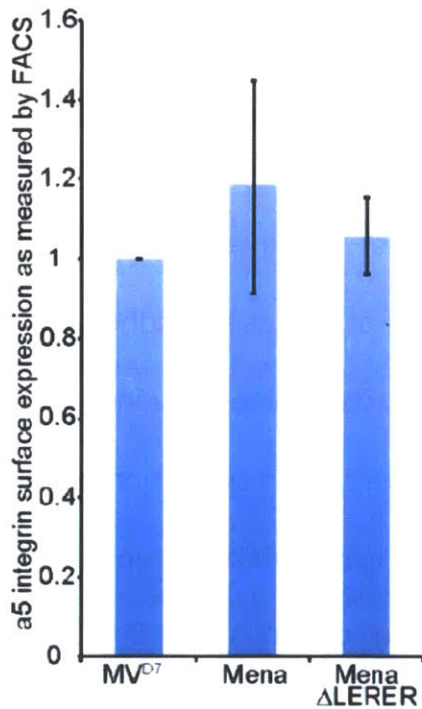
Figure B-4: FACS analysis of α5 surface levels in MV^{D7} cells used.

Surface α5 levels in MV^{D7} cells and MV^{D7} cells expressing EGFP-Mena and EGFP-MenaΔLERER. Cells were incubated with antibody to detect surface-exposed α5 by FACs analysis. Expression levels were normalized to fluorescence of EGFP-expressing MV^{D7} cells, and averaged over three experiments. Error bars indicate mean ± SEM. P < 0.05.

B.3.4. The Mena:α5 interaction enhances the efficiency of cell spreading

We used established assays for α5β1 function in fibroblasts to test the hypothesis that adhesion-driven dynamics of the Mena:α5 complex have functional consequences. Fibroblast spreading on FN initiates the binding of integrins to FN, and rapid formation of actin polymerization–driven, adhesion-independent membrane extensions, followed by a distinct phase during which adhesions form dynamically and provide the traction required for further spreading (Zhang et al., 2008). We examined cell spreading on FN by measuring the area of MV^{D7}, MV^{D7}+EGFP-Mena, or MV^{D7}+EGFP-MenaΔLERER cells 30 min after plating on FN-coated coverslips. MV^{D7} cells expressing EGFP-Mena were significantly more spread compared with both MV^{D7} cells and MV^{D7}+EGFP-MenaΔLERER cells, which spread equivalently. Therefore, adhesion-induced increases in the α5:Mena complex correlate with increased spreading on FN, supporting the possibility that direct interaction between α5 and Mena is required for optimal cell spreading.

B.3.5. Mena concentrates α5 and increases signaling within FAs

To determine whether Mena affects the amount of α5 within adhesions and signaling downstream of α5β1, we used immunofluorescence to measure the amount of α5, FAK phosphorylated at tyrosine 397 (pFAK397), paxillin phosphorylated at residue 118 (pPAX118), and global tyrosine phosphorylation (pY) specifically in Mena or MenaΔLERER containing peripheral adhesions in MV^{D7} cells. The signal intensity of α5 in FAs positive for EGFP-Mena was

significantly higher than in EGFP-MenaΔLERER FAs, which indicates that FAs

containing Mena capable of binding α5 have higher concentrations of α5.

Significantly higher levels of pFAK397, pPAX118 (Fig. B-5), and pY (not

depicted, $P < 0.001$) were observed in adhesions containing EGFP-Mena,

compared to EGFP-MenaΔLERER FAs. No significant differences were observed

(by either immunofluorescence or Western blotting) in levels of phosphor-FAK

(pFAK), phospho-paxillin (pPax), or pY throughout the whole cell between MV^{D7}

cells expressing EGFP-Mena versus EGFP-MenaΔLERER (unpublished data);

this indicates that differences in α5 and downstream signaling are spatially
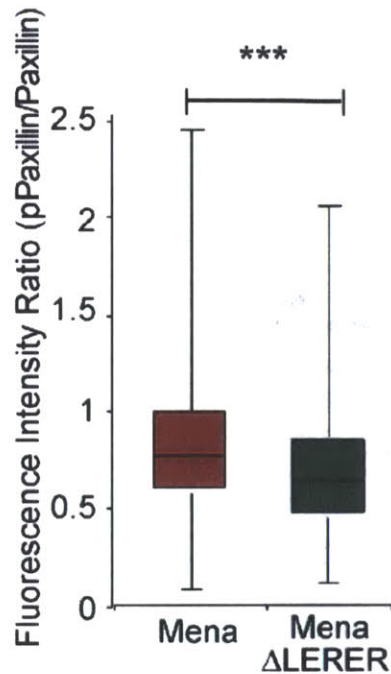
restricted to Mena-containing adhesions.

