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Zyxin Regulates Epithelial-Mesenchymal Transition by Mediating Actin-Membrane Linkages at Cell-Cell Junctions

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ZYXIN REGULATES EPITHELIAL-MESENCHYMAL TRANSITION BY MEDIATING ACTIN-MEMBRANE LINKAGES AT CELL-CELL JUNCTIONS

by

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This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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ABSTRACT

ZYXIN REGULATES EPITHELIAL-MESENCHYMAL TRANSITION BY MEDIATING ACTIN-MEMBRANE LINKAGES AT CELL-CELL JUNCTIONS

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Development is punctuated by morphogenetic rearrangements of epithelial tissues, including complete detachment of individual motile cells during epithelial-mesenchymal transition (EMT). Dramatic actin rearrangements occur as cell-cell junctions are dismantled and cells become independently motile during EMT. Characterizing dynamic actin rearrangements and identifying actin machinery driving these rearrangements is essential for understanding basic mechanisms of cell-cell junction remodeling; yet, neither the precise series of actin rearrangements at cell-cell junctions that accompany EMT, nor the machinery that controls actin rearrangement during EMT, have been identified. This work represents a detailed study of junctional actin reorganization in cells undergoing EMT, identifies actin regulatory proteins that control this actin reorganization, and defines the specific function of one regulatory protein,

zyxin, in EMT. Using immunofluorescence and live cell imaging of HGF induced scattering of MDCK cells, dynamic actin rearrangement events occurring during EMT are characterized. Junctional actin characteristic of cell-cell adherent cells is rearranged into contractile medial actin networks linked to the junctional membrane in the initial steps of cell scattering. This actin rearrangement is accompanied by dynamic redistribution of specific actin regulatory proteins, namely α-actinin and zyxin-VASP complexes. α-Actinin mediates higher order structure of junctional actin. Zyxin-VASP complexes mediate linkage of dynamic medial actin networks to adherens junction membranes. Zyxin regulation of actin-membrane linkage controls whether cell migration during EMT occurs independently in solitary cells or is coordinated through tissues. The functional analysis employed here uses novel, quantitative methods that define specific cellular EMT 'phenotypes' to reveal the precise role of zyxin in EMT. Constitutive active zyxin mutants exhibit persistent actin-membrane linkages and a scattering phenotype in which cells migrate without loss of cell-cell adhesion. Zyxin is proposed to regulate EMT progression by regulating disruption or maintenance of actin membrane linkages at cell-cell junctions. Zyxin alters the ability of cells to fully detach and migrate independently during EMT and may be an important regulator of morphogenetic plasticity.

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Chapter 1. INTRODUCTION

Assembly and disassembly of cell-cell junctions is tightly regulated during tissue morphogenesis and homeostasis, as well as in pathological states such as cancer metastasis (Gumbiner, 2005; Mege et al., 2006). Cadherin mediated assembly of adherens junctions drives cell-cell adhesion, enabling cells to function in a coordinated manner as epithelial sheets, tissues and organs (Gumbiner, 1996). The ability of epithelial cells to respond to changing environmental conditions depends on coordination of cell-cell adhesion with cytoskeletal actin organization to alter cell adhesion, migration and spreading properties (Montell, 2008). Complex biochemical interactions between cell-cell junction proteins, cell signaling pathways and the actin cytoskeleton are indicated by the wide range of morphogenetic responses downstream of adherens junctions, such as cell sorting and tissue cohesion (Tepass, 1999; Tepass et al., 2000), neural tube formation (Duband et al., 1995), dorsal cleft (Gates et al., 2007) and wound closure (Poujade et al., 2007), sheet invagination and tube formation (Lubarsky and Krasnow, 2003), and convergence-extension movements (Montell, 2008). In these processes the actin cytoskeleton is actively remodeled while cell-cell adhesion remains intact (Miyoshi and Takai, 2008). An important goal of cell biology is to identify key regulators coordinating interactions between adherens junctions, cell signaling pathways and the actin cytoskeleton which enable cells to maintain stable cell-cell contacts while responding morphogenetically to changing cellular environments.

During developmental morphogenetic rearrangements as well as in cancer metastasis, cell-cell adhesions are disassembled as individual cells detach from epithelial tissues and migrate to distant sites (Thiery, 2003; Mege *et al.*, 2006), in a process termed

epithelial-mesenchymal transition (EMT). EMT is the process by which cell-cell adherent, polarized epithelial cells disrupt cell-cell adhesions and assume a migratory mesenchymal phenotype. Actin plays fundamental but differing roles in cell-cell adhesion (Vasioukhin et al., 2000; Vasioukhin and Fuchs, 2001) and migration (Pollard and Borisy, 2003), so cells undergoing EMT make dramatic structural changes to the actin cytoskeleton. The ability of cells to reorganize the actin cytoskeleton is essential for morphogenetic responses to different cellular environments, yet characterization of the structural actin reorganization events at cell-cell junctions during EMT has not been performed. In this study I characterize junctional actin reorganization during EMT, identify regulatory proteins driving this process, and define a specific role for the actin regulator, zyxin, in EMT.

Using MDCK cells, a well-established epithelial tissue culture model (Behrens et al., 1985), induced to undergo EMT with hepatocyte growth factor/scatter factor (Stoker and Perryman, 1985), I demonstrate zyxin mediates actin-membrane linkage of medial actin networks established early in EMT. A functional analysis of zyxin in EMT, using stable zyxin mutant expressing cell lines, shows zyxin, through its binding partner VASP, regulates whether cell migration during scattering occurs independently in solitary cells, or is coordinated between neighboring cells. I propose these effects are due to altered actin organization during EMT due to zyxin regulation of VASP function and propose a model in which zyxin-VASP complexes regulate actin-membrane linkages during EMT, thereby mediating morphogenetic rearrangements. These findings should be of wide interest to cell and developmental biologists, generating new ideas about how

morphogenetic rearrangements are controlled at the molecular and cellular level that can be tested in more complicated model systems.

Junctional plasticity during morphogenesis

Morphogenesis is the creation of ordered form during development in which the body plan of developing organisms is established by tightly regulated changes in cell shape, migration and adhesion. It is the program by which embryonic patterning to establish shape, form and function of tissues and organs is accomplished. Cell migration and junctional plasticity are essential during morphogenesis as well as in other physiological processes such as wound healing and cancer metastasis. There are two basic types of cells in the developing embryo: nonmigratory epithelial cells, which are adherent to each other, forming sheets and tubes; and loosely arranged mesenchymal cells, which migrate independently of each other. Epithelial cells arise when migratory mesenchymal cells form cell-cell adhesions, develop apical-basal polarity, and form coordinated groups of cells in the process of mesenchymal-epithelial transition (MET). During morphogenetic rearrangements epithelial cells can return to a mesenchymal state, disrupting cell-cell adhesion fully and moving independently of surrounding cells, in the reverse process of epithelial-mesenchymal transition (EMT).

EMT is essential during development for gastrulation and the formation of various tissues and organs such as heart, musculoskeletal system, craniofacial structures and peripheral nerves (Thiery, 2002). Neural crest cell delamination is a classic example of EMT (Duband et al., 1995). However, many developmental as well as homeostatic processes involve cell migrations in which cells migrate without returning to a fully mesenchymal state. In these cases, cells remain adherent to each other while migrating

individually within the epithelium or moving collectively in clusters, strands, and sheets (Friedl, 2004; Montell, 2008). Such cell movements are tightly regulated to establish embryonic form. For example, convergence-extension movements during vertebrate gastrulation elongate the embryo along its anterior-posterior axis by cells changing position with respect to one another within the epithelium. Medial migration and intercalation of mesodermal and neurectodermal cells results in mediolateral narrowing of the body axis (Keller, 2002; Wallingford *et al.*, 2002; Vervenne *et al.*, 2008). During trigeminal placode development, clusters of cells delaminate from the epithelial ectoderm, invade the mesenchyme and migrate to establish tissues elsewhere (Stark et al., 1997). During follicle development in *Drosophila*, border cells delaminate from the epithelium and migrate as a group to the oocyte, while remaining cell-cell adherent to each other, (Montell, 2008). Such variety in morphogenetic movements suggests that cells undergo EMT to varying extent along a continuum from partial to full EMT in which they undergo some, but not all, steps of EMT in different situations (Huber et al., 2005; Leroy and Mostov, 2007). How cells control the decision to begin EMT and regulate the extent of EMT response are important questions in cell biology, influencing both normal developmental physiology and pathological conditions similar to EMT such as cancer metastasis and inflammatory responses. To begin to understand how cell-cell adhesion is regulated during EMT, we must first understand how cell-cell adhesions are formed. This process has been well studied.

Structural organization of apical junctions

An important transition during mammalian embryonic development is compaction, in which loosely organized mesenchymal cells condense together and form

tight contacts with each other (Peyrieras *et al.*, 1983; Fleming *et al.*, 2001). The mesenchymal to epithelial transition occurring during compaction results in formation of polarized epithelial cell sheets in which cells spread against one another in a process mediated by the cadherin family of adhesion receptors (Takeichi et al., 1988). Polarization and establishment of tall, cuboidal epithelial cells is mechanistically analogous to the well studied process of fibroblast spreading on the extracellular matrix (Gumbiner, 1996). Spreading in both cases requires forces generated by the actin cytoskeleton. As cell junctions form, contraction of apical actin filaments which become incorporated into the junctional actin spreads cells against each other and drives cell polarization and assumption of a cuboidal morphology (Zhang et al., 2005).

Tight cell-cell contact and a polarized epithelium are established through formation of adherens junctions (AJs), tight junctions, desmosomes, and gap junctions (Miyoshi and Takai, 2008). The AJ is of particular importance because it is the first of these adhesion structures to form and it regulates formation of the other structures. Tight junctions act as a fence to physically separate apical and basal domains of polarized epithelial cells. AJs act to stabilize the epithelium by promoting tight junction formation and interacting with the cytoskeleton. AJs are associated with a denser actin structure than tight junctions, as demonstrated by electron microscopy (Miyoshi and Takai, 2008). AJ formation regulates reorganization of the actin cytoskeleton from the loosely arranged cortical actin filaments characteristic of mesenchymal cells into the tightly bundled junctional actin ring lining cell-cell junctions, typical of epithelial cells (discussed below). AJ formation drives cortical actin reorganization into junctional actin, and forces

generated by the junctional actin cytoskeleton are involved in maintenance of AJs (Quinlan and Hyatt, 1999).

It is well established that cadherin receptors mediate formation of initial cell-cell contacts and the formation of AJs (Gumbiner et al., 1988; Yap et al., 1997). The cadherin family is composed of over 70 members in mammals, and mediates adhesion between cells of the same type (Takeichi *et al.*, 1989; Miyoshi and Takai, 2008). Epithelial cadherin (E-cadherin) is the most ubiquitous. Sorting of mixed cell populations can be accomplished by variations in cadherin expression (Nose et al., 1988; Nose et al., 1990). Cadherins are single pass, transmembrane glycoproteins that bind homophilically in the extracellular domain in a Ca^{2+} dependent manner. Intercellular cadherin binding initiates AJ formation and stable cell-cell adhesion (Takeichi *et al.*, 1988; Takeichi, 1991). Cadherin extracellular domains are variable and cadherins generally bind homophilically with cadherins of the same type on adjacent cells to initiate cell-cell adhesion. Cadherin intracellular domains are highly conserved and interact with a multimolecular complex of cytoplasmic adaptor proteins including α, β and p-120 catenins. Cadherins are at or near the top of the molecular cascade resulting in adhesion; transfecting non-adherent cells with cadherin is sufficient to induce adhesion (Nagafuchi and Takeichi, 1988; Mege *et al.*, 2006) and AJ formation is required for establishment of other cell-cell adhesion structures.

Cadherin complex formation

Adams et al (1996) studied formation of the cadherin complex at nascent AJs using time lapse differential interference contrast (DIC) imaging followed by quantitative retrospective immunocytochemistry. They demonstrated that AJs are stabilized and

strengthened over a period of about 45 minutes as the cadherin complex becomes associated with the actin cytoskeleton, as measured by a shift from a detergent soluble to a detergent insoluble pool. The cadherin complex appears to be preformed in the cell and is recruited into a detergent (Triton X) insoluble pool upon initiation of cell-cell adhesion (Adams et al., 1996). Pulse chase studies of newly synthesized protein revealed that E-cadherin and β-catenin form a complex following synthesis and α-cat joins the complex as E-cad/β-cat arrives at the plasma membrane (Hinck *et al.*, 1994; Adams *et al.*, 1996). Complexes of E-cad/β-cat/α-cat can be isolated from noncontacting cells (Shore and Nelson, 1991). Prior to cell-cell junction formation cadherin complex constituents are TritonX (TX) soluble. There is a lag between initial cell-cell contact and recruitment of cadherin complex constituents into a TX insoluble pool (McNeill et al., 1993) which coincides with strengthening of adhesion between cells over 30-40 minutes after initial contact (Angres et al., 1996). If cadherin association with catenins or actin is disrupted, both TX insolubility and cell-cell adhesion are lost (Nagafuchi and Takeichi, 1989; Hirano *et al.*, 1992). These data support the view that upon AJ formation, the cadherin complex is stabilized by linkage to the actin cytoskeleton, resulting in strong adhesion between cells.

Actin cytoskeletal reorganization during cell-cell junction formation

Cadherin-mediated AJ formation and the initial steps of actin reorganization as cell-cell contacts form have been well studied. Prior to adhesion individual mesenchymal cells are circumscribed by a thick ring of loosely arranged actin filaments called a cortical actin belt (Yonemura et al., 1995). As cells become adherent this cortical belt is remodeled to circumscribe the outer border of the cell colony, forming a thin line of

tightly bundled actin, juxtaposed along cell-cell junctions. The initial steps in the transition from a mesenchymal to an epithelial actin cytoskeletal arrangement have been detailed (Adams *et al.*, 1996; Adams *et al.*, 1998) and recently the later stages have been examined (Zhang et al., 2005).

Cadherin components appear in proportionate (1:1:1) amounts and initially exist as discrete puncta along cell-cell contacts. As individual cells contact each other, lamellipodia from each cell interact, appearing to "test" the contact, after which the contact is first stabilized in small (1-2 um) areas followed by gradual zippering together of the apposed membranes (McNeill *et al.*, 1993; Adams *et al.*, 1998). Punctate structures coincident with E-cadherin puncta are visible by DIC where membranes abut. Electron microscopy indicates these puncta are spatially coincident with sites where actin bundles branching from the cortical actin cytoskeleton attach to the membrane (Yonemura *et al.*, 1995; Vasioukhin *et al.*, 2000). Puncta form within minutes after initial contact, coincident with actin recruitment, forming transient weak contacts. The initial puncta appear to move towards the outer area of the contact, with new cadherin puncta forming within the original contact site as membrane contact expands. Puncta are stabilized and zipper together as junctions mature (Vasioukhin *et al.*, 2000; Vaezi *et al.*, 2002). Larger plaques (10-100 times larger than a single punctum) of cadherin complex eventually form at either end of the contact and at the vertices of 3 or more cells (Adams et al., 1998). In mature contacts individual puncta are no longer discernable, rather cadherin stains in a continuous thin line along cell junctions and more heavily at vertices between cells (Adams et al., 1998). Likewise, actin in mature junctions is rearranged into a cable of

actin circumscribing each cell and tightly apposed to AJs (Yonemura *et al.*, 1995; Adams *et al.*, 1998).

Association with actin mediates the strengthening and stabilization of cell-cell contacts coincident with the progression from punctate to linear cadherin staining along AJs (Adams *et al.*, 1996; Adams *et al.*, 1998). In nascent junctions, bundles of actin perpendicular to the actin belt can be seen emanating from cadherin puncta lining the junction. Using DIC (differential interference contrast microscopy), Adams et al (1996, 1998) observed breakdown and reorganization of a structure which appeared to be the actin belt, coincident with formation of radial actin bundles associated with E-cadherin puncta. Phalloidin (which stains actin filaments) and E-cadherin antibody staining revealed actin bundles, perpendicular to the actin belt, directed toward individual cadherin puncta at nascent contact sites. These observations suggest that the initial steps of E-cadherin mediated adhesion result in rapid dynamic reorganization of the actin cytoskeleton to form radial actin bundles which interact with the thick belt of loosely arranged actin circumscribing individual cells. Disrupting the actin cytoskeleton with latrunculin B or cytochalasin D results in cells with weaker adhesive ability, demonstrating the importance of an intact actin cytoskeleton for strong cell-cell adhesion (Chan et al, 2004).

As junctions mature, cells become polarized, assuming a tall, cuboidal cell shape with distinct apical and basal membrane domains as the actin cytoskeleton is remodeled from a mesenchymal to an epithelial organization. Using confluent keratinocytes, a model for cadherin dependent adhesion and epithelial polarization, Zhang et al., 2005, examined the later steps of actin reorganization as cells become polarized. They divide

cell polarization into 4 steps: 1) new cell-cell contact formation, 2) stabilization of new contacts (by association with actin bundles), 3) junction maturation (as the AJ extends and punctate cadherin staining becomes linear along AJs, and actin in reorganized), and 4) acquisition of cuboidal, polarized phenotype (as thin apical actin cables contract) (Zhang et al., 2005). They demonstrated that extension of cell-cell contact and stabilization at vertices requires active cooperation between cadherin based adhesions and the force generation of actin structures. They identify two discrete pools of actin at nascent cell-cell junctions; bundled actin emanating from puncta and thin filaments of actin which become incorporated with junctional actin during AJ maturation (Zhang et al., 2005). The apically located thin filament pool cinches up to the junction until the two pools are indistinguishable, forming the line of continuous actin observed to circumscribe cells in mature epithelia. The recruitment of thin actin cables to the maturing junction is accompanied by an increase in myosin dependant contractility, and assumption of a polarized phenotype.

In summary, junction formation begins with homophilic binding of cadherin extracellular domains, which initiates a cascade of intracellular reactions resulting in cadherin complex formation and remodeling of cortical actin into the junctional actin ring. Cadherin complexes appear initially as punctate structures, associated with short, thick radial actin bundles which interact with thin, apical circumferential actin filaments. During junction maturation, cadherin complexes coalesce into plaques which localize to vertices between adjoining cells as junction lengths are extended. At the end of this process, cells become polarized and fully adherent to each other, exhibiting continuous linear cadherin staining and junctional actin tightly juxtaposed to AJs.

Actin-membrane linkage at cell-cell junctions

That junctional actin is associated with AJs is evident, but how actin is linked to junctional membranes is unclear. Since adaptor proteins that bind transmembrane cell-cell adhesion molecules also have sites for binding actin filaments, the accepted model for actin-membrane linkage has been through the mediation of adaptor proteins (Takai and Nakanishi, 2003; Gumbiner, 2005). The classic cadherin complex consists of β-catenin bound to the cytoplasmic domain of E-cadherin and, in turn, α-catenin bound to β-catenin. α-Catenin contains actin filament binding sites and also binds other adaptor proteins which bind actin, such as vinculin and α-actinin. Thus, the largely accepted model for how cadherins associate with the actin cytoskeleton has been that α-catenin links the cadherin complex to the actin cytoskeleton (Gumbiner *et al.*, 1988; Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990; Aberle *et al.*, 1994; Rimm *et al.*, 1995; Adams *et al.*, 1996; Lambert *et al.*, 2002; Perez-Moreno *et al.*, 2003; D'Souza-Schorey, 2005). However, no researcher has yet physically demonstrated the ternary complex of an adhesion molecule, an adaptor protein and actin filaments (Gates and Peifer, 2005). Rather, it was recently demonstrated that α-catenin cannot bind β-catenin and actin simultaneously (Drees et al., 2005; Yamada et al., 2005), suggesting that actin-membrane linkage is more dynamic than previously thought (Gates and Peifer, 2005).

Nevertheless, it is clear that apical junctions are linked to the actin cytoskeleton since disrupting actin filaments disrupts junctional complexes and vice versa. AJs and tight junctions are dismantled after treating cells with actin filament disrupting agents such as latrunculin B or cytochalasin D (Yamazaki *et al.*, 2007; Miyoshi and Takai, 2008). Reorganization of actin into its characteristic junctional structure of tight bundles

lining AJs is necessary for proper localization of cadherin complex components to cell-cell junctions (Quinlan and Hyatt, 1999). In Drosophila, loss of β-catenin from AJs results in disrupted cytoskeletal organization and altered cell shapes (Peifer, 1993), and defects in polarity of actin cytoskeletal structures (Cox et al., 1996). Since actin-membrane linkage at cadherin-based cell-cell adhesions has not been demonstrated directly to cadherin complexes (Yamada et al., 2005), uncharacterized mechanisms of actin-AJ interactions, perhaps independent of cadherin complexes, are likely important. Identifying actin regulatory proteins that mediate actin-membrane interactions at AJs during morphogenetic rearrangements, including EMT, is critical to understanding how junctional plasticity is regulated.

Whether the connection to actin is direct or indirect, it is clear that cadherin complex formation initiates changes in actin organization at the cell periphery and that these changes are necessary for strong cell-cell junction formation. Actin-membrane linkage is important during morphogenetic rearrangements as well. Connection of contractile medial actin networks to AJ membranes, by an unknown mechanism, has recently been demonstrated to drive apical constriction necessary for ventral furrow formation in *Drosophila* gastrulation (Martin et al., 2008). Contractile forces in individual cells are transduced across AJs and through the tissue, generating tissue organization changes at the ventral furrow. How actin-AJ connections are mediated and regulated during morphogenesis is poorly understood. Junctional actin-membrane linkage and how this actin structure is rearranged during EMT are focuses of this study.

Cellular actin machinery

The actin cytoskeleton is dynamic and its structure is altered in response to different cellular signaling environments by regulating the cellular core actin machinery. Actin filaments are double helical polymers composed of 42 KD actin monomers (Cramer, 1999). Actin filaments are polarized, with a barbed and a pointed end. Filament growth is preferentially directional by adding actin monomers to the barbed end. Actin filaments can be linear, branched and/or bundled, resulting in a wide variety of cellular actin structure. Variable actin structures result from the differing activity of cellular actin regulatory proteins which nucleate, elongate, sever, crosslink and bundle actin filaments.

Identification of cellular actin machinery by study of L. monocytogenes

Understanding regulation of actin organization in eukaryotic cells is complicated by the large number of actin regulatory systems present, the redundant role of many proteins and the dynamic state of actin structure. Therefore, identifying actin machinery and regulatory roles of individual proteins in the eukaryotic system was a daunting task. In contrast, actin-based motility of the bacterium *Listeria monocytogenes* represents a simplified model for understanding cellular regulation of actin dynamics. This system was used to identify important core actin regulatory machinery in eukaryotes. *L. monocytogenes* infects the cytoplasm of host cells and recruits the cell's actin machinery in order to build a propulsive actin comet tail. A single bacterial protein, ActA, is both necessary and sufficient to induce actin polymerization and generate bacterial motility in both cells and lysates (Tilney et al., 1992a; Tilney et al., 1992b). ActA does not directly initiate actin polymerization, but rather recruits cellular actin regulatory proteins that nucleate actin filaments and drive actin polymerization. Study of *L. monocytogenes* has

identified two important actin regulatory systems in mammalian cells, the Arp2/3 complex and ena/VASP family proteins (Skoble et al., 2001). Arp2/3 is part of the core of cellular actin regulators responsible for actin assembly, turnover and crosslinking whereas VASP is part of a higher order regulatory system responsible for modifying the primary actin structure (Scott et al., 2006). VASP improves efficiency and velocity of mobility, but, unlike Arp2/3, is not required for *Listeria* movement (Niebuhr et al., 1997), supporting the hypothesis that VASP modulates actin structure.

ActA recruits cellular actin machinery to build the bacterial comet tail through specific domains which recruit Arp2/3 or ena/VASP proteins, respectively. This suggests that cells contain endogenous proteins with similar domains which likewise function to recruit actin machinery to cellular sites. Determining how ActA recruits VASP to alter actin structure of the comet tail has led to understanding of how the cell normally recruits these proteins to specific regions where actin polymerization is ongoing. The central domain of ActA contains four proline rich repeats, (D/EFPPPPXD/E) termed FP4 or ActA repeats, which bind ena/VASP family members (Niebuhr et al., 1997). Zyxin (discussed below) is believed to be a mammalian homolog of the bacterial ActA protein, functioning to recruit ena/VASP and modulate actin dynamics at specific cellular sites (Golsteyn et al., 1997).

Numerous actin regulatory proteins have been identified since the initial *Listeria* studies, many of which are recruited to nascent AJ sites (Yap *et al.*, 1997; Vasioukhin *et al.*, 2000). These include actin nucleators such as the Arp2/3 complex and formin-1 (Kovacs *et al.*, 2002; Kobielak *et al.*, 2004), actin assembly modulators such as cortactin and VASP (Scott et al., 2006), and actin bundling proteins such as α-catenin, α-actinin

and vinculin (Otey and Carpen, 2004; Drees *et al.*, 2005). α-Actinin is an actin cross-linking protein that localizes to cell-cell junctions (Taylor and Taylor, 1994; Knudsen *et al.*, 1995) and stabilizes actin-membrane linkages (Jamora and Fuchs, 2002). Myosin II mediates tension and contractility of the actin cytoskeleton by converting the chemical energy of ATP into mechanical force (Sellers, 2000) to cause oppositely directed actin filaments to slide against one another (Ivanov, 2008). Myosin II can also act as a structural rather than a motor protein, to cross link and bundle actin filaments, and is important for the establishment of junctional actin structure during cell-cell junction formation (Zhang *et al.*, 2005; Ivanov *et al.*, 2007; Ivanov, 2008). The variety of actin regulators present at nascent AJs indicates regulation of cell-cell adhesion is tightly controlled, and stresses the importance of local actin polymerization in the regulation of cadherin-based adhesion (Chan et al., 2004).

VASP fine tunes actin structure

VASP is a member of the ena/VASP family of actin regulatory proteins (Haffner et al., 1995; Reinhard et al., 1995). That ena/VASP family members are important for proper cellular actin organization is becoming clear with ongoing research (Krause et al., 2003; Trichet et al., 2007). But, despite a number of established functions for VASP in *in vitro* studies, how ena/VASP regulates actin organization at the biochemical level and the resulting effect on cellular behavior are not yet fully understood. Actin polymerizing functions of ena/VASP proteins include actin filament nucleation (Huttelmaier et al., 1999; Samarin et al., 2003), filament detaching capability (Samarin et al., 2003), filament bundling (Schirenbeck et al., 2006), profilin and G-actin recruitment (Reinhard et al.,

1995; Walders-Harbeck et al., 2002), and anticapping activity (Bear *et al.*, 2002; Barzik *et al.*, 2005; Applewhite *et al.*, 2007).

Research suggests Ena/VASP family proteins may serve to regulate cytoskeletal structure at cadherin mediated contacts by modulating distinct modes of actin organization at AJs. Disrupting normal VASP localization results in aberrant actin structure at adhesion sites (Grevengoed et al., 2003; Scott et al., 2006). Homophilic E-cadherin binding localizes Ena to the tips of cadherin based actin bundles at nascent junctions and to lamellipodia (Scott et al., 2006). Ena/VASP is necessary at both sites, yet regulates different actin organization. Depletion of VASP only at the cadherin based tips perturbs bundles without affecting lamellipodial protrusion (Scott et al., 2006). Thus VASP can regulate assembly of different actin structures perhaps due to the different VASP regulatory environments found at these sites For example, zyxin, which recruits VASP, localizes to tips of actin bundles in AJs, but not to lamellipodia (Vasioukhin et al, 2000; Rottner et al, 2001; Hansen & Beckerle 2006).

VASP exhibits a wide variety of actin regulatory functions, making it an ideal candidate for fine tuning basic actin structure to fit specific cell needs (Scott et al., 2006; Trichet et al., 2007). Migration is an important characteristic of cells undergoing EMT. VASP has been reported to both positively and negatively regulate motility (Bear et al., 2000; Hoffman et al., 2006), and is important in cell-cell adhesion and morphogenesis (Scott *et al.*, 2006; Gates *et al.*, 2007). VASP localizes to the tips of actin bundles and the leading edges of migrating fibroblasts where it regulates filament elongation and branching, thereby generating force production and cell surface protrusiveness (Bear et al., 2000; Krause et al., 2003). *Drosophila* ena directs axon guidance and complete

zygotic/maternal loss of ena shows morphogenetic defects in epithelial zippering and dorsal closure (Gates et al., 2007). Likewise, disrupting Xena (*Xenopus* ena) elicits defects in neural plate formation and neural tube closure (Roffers-Agarwal et al., 2008). Ena mutants in *Drosophila* reduce F-actin accumulation at cell contacts (Baum and Perrimon, 2001). VASP is also important in AJ formation. It localizes to AJs in a variety of cell types: *Drosophila* epithelia (Baum and Perrimon, 2001), MDCK cells (Hansen and Beckerle, 2006), mammary cells (Scott et al., 2006) and murine keratinocytes (Vasioukhin et al., 2000). Since VASP can clearly effect changes in cytoskeletal behavior, it is a likely candidate for regulating filamentous actin organization at AJs (Baum and Perrimon, 2001), but how VASP activity is regulated in different cellular environments is not yet known. Zyxin recruits VASP to AJs and colocalizes with VASP, so it is a likely candidate for regulating VASP activity there (Vasioukhin *et al.*, 2000; Hansen and Beckerle, 2006).