| Mena | Paxillin (pY118) | Paxillin | F-actin | |
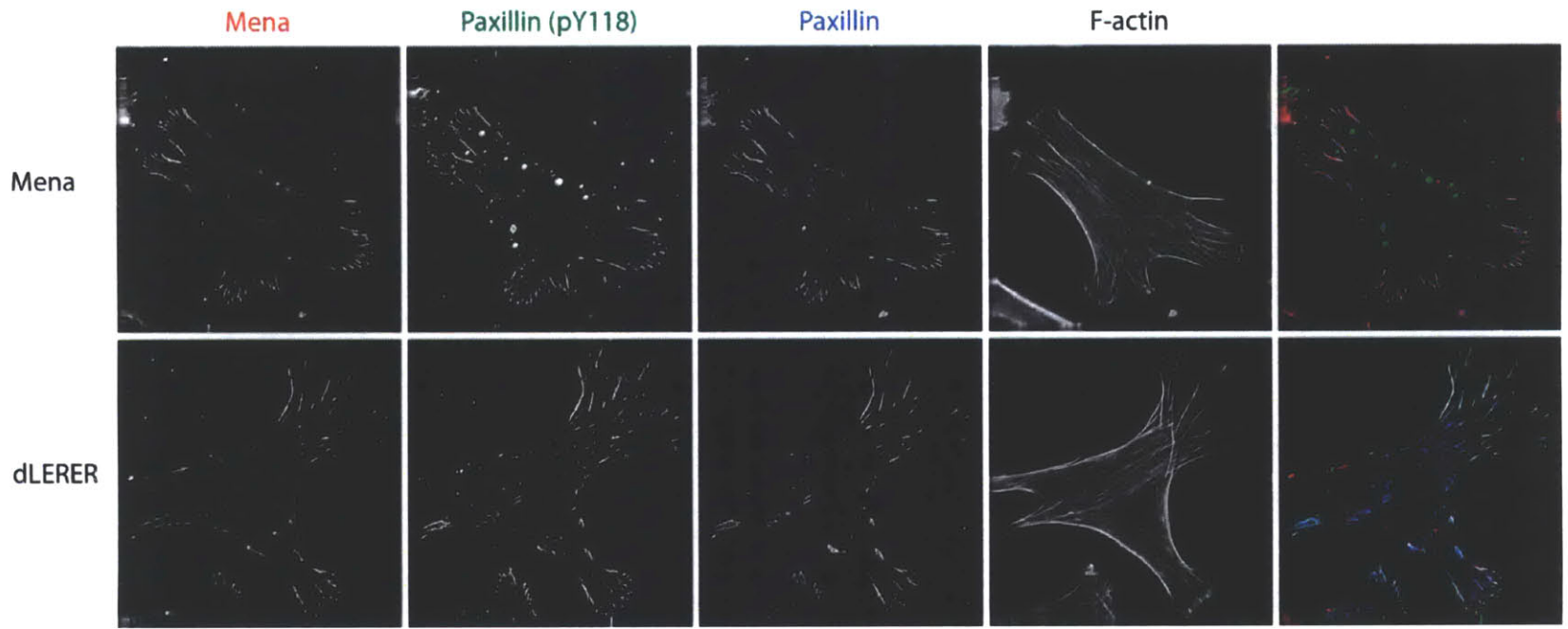
B

Figure B-5: The Mena:α5 interaction modulates adhesion signaling.
(**A**) MV^{D7} cells expressing EGFP-Mena or EGFP-MenaΔLERER were plated on 10μg/ml fibronectin, then stained for phosphorylated Paxillin (pPaxillin) and Paxillin. (**B**) The ratio of pPaxillin/Paxillin was measured in Mena and MenaΔLERER-positive adhesions. pPax118/Paxillin was significantly increased in Mena compared with MenaΔLERER-containing adhesions. ***, P < 0.001.