Zyxin regulates actin through VASP

Zyxin is an 82 KD phosphoprotein recruited to sites of cell-cell adhesion as well as cell-matrix adhesions (Crawford and Beckerle, 1991; Vasioukhin *et al.*, 2000; Hansen and Beckerle, 2006). Zyxin contains four ActA repeat domains near its amino end and three LIM domains at its carboxy-terminus (Figure 1). Zyxin also contains a binding site for α-actinin at its amino-terminus (Crawford et al., 1992). Zyxin localization to various cell sites is mediated by different domains. Zyxin localizes to AJs via its central domain (Hansen lab, unpublished data) and to focal adhesions by its LIM domains (Drees et al., 1999; Nix et al., 2001). Zyxin localization in cells is dynamic. For example, zyxin moves from AJs to localize more strongly to stress fibers when cells are mechanically stressed

(Yoshigi et al., 2005), suggesting zyxin's recruitment and regulation can be altered to meet specific cell needs. Thus zyxin is a likely candidate for a regulatory role in cell adhesion.

Zyxin is an endogenous mammalian homolog of the bacterial ActA protein (Golsteyn et al, 1997). It is important for spatial and temporal control of actin polymerization in mammals through its recruitment of VASP. The proline rich domain of the bacterial ActA protein is sufficient to recruit VASP to cellular sites and modify actin structure. Expression of the ActA repeat domain, using the inner plasma membrane localization sequence CAAX, results in mislocalization of VASP along with production of actin rich structures (Gertler *et al.,* 1996; Golsteyn *et al.,* 1997; Drees *et al.,* 2000). Zyxin contains 4 proline rich ActA repeats, and is sufficient to recruit VASP to sites of increased actin filament formation, both at AJs and focal adhesions (Hoffman *et al.,* 2003; Hansen and Beckerle, 2006; Hoffman *et al.,* 2006).

Zyxin both recruits VASP to cellular sites where zyxin localizes, and regulates VASP function. Zyxin binds ena/VASP family members via its ActA repeat domains (Golsteyn et al., 1997; Nix et al., 2001) and by its LIM domains (Moody et al., 2009). The LIM domain can also bind zyxin, resulting in a head tail interaction that acts as a negative regulatory element preventing zyxin-VASP binding under certain conditions (Moody et al., 2009). Zyxin LIM domains also appear to negatively regulate VASP function. Thus, a model for zyxin regulation of VASP activity is dependent on zyxin domains and conformation (Figure 2). Zyxin recruits VASP to sites of active actin polymerization through its ActA repeat domain, and modulates VASP activity through variable LIM binding. Zyxin folded on itself cannot recruit VASP. Open zyxin, in linear

conformation, can recruit VASP through binding the ActA repeat domain, resulting in an activated zyxin/VASP complex in which VASP is functional. But VASP bound by zyxin at both its ActA and LIM domains, results in an inactive complex in which VASP function is inhibited. Previous work demonstrating that altered zyxin function affects both the rate and strength of cell-cell junction formation in MDCK cells (Hansen & Beckerle, 2006) supports the hypothesis that zyxin regulates VASP function. In this study, cells expressing constitutive active zyxin mutants exhibit faster, stronger junction formation and increased VASP localization to AJs. These mutants lack LIM domains (Figure 3); indicating LIM domains negatively regulate VASP function and junction formation. Dominant negative mutants also lack LIM domains, but importantly, have mutated ActA repeat sites, so they cannot bind VASP. These mutants still localize to AJs however, displacing endogenous zyxin and thereby acting as a dominant negative by decreasing VASP recruitment to AJs. Slower, weaker junction formation in these cells demonstrates the necessity for VASP in normal cell-cell junction formation.

In a model in which VASP regulates cytoskeletal structure, local control of VASP activity is critical for regulation of actin organization. Zyxin is an excellent candidate for regulation of VASP function at cell-cell adhesions. Zyxin mutants require VASP binding to increase cell-cell junction formation and strengthening of adhesion (Hansen and Beckerle, 2006), indicating that zyxin's actin regulatory function contributes to cell-cell adhesion through VASP. Consistent with this, VASP activity is also required for strong cell-cell adhesion (Vasioukhin *et al.*, 2000; Scott *et al.*, 2006). Not surprisingly, effects on cell motility similar to VASP misexpression have been observed with misexpressed zyxin. Zyxin overexpression results in slower migration, and depletion, in faster

migration of fibroblasts (Hoffman et al., 2006), as observed for VASP misexpression (Bear et al., 2000; Krause et al., 2003), supporting the hypothesis that zyxin regulates VASP function. Zyxin expression has also been found to affect cancer progression. Metastasis of cancer cells resembles EMT (Thiery, 2002). Zyxin expression changes are associated with increased cancer metastasis (van der Gaag *et al.*, 2002; Sy *et al.*, 2006) indicating a possible role for zyxin in regulating EMT.

Potential role for zyxin-VASP in morphogenesis

The actin cytoskeleton is plastic and actin filaments can be rapidly remodeled in response to changing cellular signaling and extracellular conditions. Zyxin, which localizes to cell-cell contacts and binds cytoskeletal regulatory proteins such as VASP and α-actinin is an excellent candidate for mediating this reorganization. Based on this, it seems reasonable that zyxin would exhibit important roles in development. A number of recent studies indirectly support a role for zyxin in morphogenetic remodeling. Ena/VASP proteins have been shown to be required for normal development. For example, ablation of Xena (an ena/VASP family member in *Xenopus*), results in disrupted neural tube closure and neural plate defects in frogs (Roffers-Agarwal et al., 2008). This study concludes that Xena may regulate morphogenetic rearrangements by linking cytoskeletal actin to junctional membranes. Mice with disrupted ena/VASP activity exhibit defects in platelet function, nervous system development and formation of epithelial sheets (Hoffman et al., 2003). Surprisingly, no developmental defects were identified in zyxin (-/-) mice (Hoffman et al., 2003). However, mice express other proteins in the zyxin family, LPP and Trp6, as well as zyxin. These proteins exhibit significant overlap with zyxin structure and their function may mask the effects of

ablating zyxin (Hoffman et al., 2003). Interestingly, Lpp (a homologue of mammalian LPP) knockdown in zebrafish results in significant defects in neural plate structure and neural tube closure (Vervenne et al., 2008). Lpp mediates convergence-extension movements during zebrafish gastrulation. Morpholino knockdown results in embryos with a shorter body axis, broader and compressed somites, and a broader, shorter notochord. Lpp knockdown does not affect migration rate but directional migration dorsally is inhibited and cells fail to follow straight paths (Vervenne et al., 2008). The authors conclude Lpp is likely involved in actin reorganization and in PCP signaling. Zyxin may likewise exhibit important functions in development.

Epithelial invagination requires constriction of the apical cell surface, suggesting a mechanical link between AJs and actin filaments is required to create tensile force. Xena is required for apical constriction enabling cell shape changes necessary for wedge cell formation during neural tube formation (Roffers-Agarwal et al., 2008). Likewise, during *Drosophila* gastrulation, the ventral furrow forms by apical constriction of ventral cells. It was recently demonstrated that apical constriction is driven by contraction of a medial actin network, which is linked to cadherin-based junctions through an undefined mechanism (Martin et al., 2008). Connections between medial actin networks and AJs enable contractile forces in individual cells to be transduced across the tissue, driving tissue reorganization at the ventral furrow (Martin et al., 2008). This example highlights the importance of physical linkages between AJs and medial actin networks for coordination of cell movement during tissue remodeling.

MDCK tissue culture model

The ability of cells to reorganize the actin cytoskeleton is essential for morphogenetic responses to different cellular environments, yet in depth analysis of structural actin reorganization events during EMT has not been performed. This research is designed to characterize specific actin rearrangements during morphogenesis and identify a possible role for zyxin in regulating such rearrangements. I use the MDCK tissue culture model for cell-cell adhesion and simulate morphogenetic reorganization by inducing small adherent colonies of MDCK cells to undergo EMT. This model enables high resolution examination of changing actin structure, protein localization, and cellular behavior of cells in an EMT signaling environment. This reductionist approach, using induced EMT to mimic morphogenetic rearrangements, answers basic, fundamental questions about how cells use regulation of the actin cytoskeleton to control epithelial morphogenesis in a simple controlled system, eliminating confounding difficulties inherent in more complex developmental models for morphogenesis.

Madin Darby canine kidney (MDCK) cells are a well established model for studying both formation and disruption of cell-cell adhesion (Adams *et al.*, 1996; Palacios and D'Souza-Schorey, 2003; de Rooij *et al.*, 2005; Palacios *et al.*, 2005). MDCK cells are epithelial cells which, in 2D culture seeded at low confluence, form small non-migratory colonies of cell-cell adherent cells (Behrens et al., 1985). These cells can be induced to undergo EMT by addition of hepatocyte growth factor/scatter factor (HGF) (Stoker and Perryman, 1985). HGF is a growth factor that binds and activates the MET receptor (Weidner et al., 1993). MET is a receptor tyrosine kinase (RTK) that drives EMT when activated by its ligand, HGF. MET activation has been implicated in cancer

progression in a number of different cancer types (Hurle et al., 2005), driving changes in cell adhesion, migration and invasion, that are hallmarks of EMT (Parr and Jiang, 2001). Individual MDCK cells assume a migratory, fibroblastic phenotype as cells in colonies disrupt cell-cell adhesion and scatter in response to HGF treatment (Stoker and Perryman, 1985; Palacios and D'Souza-Schorey, 2003). Therefore HGF induced scattering of MDCK cells is an ideal model to study cellular regulation of loss of cell-cell adhesion and its implications for morphogenetic rearrangements as well as cancer progression in epithelial tissues.

Since actin plays fundamental roles in both cell-cell adhesion and migration, cells undergoing EMT must make dramatic structural changes to the actin cytoskeleton to support these differing functional roles. It has been generally observed that actin is significantly remodeled in MDCK cells induced to scatter (Palacios and D'Souza-Schorey, 2003). Actin changes from its typical epithelial organization of actin cables tightly associated with AJs, to a typical mesenchymal actin organization of protruding lamellipodia with a loosely arranged network of actin filaments circumscribing individual cells. Actin reorganization at the periphery of colonies during HGF induced EMT has been more closely examined. The first morphological response to HGF is formation of filopodia and lamellipodia at the cell edges, coincident with cell flattening (Royal et al., 2000) in the first ½ hour after HGF treatment. However, a detailed analysis of actin remodeling at cell-cell junctions in adherent cells during EMT has not been performed.

Summary of literature review and Specific aims

Cadherin mediated calcium dependent cell-cell adhesion is tightly coordinated with the actin cytoskeleton to regulate cellular response to environmental stimuli resulting in cell shape changes, migration and changes in adhesion (Mege et al., 2006). Morphogenetic cell movements require reorganization of the actin cytoskeleton and changes in cell-cell adhesion. During morphogenesis and cancer metastasis cell-cell adhesions are disassembled when individual cells detach from epithelial tissues and migrate to distant sites in the process of EMT (Thiery, 2003). Cell adhesion and migration are not always mutually exclusive but rather are coupled during morphogenetic movements. For example, during gastrulation (Keller, 2002), neural tube formation (Duband et al., 1995), dorsal cleft (Gates et al., 2007) and wound closure (Poujade et al., 2007), sheet invagination and tube formation (Lubarsky and Krasnow, 2003), the actin cytoskeleton is actively remodeled while cell-cell adhesion remains intact (Miyoshi and Takai, 2008).

Cells undergoing EMT make dramatic structural changes to the actin cytoskeleton (Yonemura *et al.*, 1995; Vasioukhin and Fuchs, 2001; Pollard and Borisy, 2003). Actin reorganization during junction formation (Adams *et al.*, 1996; Adams *et al.*, 1998) and at non-contacting edges of cells undergoing EMT has been characterized (Royal et al., 2000). However, reorganization of actin associated with cadherin-based cell-cell adhesions during EMT has not been examined in detail. Nor is the cellular mechanism of actin linkage to junctional membranes clear. While anchoring of cadherin complexes to actin filaments via catenin proteins was previously accepted to link cadherin to actin in a stable, but static structure at AJs (Jamora and Fuchs, 2002; Kobielak and Fuchs, 2004),

this terniary complex has not been reconstituted (Gates and Peifer, 2005). It is now accepted that actin-membrane linkage in adherent cells is more dynamic than formerly thought (Drees et al., 2005; Yamada et al., 2005) and that currently unidentified proteins may mediate actin-membrane linkage.

Several actin regulatory systems have been implicated in maintaining cadherin-based adhesion (Kobielak et al., 2004; Miyoshi and Takai, 2008). The zyxin-VASP actin regulatory complex stands out as an ideal candidate for regulating actin dynamics at cell-cell adhesions. VASP has multiple described actin regulatory functions including mediating actin-membrane linkages (Reinhard et al., 2001; Trichet et al., 2007). VASP is recruited to cellular sites by binding to the proline rich ActA repeat motifs in zyxin (Golsteyn et al., 1997). Zyxin recruits VASP to focal adhesions (Drees et al., 2000) where it may serve to anchor actin filaments to the integrin complex (Drees et al., 1999). Zyxin and VASP also colocalize at AJs (Vasioukhin *et al.*, 2000; Hansen and Beckerle, 2006; Scott *et al.*, 2006) and could mediate actin-membrane linkages there as well. Consistent with this idea, zyxin mutants alter cell-cell junction formation and strengthening of adhesion in a manner that depends on the status of their proline rich VASP binding sites (Hansen and Beckerle, 2006).

I propose specific actin reorganization occurs at cell-cell adhesions during EMT, driving junction rupture and assumption of a migratory phenotype, and that zyxin mediates maintenance of actin-membrane linkage at AJs, thereby regulating EMT response. I test this by addressing the following specific aims:

1) Characterize junctional actin reorganization as MDCK cells undergo EMT
2) Identify actin regulators driving this process
3) Define the role of zyxin in regulating HGF induced EMT response.

The results of this study reveal that junctional actin is reorganized into medial contractile actin networks as individual actin cables are released from AJs. These cables remain connected to points of cell-cell adhesion in the junctional membrane to which zyxin and VASP localize conspicuously. Disruption of these connections precedes rupturing of cell-cell contacts and independent migration of individual cells. Zyxin's specific molecular function is characterized by a functional analysis. If zyxin mediated actin-membrane linkage affects maintenance of epithelial integrity then disrupting zyxin function should show differences in EMT response in HGF induced epithelial cells. Expression of zyxin mutants alters cell scattering, specifically generating phenotypes that alter whether cells migrate in a coordinated or solitary fashion, consistent with zyxin-VASP complexes mediating actin-AJ connections during EMT. The most striking phenotype occurs in cells expressing a constitutive active zyxin mutant, where cells migrate without loss of cell-cell adhesion. The data strongly implicate zyxin in stabilizing actin-AJ linkages to control whether cell motility during EMT is coordinated or occurs in separate, individual cells.

Chapter 2. ACTIN REORGANIZATION DURING EMT

Epithelial-Mesenchymal Transition (EMT) is a process in which epithelial cells lose their polarized epithelial phenotype, become migratory and assume a mesenchymal phenotype. This process is a hallmark of epithelial tumor progression, as well as occurring normally during embryonic development (Thiery, 2003). Understanding the structural events enabling this transition, as well as the regulatory events controlling EMT response, is important to our understanding of cancer progression, embryonic development, and homeostasis.

HGF induced MDCK cell scattering

I used the tissue culture model of discrete colonies of MDCK cells undergoing HGF-induced scattering as a model system to define junctional actin rearrangements during EMT. This reductionist model enables identification of gross changes in cellular morphology and actin reorganization in a controlled experimental environment, without the confounding effects of a more complicated embryonic system. I first verified that my cultured MDCK cells undergo HGF induced scattering as has been observed by others (Royal and Park, 1995) and then identified specific stages of cell scattering. MDCK cells were seeded at low confluence on collagen coated imaging dishes, grown overnight and treated with HGF at the start of imaging. Phase contrast images were taken at 2 minute intervals for 12 hours to monitor cell scattering response. As expected, MDCK cells do not scatter without addition of HGF (Figure 4A). Though cells in untreated colonies shift position slightly within colonies over time, colonies are non-migratory and cell-cell junctions are stable. In contrast, HGF treated cells exhibit a dramatic scattering response (Figure 4B). First, all cells in each colony spread, increasing in area. Most colonies

complete cell spreading by 2 hrs after HGF treatment. After spreading, cells change shape, pinching inward to become crescent shaped. Coincident with shape changes, ruptures form along cell-cell junctions which expand to form larger intracolony spaces, leaving only small regions of cell-cell contact remaining. Finally, as cells migrate apart, retraction fibers form between points of remaining cell-cell adhesion. Retraction fibers stretch and break as cells fully separate, completing cell scattering. Colonies are typically fully scattering by 6 hrs of HGF treatment.

Junctional actin is reorganized into medial actin networks

The opposing processes of cell migration and cell-cell adhesion require different cytoskeletal actin organization. Migratory cells, which lack cell-cell adhesion, display a significantly different filamentous actin structure than cell-cell adherent epithelial cells (Yonemura *et al.*, 1995; Adams *et al.*, 1998; Pollard and Borisy, 2003), so it follows that actin filaments must be reorganized during the transition from an epithelial to a mesenchymal state. Actin reorganization during the opposite process of cell-cell junction formation is well documented for MDCK cells and keratinocytes (Adams *et al.*, 1996; Adams *et al.*, 1998; Vasioukhin *et al.*, 2000; Vaezi *et al.*, 2002; Zhang *et al.*, 2005). Actin reorganization at the periphery of colonies during HGF induced EMT has also been examined (Royal et al., 2000). However, cytoskeletal actin reorganization at AJs occurring during EMT has not been characterized. In order to identify specific actin structures occurring during the transition from an adherent to a migratory cytoskeletal actin arrangement, I examined junctional actin structure in MDCK cells undergoing HGF-induced scattering by phalloidin staining fixed cells and by direct fluorescence imaging of GFP-actin expressing cells.

Fixed cell immunofluorescence

MDCK cells were seeded at low confluence on collagen coated coverslips, grown overnight, then fixed and stained following HGF treatment for 0, 2, 4 or 6 hrs. Fixed cells were costained with phalloidin and cadherin antibodies to visualize actin rearrangement with respect to junctional membranes. (Phalloidin labels filamentous actin. Cadherin is an important component of cell-cell junctions; cadherin staining labels where cell junctions are located on the cell membrane.) As expected, in untreated colonies, cytoskeletal actin is closely associated with the junctional membrane, lining cell-cell junctions (Figure 4C). After HGF treatment, this actin structure changes dramatically. Multiple individual actin cables appear alongside cell-cell junctions. Cadherin staining demonstrates these actin cables are unattached to the junction along their lengths, though they remain anchored into the junctional membrane at their ends, in bright yellow punctate spots (Figure 4D). This colocalization does not demonstrate a direct actin linkage to the cadherin complex but does indicate individual actin cables interact with the cell-cell junctional membrane and that the observed actin puncta are not basal membrane associated focal adhesions (since focal adhesions do not contain cadherin). Further suggesting interaction with the junctional membrane, local extrusion of E-cadherin staining membrane into the cytosol demonstrates individual actin cables generate tension along the junctional membrane at anchor points (Figure 4D).

Individual actin cables are arranged into different types of actin networks within cells. Cables anchored at both ends into the junctional membrane form transcellular networks, spanning individual cells (Figure 4D). Cables can also be coordinated across multiple cells through the punctate spots of actin lining the junctional membrane, forming

actin networks spanning multiple cells (Figure 4E). Alternately, individual actin cables can be membrane anchored at only one end, converging into a hublike central actin structure at the other end to form radial actin networks within cells (Figure 4F).

The dramatic changes in cytoskeletal actin organization occurring during EMT involve crosstalk between the apical actin filaments supporting cell-cell adhesions and basal actin stress fibers, associated with focal adhesions, which are important in migration (Miyoshi and Takai, 2008). Radial actin networks may mediate this cross talk. In radial networks, individual cables are anchored to AJs at only one end and associate with focal adhesions at the opposite end, either directly to stress fibers, or through the hublike central actin structure from which apical network cables and basal stress fibers emerge (Figure 4F, also see 10D below). Like transcellular networks, radial actin networks also apply force along the junctional membrane, as evidenced by cadherin membrane extrusions (Figure 4F).

During cell scattering, cells first spread, then change shape and form intracolony spaces as cell-cell junctions begin to rupture (Figure 4B). Forces applied by medial actin networks may facilitate these shape changes. Actin puncta along the junctional membrane appear to converge at remaining points of cell-cell adhesion as intracolony spaces expand (Figure 4G). Highlighting the role of actin-membrane linkages in reinforcing cell-cell adhesion, initial ruptures in cell-cell adhesion occur between areas where punctate actin-membrane linkages are concentrated (Figure 4G).

Formation of medial actin networks composed of individual actin cables suggests that prior to scattering, AJ associated actin is arranged into a larger cable of junctional actin by tight bundling of many smaller actin cables. Upon HGF signaling, these

individual cables appear to be released from the higher order junctional actin structure, weakening the AJ through loss of actin stabilization, and forming a contractile network anchored to AJs only at specific points. The presence of parallel actin cables alongside junctions suggests these cables originate from the adherens junction associated actin as cells flatten and spread in the first stage of cell scattering. Supporting this hypothesis, junctional actin decreases as individual actin cables appear. I quantified junctional actin levels after HGF treatment in 3 ways, all of which demonstrate a decrease in junctional actin during EMT, coincident with the appearance of individual actin cables. First, using masks segmented by pixel intensity, I quantified total filamentous actin in the cell and the subset of filamentous actin associated with AJs (that colocalized with E-cadherin). Over time after HGF treatment, the ratio of actin colocalized with cadherin to total cellular actin decreases (Figure 5A). Without HGF 70% of total actin is associated with junctions and the colocalized actin mask looks essentially the same as the total actin mask, whereas after HGF treatment, junctional actin levels drop to about 20 %, coincident with the appearance of individual actin cables alongside junctions (Figure 5B). The appearance of individual actin cables correlates with cell spreading. So I next compared the intensity of junctional actin in spread verses less spread cells, stained for actin and cadherin, by pseudocoloring to quantify the intensity of actin colocalized with cadherin. In more spread cells (arrow), there is less actin at junctions (so they appear bluer) than in cells which have not yet spread (arrowhead) which are more green and yellow, indicating a higher level of junctional actin (Figure 5C). Finally, I measured actin intensity across cell-cell junctions directly by creating line intensity profiles of a line crossing the cell-cell junction perpendicularly. The line intensity profiles show actin intensity at points along

this line. Without HGF (dotted line) one high peak of actin is evident as the junction is crossed, representing the junctional actin tightly juxtaposed along AJs. In contrast, after HGF treatment, the junctional peak is reduced and multiple smaller actin peaks appear on either side of the AJ. These analyses clearly demonstrate that junctional actin is decreased coincident with the appearance of individual actin cables alongside junctions, suggesting actin cables are derived from junctional actin. Further suggesting that cable release represents the "fraying' of a larger cable, the anchor points at the ends of individual filaments can be seen most frequently near the cell vertices adjacent to the fraying contact and with decreasing frequency farther from vertices, suggesting anchor points originate at vertices and disperse along AJ lengths as cables are released.

GFP-actin live cell imaging

Fixed cell staining suggests that medial actin networks are formed dynamically from junctional actin during EMT. To verify the dynamic relationship between actin structures observed in cells fixed during EMT, I performed time lapse fluorescence imaging of MDCK cells stably expressing GFP-actin, for 10 hours after treatment with HGF. An automated stage allowed multiple points to be imaged and 10-20 time-lapse series to be generated from each independent experiment. Analysis of time series from 8 independent experiments revealed a reproducible series of actin rearrangements occurring along AJs, as cells spread prior to scattering, which substantiate the sequence of events proposed from analysis of fixed cells. Importantly, all transitional actin structures observed in fixed specimens- release of individual actin cables, transcellular and radial medial actin network formation, dispersal of puncta from vertices and reconvergence to remaining points of cell-cell adhesion as junctions rupture, intracolony space formation

and retraction fiber formation- were also observed by direct fluorescence in living cells expressing GFP-actin. Prior to HGF addition, cells display cortical actin tightly associated with AJs, as expected. Following HGF addition, individual actin cables detach from the junctional actin and, while remaining parallel to the AJ, move medially to form a transcellular actin network (Figure 6A, B). GFP fluorescence at junctions diminishes as this process continues, as would be expected as actin is lost from AJs. Kymograph analysis confirms that individual cables originate from actin associated with AJs, rather than assembling *de novo* (Figure 6C). Individual cable ends are initially anchored to AJs at cell vertices. Vertices display an increase in actin fluorescence intensity (Figure 6A), representing a burst in actin polymerization. This burst in polymerization may be due to stabilization of actin-membrane linkages at the ends of individual cables. Actin at vertices then fragments into smaller puncta, which disperse away from cell vertices, to which individual actin cables remain connected (Figure 6A, B). The resulting medial actin networks formed within individual cells interface through actin-membrane linkage points at cell-cell junctions, forming a multicellular cytoskeletal network (Figure 7A), like that observed in fixed specimens (Figure 4E). Alternatively, individual cables are integrated into convergent medial actin structures within individual cells to form radial networks (Figure 7B) which apply force along AJs as junctions rupture. Intracolony spaces form and expand, rupturing cell-cell junctions between points of actin-membrane linkage (Figure 7C), as observed in fixed cell staining. Remaining points of cell-cell adhesion merge as intracolony spaces expand and junctions rupture. These points of remaining cell-cell adhesion persist as cells separate, resulting in formation of retraction fibers between separating cells. These networks are short-lived, lasting for about 2 hrs,

and vanishing prior to colony dissociation, although puncta along AJs remain. As intracolony spaces form and expand (Figure 6B), puncta coalesce together again at points of persistent cell-cell adhesion from which retraction fibers form when cells separate. Surprisingly, even though puncta regularly merge at the base of forming retraction fibers, retraction fibers themselves do not exhibit any organized actin structure. However, the fact that puncta convergence usually coincides with RF formation suggests that actin puncta play a role in RF formation.

Individual cables apply force at actin-membrane anchor points, demonstrated by local extrusion of E-cadherin-rich membrane into the cytosol (Figure 4D and F), suggesting these cables are contractile. I examined myosin II localization during medial actin network formation by immunostaining fixed cells treated with HGF. Triple staining for cadherin, myosin and actin shows that myosin II is recruited to the individual cables of both transcellular (Figure 8A) and radial (Figure 8B) actin networks formed during HGF-induced EMT, demonstrating these cables are indeed contractile. It is interesting that individual actin cables released during EMT are contractile because recent work demonstrated that contractility of apical actin fibers is necessary for cell-cell junction formation and establishment of a polarized epithelium (Zhang *et al.*, 2005). I observe the reverse behavior during EMT, in which actin cables containing myosin are released from junctional actin as cells lose polarity and spread. Myosin contractility is also necessary for migration of cells during EMT, and has been proposed to physically wrench cell-cell junctions apart (de Rooij *et al.*, 2005). I observe that contractility of the fibers in the medial actin networks formed during EMT apply force to cell-cell membranes which could result in changes in cell shape or rupturing of cell-cell adhesion when force is

applied along junctions. However, if this force is transduced across junctions through multicellular networks, coordinated movement of cells within the epithelium could result. Such an event has been observed in developing fly embryos. During *Drosophila* gastrulation, the ventral furrow forms by apical constriction of ventral cells. It was recently demonstrated that medial actin networks, similar to the radial actin networks identified here, enable contractile forces in individual cells to be transduced across groups of multiple cells, driving epithelial tissue reorganization to form the ventral furrow during *Drosophila* gastrulation (Martin *et al.*, 2008). This apical constriction was shown to be driven by contraction of medial actin networks, attached to cadherin-based junctions through an undefined mechanism. Medial actin networks formed during MDCK cell scattering likewise apply force along cadherin based junctions during EMT.

In summary, data presented here demonstrate that the cortical actin associated with AJs is reorganized into contractile medial networks during EMT as individual actin cables, which remain linked to the junctional membrane at punctate points originating at cell vertices, are released from the higher order junctional actin. This results in formation of actin networks that can span cells, anchoring into the membrane at both ends, or interact with basal stress fibers through higher order medial actin structures. Individual released actin cables are contractile and apply force to junctions. This force can be dispersed across multiple cells through punctate linkages at AJs of multicellular networks or act within single cells.

Chapter 3. ACTIN REGULATORY PROTEINS IN EMT

Maintenance of cell-cell adhesion is important for stability of tissues, while junctional plasticity is needed for wound healing and establishment of new tissues and morphogenetic rearrangements during development. Misregulated disruption of AJ stability leads to cancer metastasis, therefore identification of cellular regulators of adhesion and the EMT program is important. That contractility of medial networks is transduced to membranes at sites of cadherin-based adhesion demonstrates that a physical linkage between actin networks and junctional membranes exists during early EMT. To identify proteins that mediate this linkage, I examined the dynamic localization during EMT of actin regulatory proteins known to localize to cell-cell junctions. I treated MDCK cells for 0, 2, 4, or 6 hrs with HGF and immunostained for α-actinin, VASP, zyxin, Arp 2/3 complex, cofilin, annexin, and villin. α-Actinin, VASP, and zyxin demonstrated interesting relocalization during EMT suggesting they may regulate observed junctional actin reorganization.