B.3.6. The Mena:α5 interaction is required for normal FN fibrillogenesis

During fibrillogenesis, $\alpha 5\beta 1$ is attached to FN as it moves centripetally along stress fibers, forming FBs and generating the required tension (Pankov et al., 2000; Danen et al., 2002). Because central $\alpha 5\beta 1$-positive FBs are absent in MV^{D7} and MenaΔLERER cells, we asked whether Mena:α5 binding is required for $\alpha 5\beta 1$-dependent FN fibrillogenesis. Parental MV^{D7} cells and MV^{D7} cells expressing EGFP-Mena, or EGFP-MenaΔLERER were plated overnight on vitronectin, and then fluorescent FN was added to the media (depleted of any pre-existing FN) for 4 hours. Cells were fixed and stained to identify FN fibrils (Fig. B-6). MV^{D7}+EGFP-Mena cells generated typical FN fibrils aligned with stress fibers and FBs, whereas parental MV^{D7} cells and MV^{D7} cells expressing either EGFP-MenaΔLERER formed significantly less fibrillar FN, which suggests that the interaction between Mena and α5 is critical for efficient fibrillogenesis.

A

F-actin   Fibronectin

MVD7

Mena

dLERER

B

% Cell Area with FN Fibers

6
5
4
3
2
1
0

*** ***

MV^{D7}   Mena   Mena
                   ΔLERER

C

Total FN per cell

1.2
1
0.8
0.6
0.4
0.2
0

*** ***

MV^{D7}   Mena   Mena
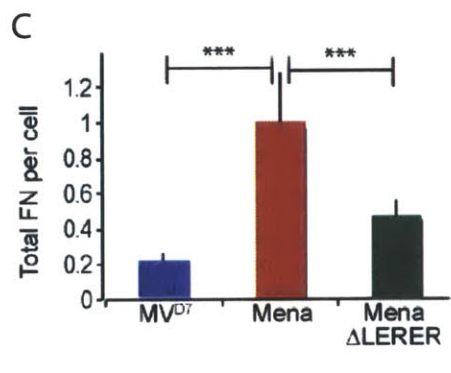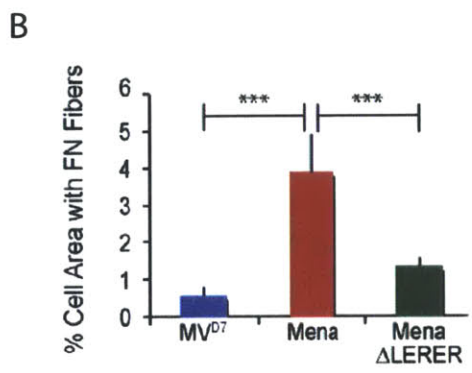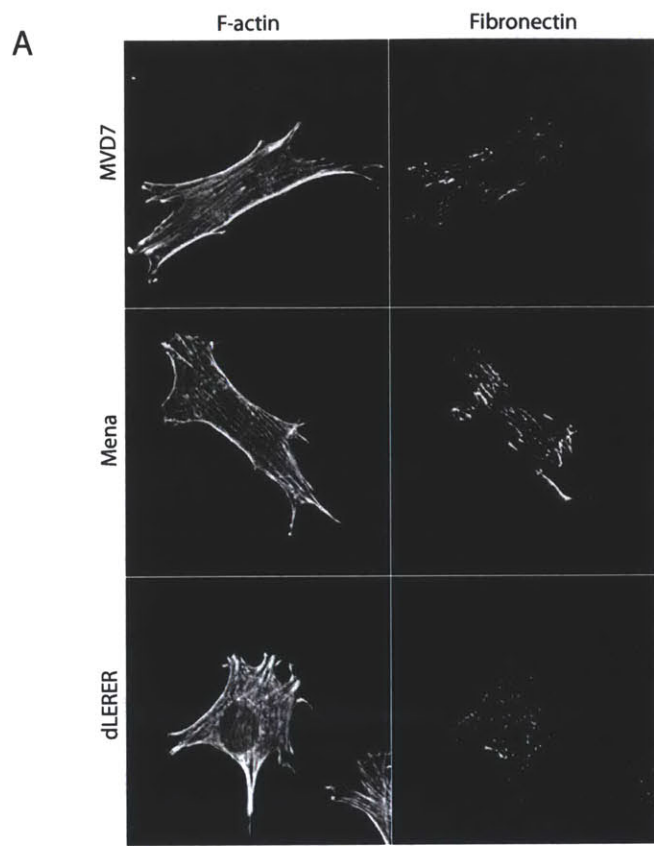   ΔLERER