Dynamic localization of actin regulatory proteins

Actin bundling proteins function to crosslink individual actin filaments together, forming thicker cables. α-Actinin is an actin bundling protein that localizes to AJs (Knudsen et al., 1995) and may assist in maintaining higher order actin structure along AJs by bundling actin filaments (Jamora and Fuchs, 2002). Since the tight bundling of actin cables apposed along cell junctions is lost as medial actin networks form, I wondered if a-actinin and VASP may play a role in this process. I tested this by examining dynamic relocalization of these proteins during EMT. As expected, in untreated cells α-actinin localizes along AJs (Figure 9A). Two hrs after HGF treatment,

α-actinin is completely displaced from AJs, coincident with the appearance of individual actin cables alongside cell-cell junctions (Figure 9A), suggesting α-actinin may indeed function to maintain higher order actin structure observed at AJs of unstimulated cells. VASP also exhibits actin bundling activity (Bachmann et al., 1999; Schirenbeck et al., 2006) and localizes to cell-cell adhesions (Vasioukhin *et al.*, 2000; Hansen and Beckerle, 2006; Scott *et al.*, 2006), so may play a role in maintenance of higher order junctional actin structure as well. Like α-actinin, VASP uniformly localizes along cadherin based cell-cell junctions in untreated cells (Figure 9B). But unlike α-actinin, HGF treatment induces striking redistribution of VASP from AJ lengths to discrete puncta localized along AJs and at the ends of individual actin cables (Figure 9B). Thus, during EMT, VASP may mediate anchoring of individual actin cables to the junctional membrane. In contrast, the absence of α-actinin staining at points of actin-membrane linkage indicates that it does not mediate actin-membrane linkage during EMT.

Zyxin is proposed to mediate actin-membrane linkages at AJs, through VASP binding. Zyxin localizes to cell-cell adhesion sites (Vasioukhin *et al.*, 2000; Hansen and Beckerle, 2006) and zyxin affects actin organization by recruiting VASP to cellular sites (Crawford and Beckerle, 1991; Golsteyn *et al.*, 1997; Drees *et al.*, 2000; Hansen and Beckerle, 2006). I next examined zyxin localization during EMT to determine if zyxin also dynamically relocalizes. In mature adherent colonies, untreated with HGF, zyxin weakly, but uniformly lines AJs, with no puncta evident (Figure 9C). As expected, since zyxin forms a complex with VASP, zyxin exhibits localization dynamics identical to those of VASP upon HGF treatment (Figure 9D). After HGF treatment, zyxin relocalizes into discrete puncta along AJs. Zyxin puncta do not occur uniformly along the length of

AJs. Rather, puncta tend to localize at or near vertices (Figure 9C, D). In contrast, cadherin continues to line AJs after HGF treatment, but also appears in bright punctate spots near vertices (Figure 9C). Zyxin colocalizes only with cadherin puncta and is not evident along AJs exhibiting uniform, linear cadherin staining (Figure 9C). Interestingly, cell-cell junctions are distorted in regions where zyxin puncta are observed (Figure 9C), suggesting these puncta link contractile actin to the membrane. Indeed, zyxin puncta localize precisely where ends of individual actin cables terminate at AJs (Figures 10A-D) in transcellular, multicellular and radial actin networks. These data suggest that zyxin puncta represent a novel response to HGF signaling, anchoring released individual actin cables to the junctional membrane at discreet points.

The cadherin associated, zyxin-VASP rich actin puncta that appear in response to HGF treatment, but preceding junction rupture (Figure 4G and 10B), are reminiscent of those observed during junction formation (Hansen and Beckerle, 2006). This suggests that cadherin/actin puncta in nascent junctions, observed to coalesce into plaques at cell vertices (Adams *et al.*, 1996; Adams *et al.*, 1998), may not be transitory structures, but may persist, though hidden in mature junctions, representing strong points of cell-cell adhesion which anchor cytoskeletal actin filament ends into the junctional membrane at vertices. The fact that these hypothesized persistent puncta are not visible in mature adherent colonies could be due to their concentration at vertices, which stain brightly for cadherin and actin and could mask the presence of individual puncta. Using a cadherin-based cell-cell adhesion model in which cadherin transfected Cho cells adhere to cadherin coated substrate, Scott et al. (2006) identified two pools of ena/VASP recruitment to cadherin-based cell-cell adhesions, one of which localized with cadherin macroclusters.

This pool was associated with prominent actin bundles, which terminated at the cadherin macroclusters and traversed the cells. They suggest that ena/VASP proteins serve as regulators of different cytoskeletal actin structure through distinct modes of actin organization at cell-cell contacts. VASP has been proposed to mediate actin-membrane linkages (Samarin et al., 2003; Trichet et al., 2007) consistent with its observed localization to persistent membrane anchor points at sites of cadherin-based adhesion during HGF induced EMT.

Interestingly, zyxin has binding sites for both VASP and α-actinin (Figure 1), indicating it may play important regulatory roles in mediating actin-membrane linkage both in unstimulated and in scattering cells, through different mechanisms. α-Actinin has been proposed to link actin to membranes at cell-cell junctions through binding the cadherin complex protein α-catenin (Knudsen et al., 1995). However, efforts to reconstitute this quaternary complex of cadherin-α-catenin-α-actinin-actin have been unsuccessful (Yamada et al., 2005). Zyxin, which localizes uniformly along cell-cell junctions in unstimulated adherent cells, could mediate linkage of bundled actin cables, along their length, to the junctional membrane through binding of α-actinin. During HGF induced EMT, as actin is reorganized into medial actin networks, α-actinin, zyxin and VASP are all lost from junction lengths while only zyxin and VASP relocalize to punctate spots along AJs, where ends of individual actin cables terminate. This suggests zyxin, through VASP, mediates end-on membrane anchoring of individual actin cables during EMT. The multiple actin regulatory functions of VASP, including actin filament nucleation and polymerization as well as filament detaching capability (Samarin et al., 2003; Trichet et al., 2007), could regulate whether actin-membrane linkage of individual

actin cables is sustained or disassembled, depending on VASP function. Since zyxin is proposed to regulate VASP function, altering zyxin function could result in altering regulation of actin-membrane linkage during EMT.

Chapter 4. FUNCTIONAL ANALYSIS OF ZYXIN

Zyxin's dynamic localization to puncta abutting the ends of medial actin networks suggests it functions in regulating actin rearrangements during EMT. If zyxin-VASP complexes mediate actin-AJ linkages, perturbation of zyxin function would alter the ability of cells to scatter. To test this, I perturbed zyxin function by developing MDCK cell lines stably expressing previously characterized zyxin mutants (Hansen and Beckerle, 2006). These mutants (Figure 3) lack the regulatory LIM domains, which mediate a head-tail interaction that prevents VASP binding under certain conditions (Moody et al, 2009) and are proposed to regulate VASP function (Figure 2). Zyxin binds VASP via its four ActA repeat domains (Golsteyn et al., 1997). Constitutively active zyxin (zyxinΔLIM) contains functional VASP binding motifs but no regulatory LIM domains to inhibit VASP binding or function (Figure 3). Thus zyxinΔLIM is a constitutive active mutant because it recruits VASP to cell-cell junctions but cannot negatively regulate VASP function. Previous work characterizing the effect of mutant zyxin expression on cell-cell junction formation demonstrated that, as expected, zyxinΔLIM drives stronger cell-cell adhesion and increases incorporation of VASP into actin structures at cell-cell junctions. In contrast, expression of dominant-negative zyxin (zyxin4AΔLIM), where VASP binding motifs are mutated, exerts the opposite effect, exhibiting decreased VASP recruitment to AJs and slower, weaker junction formation (Hansen and Beckerle, 2006). Zyxin4AΔLIM is proposed to act as a dominant negative since it localizes to cell-cell junctions, displacing endogenous zyxin, but is unable to recruit VASP. Zyxin is known to localize to focal adhesions as well as cell-cell junctions, where it has been demonstrated to affect migration, spreading and stress fiber

morphology (Drees *et al.*, 1999; Yoshigi *et al.*, 2005). However, expression of these LIM deletion mutants should not alter zyxin function at the level of focal adhesions. LIM domains drive localization of zyxin to focal adhesions (Nix *et al.*, 2001), so zyxinΔLIM and zyxin4AΔLIM mutants, both lacking LIM domains, do not localize to focal adhesions. Cells expressing these constructs exhibit normal focal adhesion structure (Hansen and Beckerle, 2006).

In addition to the deletion mutants described above, I also created a cell line expressing full length zyxin (zyxin). Zyxin overexpressing cells have been previously shown to undergo normal cell-cell junction formation (Hansen and Beckerle, 2006). Full length zyxin and parental MDCK cells were analyzed as controls in the following experiments. I also created zyxin knockdown lines using plasmid-based short hairpin (sh) RNA expression against canine zyxin. Plasmid-based shRNA knockdown allowed development of stable cell lines with reduced zyxin protein levels. Zyxin knockdown by three plasmid variations was verified by Western blot, revealing that two lines successfully lowered zyxin levels significantly below those of control MDCK cells (figure 11F). These two lines were used for further analysis. Their results are averaged and reported as shZyxin.

Zyxin mutant expression alters cell scattering behavior

I reasoned that altering zyxin function at cell-cell junctions should alter scattering behavior if zyxin is involved in regulating EMT response through mediating actin-AJ linkage of medial actin networks. To define effects of mutant zyxin expression on cellular behavior during EMT, I performed phase contrast time-lapse imaging of each cell line every two minutes over 10 hours after HGF addition, and compared scattering response.

Interestingly, parental MDCK cells, full length zyxin, dominant negative and zyxin knockdown lines exhibit similar normal scattering responses: cell spreading, cell shape changes, intracolony space formation, and formation of numerous retraction fibers that break as cells separate, were observed in sequence and within similar time frames (Figure 11A-D). In contrast, zyxinΔLIM expressing cells often exhibit a different EMT phenotype. Scattering in zyxinΔLIM cells is often delayed (Figure 11E) and morphologically distinct (Figure 12A): cells become migratory but fail to fully detach from one another, forming long and persistent retraction fibers.

In order to reveal zyxin's molecular function in cell scattering, specific scattering 'phenotypes' generated by zyxin mutant expression were quantitatively ascertained. Data from over 100 colonies, from at least 3 independent experiments, were analyzed to determine average values for each cell line. To ensure differences in EMT behavior were not a result of experimental variability, I co-cultured control MDCK cells with zyxinΔLIM or zyxin4AΔLIM cells, and identified colonies expressing zyxin mutants by DSred fluorescence, prior to initiation of the experiment. Expected differences in scattering behavior were observed in co-cultured cell lines.

To quantify differences in EMT response, I first measured the point of EMT initiation, the earliest point at which colonies begin to disrupt cell-cell contacts in response to HGF. I hypothesized that zyxin4AΔLIM cells, the line with weaker adhesion, would initiate scattering more rapidly than control MDCK cells or cells expressing full length zyxin; whereas constitutive active zyxinΔLIM colonies would initiate scattering more slowly. I also expected shZyxin response to mimic the dominant negative zyxin4AΔLIM response. I plotted EMT initiation point for all colonies and compared the

EMT initiation curves from each cell line. All cell lines exhibit a similar sigmoid curve shape, but as expected, the zyxinΔLIM curve is offset to the right, indicating these colonies exhibit delayed initiation of scattering. I curve fit these data and calculated the EMT initiation $t_{1/2}$ for each cell line (Figures 12 and 13). MDCK, zyxin, and zyxin4AΔLIM cells initiate EMT at similar rates, with EMT initiation $t_{1/2}$ values of 192, 181, and 191 min, respectively. In contrast, zyxinΔLIM cells initiate significantly later, with an initiation $t_{1/2}$ of 248 min, confirming the qualitative observation that scattering of zyxinΔLIM cells is delayed.

Surprisingly, dominant negative zyxin expressing cells did not exhibit the inverse response of a curve offset to the left, representing more rapid initiation of scattering. The zyxin4AΔLIM EMT initiation curve is the same as for control cell lines, MDCK and zyxin (Figure 12B). However, shZyxin cells do show the expected more rapid EMT initiation response. The shZyxin curve is slightly offset to the left (Figure 12B) and the EMT initiation $t_{1/2}$ of shZyxin (162 min) is significantly lower than control cell lines (Figure 12E).

I also compared average EMT initiation point for each cell line. This analysis gave similar results to the initiation halftime analysis (Figure 12C). These analyses suggest the zyxin4AΔLIM effect, if any, is insufficient to alter EMT initiation $t_{1/2}$, or, in other words, lack of VASP function at cell-cell adhesions does not increase the rate of EMT initiation. In contrast, zyxin knockdown is sufficient to increase initiation of scattering. This may be because the zyxin4AΔLIM construct is deficient for VASP binding but retains normal α-actinin binding function (Figure 3), whereas shZyxin knockdown cells are deficient in all zyxin function. This suggests α-actinin may play a

role in EMT initiation. Cell spreading analysis (below) also supports this conclusion. Another possible explanation for the different response in the dominant negative and zyxin knockdown lines is that shZyxin lines have zyxin depleted throughout the cell, including at focal adhesions, whereas in zyxin4AΔLIM cells, zyxin function at focal adhesions is presumably normal. Lack of zyxin function at focal adhesions in shZyxin cells may enable more rapid initiation of scattering response, followed by normal completion of scattering. Recent work in zyxin depleted cardiac precursor cells and normal murine mammary gland cells demonstrated that zyxin is relocalized from focal adhesions to basal stress fibers during EMT and an increase in stress fiber formation was required for normal scattering (Mori *et al.*, 2009). In contrast to my findings, in which zyxin knockdown cells initiate scattering more rapidly, followed by normal scattering behavior, Mori et al, (2009) found zyxin depletion abrogates EMT response. These differences could be due to differences in protocol. Mori et al, (2009) used TGF-β1 induced EMT which may induce different response than HGF. Also, Mori et al examined EMT in normal murine mammary gland (NMuMG) cells and cardiac atrioventricular canal cells whereas I use MDCK cells. The role of zyxin in EMT regulation may vary in these different cell lines. Most importantly, Mori et al., focus on zyxin regulation of EMT at the level of focal adhesions and stress fibers, whereas this study examines apical actin reorganization and altered zyxin function only at the level of cell-cell adhesions.

Constitutive active zyxinΔLIM expression at cell-cell junctions significantly alters cell scattering behavior (Figures 11E and 12A). Comparison of EMT initiation points demonstrates scattering is delayed in response to constitutive active zyxin expression, nevertheless, 90% of zyxinΔLIM colonies do still undergo scattering. This is only slightly

decreased compared to the other cell lines in which only 0-5% of colonies fail to scatter (Figure 12B). However scattering of zyxinΔLIM cells is frequently incomplete. I examined differences in scattering response following EMT initiation by counting instances of colonies exhibiting incomplete scattering (referred to as partial EMT) verses normal scattering. In normal scattering individual cells change shape, form intracolony spaces, become migratory, and detach from colonies (Figure 11A). In the partial EMT scattering phenotype (Figure 12A), scattering is initiated but does not occur generally throughout the colony. Intracolony spaces rarely form and entire colonies, rather than singe cells, become elongated and migrate collectively, with individual cells rarely detaching. Forty percent of zyxinΔLIM colonies exhibit partial scattering, failing to complete normal scattering after initiating EMT, compared to 3-9% in other cell lines (Figure 12D, E). This demonstrates that zyxinΔLIM cells are less likely to break cell-cell adhesions during EMT, even though they do become migratory. De Rooij *et al.* (2005) proposed HGF induced scattering in MDCK cells is not due to weakening of the AJ, (since cadherin levels remain stable until cells actually separate, and scattering cells retain the ability to reform junctions, indicating cadherin is not down regulated prior to junction rupture); but rather, is due to myosin dependent contractile force induced by migratory stimuli. In short, they propose cell scattering results from migratory forces physically wrenching AJs apart. Partial scattering of zyxinΔLIM cells demonstrates that cells can be migratory but fail to disrupt cell-cell adhesion, thus migratory forces alone are insufficient for junction rupture. This suggests that constitutive active zyxin activity stabilizes cell-cell adhesion or decreases migratory force.

EMT involves cell spreading, loss of cell-cell adhesion and increased migration (Mege et al., 2006). Therefore, zyxin mutant expression must alter cell scattering response through one or more of these processes. To identify which of these processes are affected by zyxin mutant expression I analyzed each process independently.

Zyxin functions in scattering independently of cell migration

Differences in scattering could result from altered migration rates between cell lines. For example, cells with slower migration rates could be expected to scatter more slowly. Altered zyxin function has been demonstrated to affect migration rate. Genetic ablation of zyxin results in increased cell migration in mouse fibroblasts (Hoffman et al., 2006), as does ablation of VASP (Bear et al., 2000). However, zyxin's effect on migration in these studies is exerted at the focal adhesion level. Zyxin LIM domains target zyxin to focal adhesions (Nix et al., 2001). Our zyxin mutants, which lack LIM domains, do not localize to focal adhesions and do not disrupt focal adhesion structure (Hansen and Beckerle, 2006), so I did not expect to observe an effect on migration rate in our constitutive active and dominant negative zyxin cell lines.

To assess whether zyxin mutant expression alters scattering by altering cell migration, I determined the migration rate of each cell line by measuring the surface area covered by migratory sheets as a function of time, using wound healing assays. The results show zyxin mutant cell lines migrate at similar rates, and all are slightly slower than parental MDCK cells (Figure 14A). Thus migration rate does not account for differences in EMT initiation $t_{1/2}$ nor scattering phenotype. In fact, zyxinΔLIM cells, which exhibit delayed initiation and partial scattering, migrate slightly faster than other cell lines, and shZyxin knockdown cells migrate more slowly but initiate faster,

demonstrating EMT initiation is independent of migration rate . Further, these results show that zyxinΔLIM cells are fully competent to initiate cell migration during EMT. Their increased frequency of partial EMT response, suggests that during scattering, zyxinΔLIM cells initiate migratory behavior but are less able to disrupt cell-cell adhesion and migrate independently.

It has been proposed that HGF induced scattering in MDCK cells is primarily a result of myosin dependent contractile force induced by initiation of migration, effectively wrenching junctions apart (de Rooij et al., 2005). If so, we would expect cells with increased migration rate to be able to scatter faster, and those with slower migration to scatter more slowly, thus, all the transfected cell lines tested should initiate scattering more slowly than MDCK controls, since all exhibit slightly slower migration. Yet, the observed effect on scattering is independent of migration. Only zyxinΔLIM cells exhibit delayed initiation of scattering, indicating another event besides migration forces is required for dissociation of cell-cell contacts during EMT. Given zyxin's localization at the ends of individual released actin cables during early EMT (Figure 10), that activity is likely control of actin-membrane linkages at AJs. I propose an additional step in rupturing of cell-cell junctions during EMT, in which actin is remodeled along AJs as individual actin cables are released and form medial actin networks, thereby weakening junctions and connecting the membrane to contractile actin structures, enabling junction rupture and cell scattering once migratory, contractile forces begin to operate. Interestingly, while analyzing wound healing images, it was observed than cell-cell adhesions were frequently ruptured near the wound edge in zyxin4AΔLIM cells, resulting in intracolony spaces forming during wound healing (Figure 14B, C). This indicates cell-

cell junctions are more easily disrupted in this cell line and suggests differences in cell-cell adhesion properties could account for differences in scattering observed between cell lines. Zyxin4AΔLIM cells were more spread during wound healing as well, indicating zyxin mutant expression could also affect EMT response by altering cell spreading.

Zyxin functions in scattering independently of cell spreading

Since cell spreading is the first step in scattering response, I next examined whether differences in cell spreading might account for changes in scattering observed in cells expressing zyxin mutants. I hypothesized that cells that are more compact initially may exhibit delayed EMT initiation since it may take longer for compact cells to spread fully. Cell spreading was quantified (Figure 15A) by measuring the increase in surface area covered by colonies of cells prior to initiating scattering. Initial average area per cell and maximal area reached before EMT initiation were determined for each cell line. Zyxin4AΔLIM cells exhibit significantly greater initial and maximal areas than parental MDCK cells or zyxin cells (Figure 15A). As expected, zyxin knockdown cell lines mimic dominant negative zyxin expression, exhibiting increased cell area, both initially and after spreading. The more dramatic spreading effects observed in shZyxin cells may be due to loss of both α-actinin and VASP recruitment by zyxin to cell-cell adhesions, whereas dominant negative zyxin cells only lack VASP. In contrast, as expected, zyxinΔLIM cells show the reverse effect in which initial and maximal cell area are decreased. Interestingly, even though zyxinΔLIM cell lines exhibit significantly smaller cell area both initially and after spreading, they exhibit a greater fold area increase during spreading than the other cell lines (3.4 fold increase in area compared to 2-2.9) (Figure 15B). This highlights the compactness of zyxinΔLIM cell lines. The increased

compactness of zyxinΔLIM and decreased compactness of zyxin4AΔLIM and zyxin knockdown lines, support a role for zyxin in modulating strength of cell-cell adhesion through VASP.

In addition to quantification of average cell area, the initial phenotype of colonies in each cell line was also characterized as compact, semi-compact or normal. 'Normal' phenotype cells exhibit typical MDCK cobblestone morphology in which cells are somewhat flat such that individual cells are readily discernable and the number of cells in the colony can be easily counted at time point 0, prior to any further flattening in response to HGF treatment. 'Semi-compact' colonies are somewhat rounded and exhibit regions of compactness where individual cells cannot be discerned, but in the majority of the colony cells are easily counted. In 'compact' colonies cells are tightly adherent to each other so that colonies are rounded and no individual cells can be distinguished until after cells have spread in response to HGF treatment. This analysis gave similar results to the initial area analysis. As expected since zyxinΔLIM cells exhibit decreased average initial cell area, zyxinΔLIM cells also exhibit a greater proportion of compact colonies than the other cell lines (Figure 15C), indicating that zyxinΔLIM cells are more tightly adherent to each other.

Increased compactness of colonies may limit subsequent cellular behavior associated with EMT; thus, spreading defects in zyxinΔLIM cells might account for their delayed EMT initiation. If so, initiation of EMT would inversely correlate with initial cell area and compact cells would initiate EMT later while more spread cells would initiate sooner. To test this, I measured the correlation between average initial cell area and time of EMT initiation for individual colonies from each cell line. R^2 values indicate no

correlation between initial area and EMT initiation point, demonstrating colonies from all cell lines initiate EMT independently of their extent of initial spreading (Figure 16). Cells of varying compactness initiate at the same time, and cells initiating at different times can have the same initial area. Thus, though zyxin mutant expression does alter cell spreading properties, initial colony compactness does not account for the observed differences in scattering behavior among cell lines.

Zyxin mutants induce defects in disassembly of cell-cell adhesion

Since neither migration nor differences in cell spreading account for EMT scattering phenotypes I next tested if differences in cell-cell adhesion could account for observed differences in scattering response between zyxin cell lines. Actin strengthens cell-cell adhesion, so I reasoned defects in regulation of actin-membrane linkage could result in alterations in cell-cell junction disassembly. Zyxin mediates linkage of actin cables to junctional membranes, so zyxin function may regulate maintenance of cell-cell adhesions during EMT, enabling coordinated movement of cells within the epithelium and preventing disruption of actin-membrane linkage and detachment of individual cells. I reasoned that a more detailed analysis of scattering 'phenotypes' of zyxin cell lines undergoing EMT would reveal whether defects in regulation of actin-membrane linkage result in alterations in cell-cell junction disassembly. I quantified cell-cell junction rupture events by measuring the frequency of intracolony space (ICS) formation following HGF treatment in each cell line.

The number of ICS formed in each colony over 7 hours of HGF treatment was divided by the number of cells in the colony to normalize values, enabling comparison of ICS formation in colonies of differing size. ICS formation is markedly reduced in

zyxinΔLIM cells (0.11 ICS/cell) and increased in zyxin4AΔLIM cells (0.68 ICS/cell), compared to parental MDCK cells (0.29 ICS/cell) (Figure 17A). The increase in ICS formation in zyxin4AΔLIM cells recapitulates ICS formation noted in wound healing experiments (Figure 14C). Surprisingly, shZyxin lines exhibit normal ICS formation (0.26 ICS/cell) and likewise did not exhibit increased ICS formation in wounded sheets, in contrast to zyxin4AΔLIM cells. This may be due to a disruption of stress fiber formation. Mori et al., (2009) demonstrated that zyxin mediates increased stress fiber formation during EMT in NMuMG cells. I observe that stress fibers interact with apical released actin fibers in radial networks (Figures 4F and 10B), generating force resulting in disruption of cell-cell adhesion and formation of ICS. Disrupted interaction of focal adhesion based stress fibers and apical medial actin networks in shZyxin lines could account for the failure to observe increased ICS formation in KD lines. Effects on stress fiber formation were not examined in this study. This research examines effects of zyxin mutant expression at the level of cell-cell junctions. The observed differences between constitutive active and dominant negative zyxin expression in cell-cell junction rupture phenotype are consistent with zyxin mutants affecting scattering by regulating actin-membrane linkage at cell-cell adhesions.

To further quantify junction rupture events, I next quantified retraction fiber (RF) behavior. Since RFs form from persistent points of cell-cell adhesion between separating cells, I reasoned if zyxin mutant expression indeed affects scattering through regulating actin-membrane linkage at cell-cell adhesions, then morphology and behavior of RFs would be altered. To test this, RF formation was measured over 7 hrs of HGF treatment. Consistent with the observation that zyxinΔLIM cells are less likely to rupture cell-cell

contacts, zyxinΔLIM cells produce significantly fewer RFs (Figure 17B). In fact, 62% of zyxinΔLIM colonies form no RFs in this time, whereas only 4-6% of colonies from the other cell lines fail to form any RFs (Figure 17C). RF morphology and behavior in zyxinΔLIM cells are also markedly different. RFs of increased length and persistence are clearly evident by time lapse imaging (Figure 12A) as compared to other lines analyzed (Figure 11A-D). Comparison of maximal RF length in each cell line demonstrates that these structures become much longer in zyxinΔLIM cells than in the other cell lines (Figures 18). The average maximum RF length was 62 µm (3.5 µm, SEM) for zyxinΔLIM, compared to 37 and 39 µm, (1.1 and 1.8 SEM, respectively) for MDCK and zyxin. Zyxin4AΔLIM and shZyxin cells exhibit a shorter average RF length of 29 µm (1.0 and 1.1 SEM, respectively). Quantitative analysis of temporal persistence of RFs (Figure 19) demonstrates that RFs also resist breaking in zyxinΔLIM cells, connecting cells for longer time periods. Long and persistent RFs are indicative of stronger cell-cell adhesion points, which are not readily disrupted. These results highlight zyxin function in maintaining sites of cell-cell interaction. EMT 'phenotypes' resulting from zyxin mutant expression, taken with zyxin localization during scattering implicate zyxin in regulating maintenance of actin-membrane linkages at cell-cell contacts during EMT.

Chapter 5. DISCUSSION

Morphogenetic remodeling of epithelial tissues plays a fundamental role in normal tissue homeostasis, embryonic development, and disease progression. Full EMT, in which cells completely detach from an epithelium and migrate independently to establish new tissues elsewhere, as during neural crest cell delamination (Duband et al., 1995), is only one example of a wide variety of morphogenetic rearrangements that remodel epithelial tissues during development. Epithelial plasticity also encompasses morphogenetic rearrangements in which individual cell movements are coordinated within epithelial tissues and occur without full detachment of individual cells, in events such as apical constriction, convergence-extension, and tubulogenesis. The range of epithelial plasticity changes observed during morphogenesis may represent cells and tissues attaining different levels of progression along a series of events from partial to full EMT (Huber et al., 2005; Leroy and Mostov, 2007). A key feature that distinguishes partial and full EMT is whether actin-based motility is transduced across cell-cell contacts through medial actin networks spanning multiple cells, resulting in collective cell movements, or whether cell junctions are disrupted and cells migrate independently. A major observation of work reported here is that zyxin regulates whether cell motility is independent (full EMT) or coordinated within tissues (partial EMT), by mediating actin-membrane linkages through zyxin-VASP complexes.

Model of junctional actin rearrangement during EMT

Apical constriction movements during gastrulation in *Drosophila* are dependent on formation of contractile medial actin networks coordinated through AJs (Martin et al., 2008), though the mechanism of membrane attachment remains unknown. We

demonstrate here that medial actin networks of mammalian epithelial cells undergoing morphogenetic rearrangements are derived from junctional actin and are membrane linked through zyxin-VASP complexes. In this model for actin rearrangement during EMT, AJ associated actin is first reorganized into medial contractile actin networks, which facilitate coordinated cell movement, as occurs in partial EMT, followed by disruption of actin-membrane linkages at points of cell-cell adhesion, enabling delamination of individual cells from colonies (Figure 20). After HGF addition, actin crosslinking factors α-actinin and VASP are displaced from cell-cell junctions (Figure 9), coincident with detachment of individual actin cables from AJ associated actin (Figures 4, 9B and 10). These cables are reorganized into medial contractile networks (Figures 4 and 8) in which individual actin cables retain specific actin-membrane linkages, through zyxin-VASP complexes (Figure 9D and 10). These linkages coordinate actin networks across multiple cells (Figures 4E and 7A). Membrane linked actin networks transduce myosin-dependent contractile forces to cell-cell junctions, as evidenced by local membrane deformations at sites of actin-membrane linkage (Figures 4D, F, and 8A, B).