Figure B-6: FN fibrillogenesis in MV^{D7} cells is rescued by EGFP-Mena but not EGFP-Mena ΔLERER.

(A) MV^{D7} cells expressing EGFP-Mena or EGFPMena ΔLERER were plated overnight on vitronectin and incubated for 4 hours before fixation with 10 μg/ml of fluorescently tagged FN. Bar, 10 μm. (B) Percentage of cell area containing FN fibrils. (C) Total amount of FN within fibrils per cell. Error bars indicate mean ± SEM. ***, P < 0.001.

B.4. Conclusions

Cell motility is a highly regulated, dynamic process that requires continual remodeling of the cytoskeleton as well as cell–cell and cell–matrix adhesions. Involvement of Ena/VASP in these processes has been demonstrated in a wide range of systems. Although Ena/VASP influences cellular protrusion dynamics by regulating actin polymerization through a mechanism of emerging focus (Bear & Gertler, 2009; Hansen & Mullins, 2010), how Ena/VASP affects adhesion is not well understood. Here we identify a direct connection between Mena and α5, and document that it is required for fibroblast spreading on FN, FB formation, and FN fibrillogenesis. We conclude that the Mena:α5 interaction contributes to the physiological function of fibroblasts, which secrete and remodel ECM to perform essential functions.

In addition to these roles in inside-out regulation of α5β1, the Mena:α5 complex is also regulated by, and necessary for, outside-in signaling by α5β1. Mena binding to α5 also causes formation of FAs with higher concentrations of α5; this may reflect enhanced α5β1 clustering and binding to FN via increased avidity, though further work is needed to test this possibility. Mena binding to α5 is also necessary for signaling downstream of α5β1, as indicated by reductions in pFAK397, pPAX118, and global pY in adhesions that contain EGFP-MenaΔLERER relative to those containing EGFP-Mena. Based on these findings, we propose that Mena is a key modulator of α5β1-mediated bidirectional signaling between ECM and the actin cytoskeleton.

Mena binds to α5 via the LERER repeat, a region spanning 91 or 121

144

amino acids with 13 or 15 repeats of the five-residue LERER motif in mouse and human, respectively. Whether each repeat binds an $\alpha5$ tail is unknown, but multiple $\alpha5$ tails could bind LERER repeats within each subunit of a Mena tetramer, raising the possibility that Mena clusters $\alpha5\beta1$, thereby strengthening FN binding by increased avidity.

Despite its role in fibrillogenesis, Mena is barely detectable in FBs compared with FAs, as are two other molecules important for fibrillogenesis: FAK (Ilić et al., 2004) and ILK (Zamir et al., 2000; Vouret-Craviari et al., 2004; Stanchi et al., 2009). Mena may cluster $\alpha5\beta1$and strengthen FN binding within FAs before $\alpha5\beta1$:FN complexes begin moving toward central FBs. Alternatively, Mena:$\alpha5$ interactions could target FAs for maturation by changing $\alpha5$ dynamics and stability within FAs.

The LERER repeat is not found in VASP, EVL, or the invertebrate and *Dictyostelium discoideum* Ena/VASP orthologues. Interestingly, FN, $\alpha5\beta1$, and the Mena LERER repeat are all vertebrate-specific adaptations (Whittaker et al., 2006), which suggests that they coevolved. The Mena:$\alpha5$ interaction is highly regulated: loss of adhesion reduces the interaction whereas acute FN binding increases levels of the complex and the residence time of Mena within FAs. And though VASP does not bind any integrin subunit directly, it does promote inside-out activation of $\beta1$- and $\beta2$-containing integrins indirectly, via adaptor or signaling intermediates (Deevi et al., 2010). VASP functions in cross-regulation between $\alpha V\beta3$ and $\alpha5\beta1$ (Worth et al., 2010): loss of $\beta3$ function reduces phosphorylation of a PKA-dependent site within VASP near its EVH1 domain,

allowing it to bind FP4 repeats within RIAM, an adaptor that mediates Rap-GTPase-driven integrin activation (Lafuente et al., 2004). The VASP–RIAM complex associates with the β subunit–binding protein talin (Anthis and Campbell, 2011), causing α5β1 activation at peripheral adhesions (Worth et al., 2010); however, RIAM can also promote integrin activation by talin independently of Ena/VASP (Lafuente et al., 2004; Lee et al., 2009). The Mena EVH1 domain binds many of the same ligands as VASP (Ball et al., 2002), connecting Mena to integrins via RIAM or other FA proteins such as vinculin and zyxin that contain EVH1-binding sites and associate with β subunits indirectly. Juxtaposition of its EVH1 domain and LERER repeat may enable Mena to connect directly to α5 and indirectly to β1 simultaneously.

The interaction with α5β1 potentially allows Mena to influence cell motility through a variety of mechanisms, including modulation of adhesion strength and changes in outside-in signaling that affect other components of the motility machinery. Additionally, Ena/VASP deficiency reduces cellular capacity to generate actin-driven protrusive forces that drive lamellipodial and filopodial extension and propulsion of the intracellular pathogen Listeria monocytogenes, even though the actin networks formed during these processes are organized differently. Expression of Mena, VASP, or EVL rescues the actin polymerization–dependent phenotypes arising from deficiency of Ena/VASP in MV[D7] cells or in primary neurons from triple Mena/VASP/EVL-null embryos (Loureiro et al., 2002; Geese et al., 2002; Applewhite et al., 2007; Dent et al., 2007). In general, Ena/VASP activity produces longer, sparsely branched filament networks; in the

absence of stabilizing interconnections, these increasingly buckle against the membrane as they elongate because of their inherent flexibility (Mogilner and Oster, 2003). By coupling its stimulatory effect on barbed end elongation with its ability to bind and potentially cluster $\alpha5\beta1$, Mena could present activated but unbound integrins right at the tips of lamellipodia and filopodia, which is consistent with the proposed "sticky fingers" mechanism for haptotaxis (Galbraith et al., 2007). In addition, through its role in FN remodeling, Mena may help form the interstitial fibrillar network that serves both as a migration substrate as well as a template that organizes growth factors and other ECM components into spatially organized cues. These cues elicit complex, coordinated responses (Hynes & Naba, 2012) when touched by the sticky fingers of cells in transit.

Recently, both $\alpha5\beta1$ (Caswell et al., 2008; Valastyan et al., 2009; Muller et al., 2009) and Mena (Philippar et al., 2008; Robinson et al., 2009; Roussos et al., 2011a) have been implicated in breast cancer invasion and metastasis through effects on EGFR (Gertler & Condeelis, 2011). During tumor progression, changes in alternative splicing produce additional, functionally distinct Mena protein isoforms coexpressed with the canonical isoform. Mena[INV], a Mena isoform expressed in a subpopulation of highly invasive, motile, and chemotactic tumor cells (Goswami et al., 2009), has been detected in breast cancer patients with invasive ductal carcinomas (Roussos et al., 2011b). Mena[INV] expression promotes tumor cell invasion and metastasis by a mechanism involving increased tumor cell sensitivity to EGF (Philippar et. al. 2008; Roussos et al., 2011a). Interestingly, EGFR is sometimes found in complexes with $\alpha5\beta1$ linked

by their mutual cytosolic binding partner, RCP (Caswell et al., 2008; Muller et al., 2009). $\alpha5\beta1$-RCP association with EGFR leads to coordinated recycling that targets $\alpha5\beta1$ and EGFR to the front of cells, promotes 3D invasion, and dysregulates signaling downstream of both receptors. The potential functional and biochemical links between Mena[INV] $\alpha5\beta1$ and EGFR during tumor progression are an important topic for further investigation.