Actomyosin-based contractility has been previously identified as necessary in EMT and has been suggested to wrench AJs apart as cells are stimulated to migrate (Palacios and D'Souza-Schorey, 2003; de Rooij *et al.*, 2005). The observations presented here, as well as those of *Drosophila* gastrulation (Martin et al., 2008), show that epithelial cells can opt to coordinate actomyosin-based contractility through AJs instead of allowing cell-cell detachment. Observation that apical constriction of ventral furrow cells in *Drosophila* gastrulation is driven by actomyosin contractility applied on a medial actin network and transduced through the tissue at points of cell-cell adhesion (Martin et al.,

2008) supports the concept that assembly of a medial contractile actin network, membrane-linked at points of cell-cell adhesion, is used to drive morphogenetic rearrangements that do not result in cell-cell detachment by transducing forces across cell-cell junctions. Our data indicate zyxin function mediates maintenance of actin-membrane linkage of medial actin networks, controlling whether actomyosin-based contractility is transduced through multiple cells, enabling coordinated cell movement within the epithelium, or results in rupture of actin-membrane linkage and separation of individual cells from the epithelium. Thus, the status of actin linkages to the junctional membrane serves as a critical factor in decisions about whether cell migration will occur in a coordinated (partial EMT) or independent (full EMT) fashion during epithelial tissue rearrangements.

Zyxin-VASP regulation of EMT

Scattering phenotypes in cells expressing zyxin mutants (Figures 11 and 12), combined with zyxin localization during scattering (Figure 10), strongly indicate that zyxin, through VASP, mediates maintenance or release of actin membrane linkage at AJs. Constitutive active zyxinΔLIM cells exhibit incomplete scattering when stimulated to undergo EMT, in which cells become migratory, but fail to disrupt cell-cell adhesions (Figure 12). The collective cell migratory behavior and persistent cell-cell adhesions (Figures 12Cand 17) observed in zyxinΔLIM cells suggest that zyxin mediates actin-membrane linkages at cell-cell contacts. Like zyxinΔLIM, zyxin4AΔLIM cells also lack LIM domains, but do not exhibit the partial EMT phenotype observed in zyxinΔLIM cells. Zyxin4AΔLIM cells cannot recruit VASP to cell-cell junctions. That zyxin mutants require intact VASP binding sites to generate the cell-cell detachment defective

phenotype shows that zyxin functions in EMT through VASP. Overexpression of full length zyxin also does not induce the partial EMT phenotype. That deletion of zyxin's LIM domains generates a mutant whose expression alters scattering differently from expression of full length zyxin implicates the LIM domain region of zyxin in regulating actin-membrane linkages. The Hansen lab recently demonstrated that the LIM domain acts as a negative regulatory element by mediating a head-tail interaction preventing zyxin-VASP binding under certain conditions (Moody et al., 2009). Ongoing work indicates simultaneous LIM binding to VASP inhibits VASP activity as well. Thus zyxin can regulate VASP activity, and thereby actin structure, through variable binding to VASP. Zyxin phosphorylation alters the availability of zyxin LIM domains to associate with binding partners (Hirota et al., 2000) and could control this autoregulatory mechanism. A detailed understanding of zyxin LIM domain-mediated autoregulation will provide molecular insight into how zyxin may regulate actin-membrane linkages, and thus EMT response. ShZyxin, like zyxin4AΔLIM, does not induce a partial scattering phenotype. However shZyxin expression does increase cell spreading (Figure 15A) and exhibits more rapid EMT initiation (Figure 12A, B). ShZyxin disrupts both α-actinin and VASP recruitment to AJs. Based on the increased effect of shZyxin expression on cell spreading, and the observed decrease in EMT $t_{1/2,}$ zyxin recruitment of α-actinin appears to be more important for cell spreading and initiation of scattering; while zyxin regulation of VASP activity is more important for regulating actin-membrane linkage at cell-cell adhesions and subsequent scattering behavior.

Recent *in vivo* studies of morphogenesis support the results of this tissue culture study. This study demonstrates zyxin functions to mediate coordinated cell movements

suggesting zyxin may regulate collective cell migrations during morphogenesis. Closure of the neural tube is a vital step during embryonic development which requires coordinated movement of opposite sides of the neural plate, to fuse and form the neural tube. Consistent with zyxin-VASP complexes mediating coordinated cell movements, ablation of Xena (a VASP family member) results in disrupted neural tube closure and neural plate defects in frogs (Roffers-Agarwal et al., 2008). Likewise, during embryonic convergence-extension movements cells migrate without fully disrupting cell-cell adhesion, changing their relative position within the epithelium, to converge at the midline of the embryo. These movements cause lateral narrowing of the embryo and extension of the head-tail axis. Knockdown of LPP (a close zyxin relative) results in convergence-extension defects in zebrafish in which embryos exhibit shorter and wider body axes than normal (Vervenne et al., 2008). This suggests LPP may regulate maintenance of actin-membrane linkage needed for collective cell migration, similar to our proposed role for zyxin. Metastasis of cancer cells resembles EMT and cancer metastasis is thought to result from misregulation of cellular EMT pathways (Thiery, 2002). Zyxin expression changes are associated with cancer progression (van der Gaag *et al.*, 2002; Sy *et al.*, 2006), indicating that zyxin may regulate cellular decision to undergo EMT, perhaps through the same mechanism proposed for its regulation of tissue remodeling in development. Studies of effects of zyxin mutant expression in trigeminal placode development in the chick are ongoing to demonstrate zyxin function in a developmental model.

Actin-membrane linkage

Identification of proteins that link actin filaments to cell-cell adhesions is a major question in cell biology. Transduction of tension forces from the medial actin network to the membrane (Figure 4D, F) demonstrates robust actin-membrane linkages. The ability of cadherin complexes to directly bind actin filaments, thus linking the AJ to the actin cytoskeleton, has been recently scrutinized concluding actin cables do not directly link to the cadherin complex (Gates and Peifer, 2005). Zyxin's defined interaction with α-actinin (Crawford et al., 1992) could dock zyxin-VASP complexes to AJs, perhaps even to cadherin complexes (Knudsen et al., 1995). This is not our view, however, as the α-actinin binding site in zyxin is dispensable for zyxin function in cell-cell adhesion (Hansen and Beckerle, 2008). Further, α-actinin is absent from points of actin-membrane linkage during EMT. While both localization and functional data presented here strongly implicate zyxin-VASP complexes in mediating actin-membrane interfaces at AJs during EMT, we do not suggest this occurs directly through interactions between zyxin-VASP and cadherin complexes. Identifying how zyxin docks with the AJ membrane remains to be determined and will provide important insight into the molecular basis of adhesion and tissue remodeling. Ongoing work in our lab has recently identified nectin as a potential membrane protein recruiting zyxin to AJs. Both cadherins and nectins localize to AJs and mediate interaction with the actin cytoskeleton through binding of adapter proteins on the cytoplasmic tail (Takai and Nakanishi, 2003; Gumbiner, 2005). Nectins are the first transmembrane proteins to initiate cell-cell adhesion, and they recruit cadherins to establish AJs (Takai et al., 2003). Interestingly, nectins are associated with actin bundles in a one to one ratio in testicular AJs and nectins are identified as important in coupling

cortical actin to junctional membranes (Ozaki-Kuroda et al., 2002). Zyxin could be an intermediary in this complex, regulating actin filament linkage to the junctional membrane through binding nectin.

Implications for cell-cell junction formation

Importantly, actin-membrane linkages described here appear to be more than specialized structures observed only during EMT. Structure and protein content of punctate actin-membrane linkages (Figures 4 and 9) and actin association with these puncta (Figures 4G and 10B) are strikingly similar to structures observed during cell-cell junction formation (Adams *et al.*, 1996; Adams *et al.*, 1998; Vasioukhin *et al.*, 2000; Vaezi *et al.*, 2002; Zhang *et al.*, 2005; Hansen and Beckerle, 2006; Scott *et al.*, 2006). By extension, actin rearrangements observed during early EMT could occur as a precise reverse of events seen during formation of cell-cell junctions. In nascent junctions, points of adhesion are formed in connection with a medial contractile actin network (Adams *et al.*, 1996; Adams *et al.*, 1998). In a myosin II dependent manner, individual filaments of the medial network become tethered parallel to the AJ (Zhang et al., 2005), an event likely facilitated by actin bundling proteins like α–actinin and VASP . Initial points of actin-membrane linkage then coalesce into cell vertices (Adams et al., 1998). We observe the reverse of these actin rearrangement events during HGF-induced EMT as individual cables are released from AJ-associated actin and form medial actin networks, as punctate actin-membrane linkage points disperse from vertices. Thus, assembly of a contractile medial actin network linked to points of cell-cell adhesion occurs during the transition between epithelial and mesenchymal states, in either direction (Figure 20).

Work presented here provides important new insight into the molecular basis of how cells physically drive the cellular behavior of EMT. This research identifies zyxin mediating actin-membrane linkages during EMT and demonstrates its role in coordinating cellular movements during this process, increasing our molecular understanding of how distinct types of epithelial tissue rearrangements are regulated. Full understanding of a cell biological process requires a detailed description, the identification of required components (proteins), and definition of protein function and mechanism. While much remains to be elucidated for the molecular basis of morphogenetic rearrangements, work reported here provides a detailed and testable model for how diverse morphogenetic events can be controlled through a small number of actin regulatory machines.

Chapter 6. MATERIALS AND METHODS

Cell culture

MDCK-G cells were cultured in DMEM supplemented with 10% FBS. Stable cell lines were generated by transfecting plasmids (pEGFP-N2 containing actin, pDSred2.C1 containing zyxin, zyxinΔLIM, or zyxin4AΔLIM, and pRNAT-CMV3.1/neo containing sh zyxin) into MDCK cells using Effectene reagent (Qiagen). Stable, polyclonal cell lines were generated by selection with G418 (10 µl/ml DMEM) (Fisher Biotech), followed by subcloning of fluorescent colonies. The pRNAT-CMV3.1/neo plasmids contained the following sequences that generate shRNA transcripts complementary to canine zyxin: shZyxin1 = GGATCCTATTGGACTTGTGACTGAACG, shZyxin2=GATCCTCTCTCCGCTGCCATCTAAGG, and shZyxin3 = GGATCCTCTCTCCGCTGCCATCTAAGG. In order to determine zyxin levels in MDCK cells stably transfected with pRNAT-CMV3.1/neo plasmids, equal amounts of protein in cell extracts from each cell line were separated in SDS-polyacrylamide gels, transferred to PVDF, and subjected to Western blot analysis using enhanced chemiluminescence (ECL, Pierce) for antibody-antigen binding detection. Blots were quantified by densitometry. Zyxin knockdown lines 2 and 3 were used for subsequent analysis. Scattering of MDCK cells was induced using conditioned medium containing HGF. MRC-5 cells, cultured in DMEM +10% FBS, were used to generate conditioned medium containing HGF. Each lot of conditioned medium was assayed by titration for activity in inducing scattering of MDCK cells and diluted appropriately. Twenty percent conditioned medium was used to induce scattering in all experiments.

Immunofluorescence

Cells were seeded at low confluence on collagen-coated coverslips. HGF media was added, following overnight incubation, at 2 hr intervals for 6 hrs. Cells were then washed with ice-cold Ringer's saline containing 1.8 mM $CaCl_2$, fixed on ice for 20 min with 4% paraformaldehyde in PBS, and blocked with PBS supplemented with 0.2% bovine serum albumin 50 mM NH_4Cl, 0.5% Triton X-100, and 0.5% normal goat serum. Cells were stained with primary antibodies against zyxin (B38), E-cadherin (3G8), VASP (BD Biosciences), Myosin IIA (Novus Biologicals), or α-actinin (Abcam, Sigma) or with Alexa Fluor 488-conjugated Phalloidin (Molecular Probes). Secondary antibodies used were goat anti-mouse or -rabbit antibodies conjugated to Alexa-488, -546, or -633. Coverslips were mounted in VectaShield (Vector) and viewed under an Olympus inverted IX-81 microscope with a 40X/0.75 air objective lens. Images were acquired with a Hamamatsu Orca-ER digital camera and analyzed using Slidebook software.

Live cell imaging

HGF induced scattering movies

To visualize MDCK cell scattering, 10,000 cells were seeded on collagen coated DeltaT (Bioptechs) imaging dishes in 1 ml DMEM +10% FBS and cultured overnight. Prior to imaging, media was changed to 1.5 ml HEPES buffered DMEM +10% FBS. A heated microscope stage using Delta T4 temperature regulation system (Bioptechs) maintained temperature at 36.5 C. Multiple (10-22) positions were imaged during each experiment. 300 µl of HGF media was added immediately prior to initiating imaging (20% v/v HGF to imaging media). Initial fluorescent images of the DSred tag expressed by our zyxin mutants were taken prior to initiating the time-lapse series to verify plasmid

expression in the colonies, after which, bright light images of each colony were taken. Phase contrast images of cell scattering were taken at 16X magnification using a 10X (0.30 aperture) objective and 1.6X slider, with 30 ms exposure time. Fluorescence time-lapse imaging was performed using a 40X (0.75) objective. Bright light images were recorded every 2 minutes for 12 hrs and fluorescent images every 3 minutes. The first 10 hours of each series was used for analysis, with the remaining time demonstrating the continued health of the culture. To ensure differences in EMT behavior were not a result of experimental variability, we co-cultured control MDCK cells with zyxinΔLIM, or zyxin4AΔLIM cells and identified colonies expressing zyxin mutants by DSred fluorescence, prior to initiation of the experiment. We observed the expected differences in scattering behavior in co-cultured cell lines.

Wound healing movies

Wound healing movies were generated by plating 500,000 cells on collagen coated DeltaT dishes in DMEM+10% FBS and culturing to 100% confluence in the presence of 20% HGF and grown overnight. Wounding was performed by scraping a 1000 µl pipette tip across the cells. HEPES buffered DMEM +10% FBS, with 20% HGF conditioned medium, was perfused into the culture. Images of wound closing were taken every 2 minutes for 6 hours using a 10X/0.30 air objective.

Quantitative analysis

Imaging analyses were performed using Slidebook software and Microsoft Excel. Fixed cells immunostained for E-cadherin and phalloidin were subjected to the following analyses:

Actin localization ratio

Actin localization ratio represents the ratio of F-actin colocalized with E-cadherin, divided by the total F-actin fluorescence in each image. This ratio was determined at 0, 2, 4, and 6 hrs after HGF treatment to measure changes in actin localization with respect to AJs over time. Five to ten images, of multiple colonies, were analyzed for each cell line using masks segmented by pixel intensity.

Actin/E-cadherin ratio

The ratio of actin to E-cadherin measures the amount of F-actin colocalized with E-cadherin (i.e., localized at AJs). The amount of actin present at AJs was displayed as a pseudocolor image of actin intensity gated by cadherin intensity. This ratio was compared in colonies with cells showing variable EMT spreading response to compare actin levels in spread vs. not yet spread cells.