B.5. Material and Methods

Microscopy

Cells were fixed for 20 min in 4% paraformaldehyde in PHEM buffer warmed to 37°C, and then permeabilized in 0.2% Triton X-100 and blocked in 10% donkey serum. Primary antibodies used for immunofluorescence include α5 integrin (1928; Millipore), integrin αv [RMV-7] (ab63490; Abcam), integrin α6 [GoH3] (ab105669; Abcam), vinculin (Sigma-Aldrich), Mena, GFP (JL-8; Takara Bio Inc.), paxillin (610052; BD), Rab7 (9367S; Cell Signaling Technology), Rab11 (5589; Cell Signaling Technology), and EEA1 (3288S; Cell Signaling Technology). F-actin was stained with Alexa Fluor 647 and Alexa Fluor 350 Phalloidin (Invitrogen). Fluorochromes on secondary antibodies included Alexa Fluor 568, Alexa Fluor 488, Alexa Fluor 647, and Alexa Fluor 350 (Jackson ImmunoResearch Laboratories, Inc.). Cells were mounted in mounting media containing 90% glycerol and n-propyl-gallate, and imaged at room temperature. Z series of images were taken on a DeltaVision microscope (Applied Precision) using SoftWoRx acquisition software (Applied Precision), a 60× 1.3 NA Plan-Apochromat objective lens (Olympus), and a camera (CoolSNAP HQ; Photometrics). Images were deconvolved using Deltavision SoftWoRx software and objective specific point spread function.

Image analysis

Cell masks of cell area were made by thresholding phalloidin images. Subsequently, thresholding was done to evenly include adhesive structures between cells within these masks, and the intensity and area of these regions was measured. Pearson's coefficients of colocalization were calculated using the Intensity Correlation Analysis Plugin available for ImageJ.

Statistical analysis

The paired Student's t test was used for statistical analyses of experiments with two conditions. In the cases of three or more conditions, analysis of variance (ANOVA) was used with the least significant difference post hoc test. Significant differences are indicated throughout as: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Cell culture and plasmids

Coverslips were coated with 10 μg/ml bovine fibronectin (Sigma-Aldrich) for 2 hours at 37°C. Rat2 cells were cultured in DME supplemented with 10% fetal bovine serum and maintained at 37°C, 5% CO_2. Parental MV^{D7} cells and MV^{D7} cells expressing tagged Mena and Mena mutants were maintained at 32°C, 5% CO_2 in DME supplemented with L-Glutamine, penicillin and streptomycin, 15% fetal bovine serum, and 50 U/ml interferon γ (I-4777; Sigma-Aldrich; Bear et al., 2000). mCherry-FP4-Mito were introduced into MV^{D7} cells with use of Lonza nucleofection according to the manufacturer's instructions. pMSCV-EGFP-MenaΔLERER was cloned according to standard cloning procedures with N-terminal tags. N-terminally tagged mCherry-FP4-Mito has been described

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previously (Bear et al., 2000). N-terminally tagged EGFP-tensin (full length, chicken) was a gift from K. Yamada (National Institutes of Health, Bethesda, MD) and was introduced into Rat2 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions.

FN fibrillogenesis

FN-depleted medium was prepared as described previously (Pankov and Momchilova, 2009). In brief, 10 ml of gelatin Sepharose 4B was washed with sterile PBS three times. After removing the third wash, 10 ml of FBS was added, rocked for 30 min at room temperature, collected, aliquoted, and stored at −20°C. FN was fluorescently labeled with 549-NHS ester from Thermo Fisher Scientific (46407), as directed by the manufacturer. MV^{D7} cells were seeded on coverslips coated with 10µg/ml vitronectin from Sigma-Aldrich (V9881) and allowed to adhere overnight. Medium was replaced with FN-depleted growth medium containing 10 µg/ml fluorescently labeled FN and incubated at 32°C for 4 h. Cells were then fixed and immunostained.

α5 integrin surface levels

For assessment of α5 integrin surface levels, MV^{D7} fibroblasts were incubated on ice in 1% BSA, 2 mM EDTA in PBS with biotinylated α5 integrin antibody (557446; BD) or biotinylated rat IgG (012-060-003; Jackson ImmunoResearch Laboratories, Inc.) for 30 min. Cells were washed and incubated for 30 min on ice with APC streptavidin (554067; BD) and propidium iodide. Cells were washed, resuspended, and directly analyzed on a flow cytometer (FACSCalibur; BD).

B.6. References

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