Line intensity profiles

Line intensity profiles display F-actin intensity along a linear region of interest one pixel wide, perpendicular to and spanning a cell-cell junction. Line intensity profiles were compared in colonies with and without HGF treatment to visualize F-actin localization at junctions and alongside junctions.

Phase contrast live-cell imaging movies were subjected to the following analyses:

Scattering initiation point

The initial point at which cell scattering begins was determined by identifying the time (in minutes) at which cells within a single colony first exhibit a morphological response to HGF addition. Specifically, when two events (cell shape changes to form crescent shaped cells, intracolony space or retraction fiber formation, and/or cell

separation) occurred in different cells within 40 minutes of each other, followed thereafter by increased frequency of such events throughout the colony over the course of the next 60 minutes, a colony was deemed to have initiated. The time point at which the second initial event first occurred is the EMT initiation point. Experiments were analyzed by several individuals working independently to ensure standardization of results. Scattering initiation was compared by plotting the percent of initiated colonies of each cell line as a function of time over 10 hrs in 2 min intervals. Data were curve-fitted by Weibull regression and p-values generated by Yates chi square analysis. p-Values compare curves of mutant zyxin expressing cells to the wildtype MDCK response. The scattering initiation halftime was calculated by curve fitting the data and calculating the time at which 50% of colonies were initiated (Figure 12). The distribution of EMT initiation points approximated a normal distribution. Average EMT initiation times and corresponding p-values were also calculated using two tailed t-test to compare cell line values to MDCK (Figure 13).

Migration rate

The rate of migration of each cell line was calculated by measuring the surface area covered by migratory sheets at ½ hr intervals over 6 hours and calculating the slope of area increase over time. Migration rate is presented as the rate of area increase (μm^2 per minute). This method eliminates variation resulting from uneven movement of cells at the wound front.

Cell spreading

The amount of cell spreading was determined by measuring initial cell area prior to spreading and comparing to cell area at maximal spreading. Initial cell area was

determined by measuring the area of each colony imaged at the start of the experiment, at 0 min after HGF addition, and at maximum spreading before cells begin to scatter. Colony area values were divided by the number of cells in the colony and average cell areas were compared to account for variation in colony size. All colonies analyzed were 3-30 cells in size. Values for colonies 5-15 cells in size were compared to values for all colonies 3-30 cells in size, and demonstrated no significant difference, indicating larger colonies do not skew the data. Average maximum cell area determinations were calculated from the maximum colony area reached prior to initiation of scattering, measured for each colony at subsequent 20 min intervals. Maximal area is generally reached at about 2 hours after HGF treatment. Values were compared by SEM.

Initial and EMT phenotype

The initial phenotype for each colony in each cell line was determined by assigning an initial colony phenotype of normal, semi-compact or compact. Cells in 'normal' colonies are somewhat flat, such that individual cells are readily discernable and the number of cells in the colony can be easily counted at time point 0, prior to any further flattening in response to HGF treatment. 'Semi-compact' colonies exhibit regions of compactness where individual cells cannot be discerned, but in the majority of the colony cells are easily counted. In 'compact' colonies cells are tightly adherent to each other so that colonies are rounded up and no individual cells can be distinguished until after cells have spread in response to HGF treatment. The proportion of colonies in each cell line exhibiting each phenotype was calculated.

The scattering phenotype for each colony analyzed was determined by binning EMT response as full or partial EMT. Full EMT response was defined as colonies

undergoing the typical scattering response to HGF treatment. These colonies exhibit multiple events of ICS and RF formation, elongated cell shape, and separation of cells from colonies within 8 hrs of HGF treatment. EMT initiation in one region of the colony must be followed by initiation and separation events occurring generally throughout the colony in a continuous, smooth fashion. Though cell separation may be complete or cells may still exhibit RFs within the period analyzed, individual cells are clearly separating off in regions throughout the colony. Partial EMT response was defined as colonies that initiate, but initiation is not followed by general scattering throughout the colony. Colonies rarely form ICS and RFs, and few to no cells detach. Instead, cells migrate as contorted colonies which remain cell-cell adherent. A small percentage of colonies (0-5%) fail to initiate and were binned as not responding to HGF. The percent of colonies in each cell line exhibiting normal vs. partial EMT was determined and p-values calculated from z-scores comparing values to control MDCK.

Retraction fiber and Intracolony space formation

The number of RFs and ICSs formed in each colony analyzed was counted during the first 7 hours following HGF treatment. Values were normalized by dividing by the number of cells in the colony and RF/cell and ICS/cell were compared for each cell line. Maximum length reached and temporal persistence of each RF was also measured. (Only RFs at least 10μm long were included since RFs shorter than this are difficult to identify. However, note that zyxin4AΔLIM frequently exhibit RFs shorter than 10 μm, compared to the other cell lines, thus the reported value for number of RFs is artificially low for this line). p-Values comparing means for RF length and duration were calculated using 2 sided t-tests. The null hypothesis for two tailed t-tests is cell line value is the same as the

value for MDCK. The alternative hypothesis is that the cell line value is greater or smaller than MDCK.

Statistical analysis

Most results are means +/- standard error of the mean (SEM). Results from zyxin construct and knockdown lines were compared to MDCK values. MDCK values were considered to equal accepted population values and zyxin construct values were tested to be different than MDCK, with H_0 = zyxin mutant value is the same as the MDCK value and H_A = zyxin mutant value is greater or lesser than the MDCK value. p-Values for average values were calculated using two-tailed analysis. EMT initiation $t_{1/2}$ curve shapes were compared using Weibull regression analysis and calculating p-values from Yates chi-square values comparing curves. p-Values comparing percent phenotype values were generated using a z-test for proportions and 2 tailed p-value analysis. P-values < 0.05 are considered to be statistically significant.

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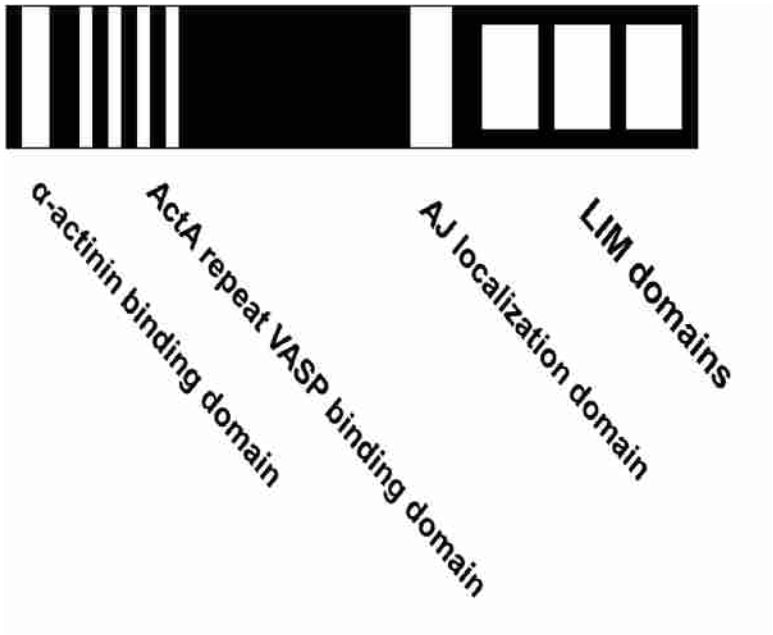
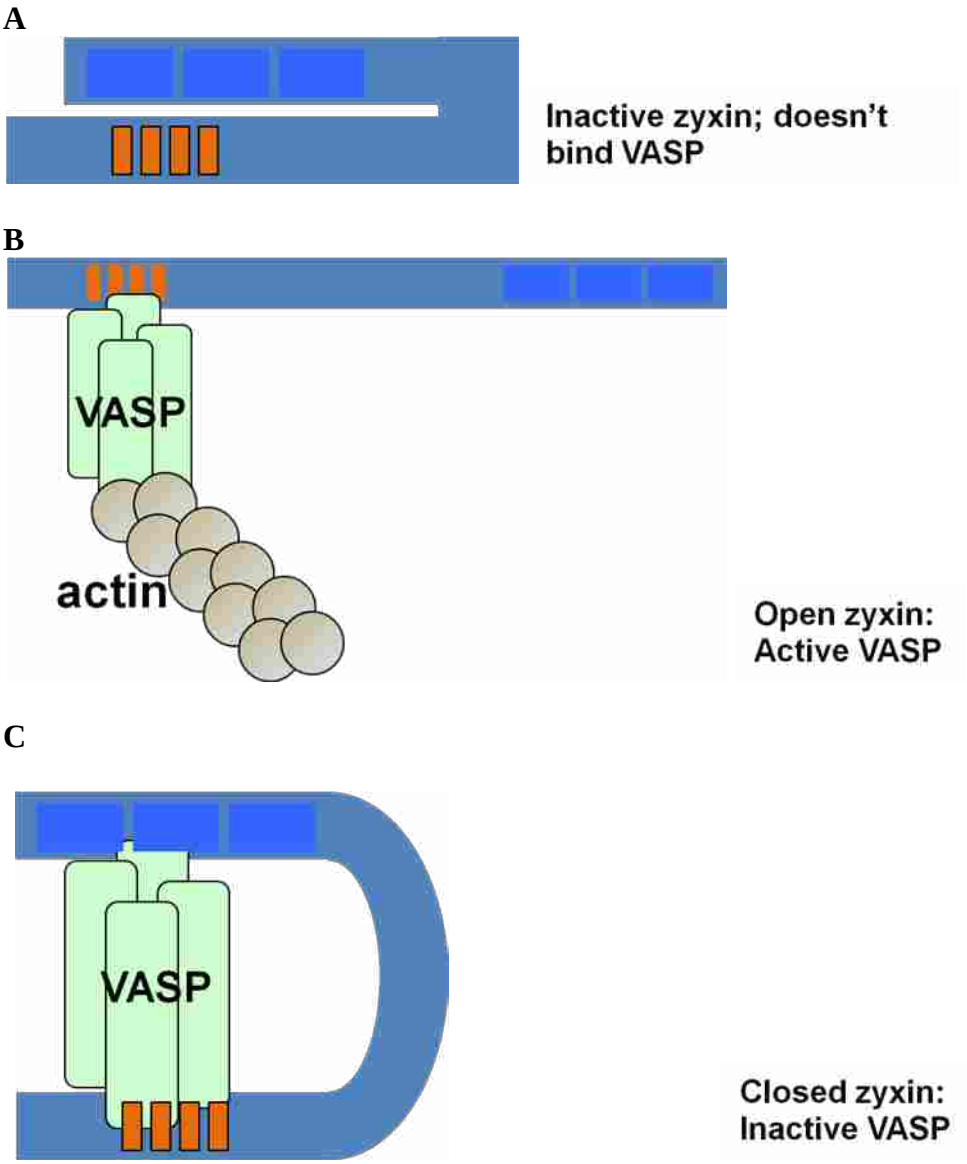
FIGURES

Figure 1: Schematic of zyxin domains



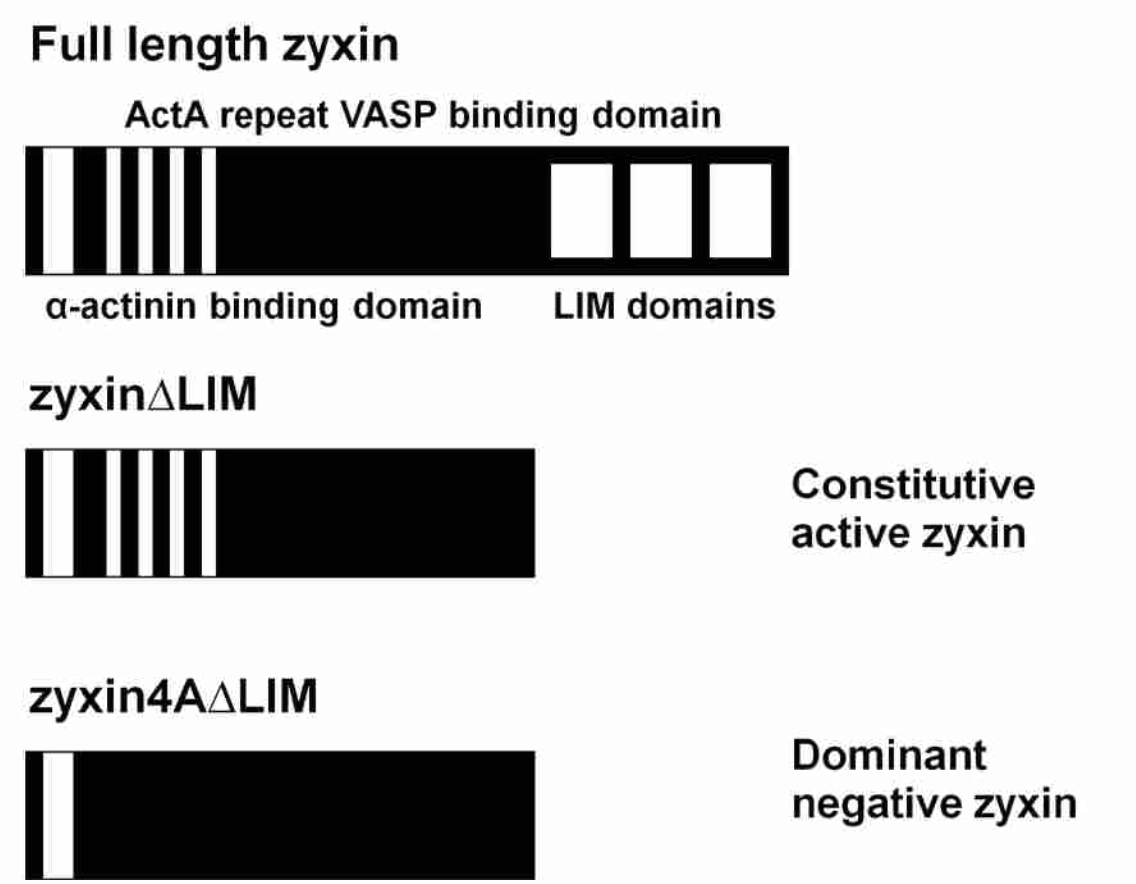
Zyxin contains an α-actinin binding site near its amino-terminus. This is followed by 4 ActA repeat sites, responsible for VASP binding. The central domain contains a junctional localization domain near its carboxy end. The carboxy terminus contains 3 copies of the LIM domain.

Figure 2: Model of zyxin regulation of VASP



Model for LIM domain regulation of VASP binding and function. (A) Under certain conditions LIM domains bind zyxin in an auto-inhibitory conformation preventing VASP binding. (B) In open conformation, zyxin ActA repeat domains bind VASP which interacts with actin filaments. (C) When zyxin binds VASP at both the actA repeat site and the LIM domain, in a closed zyxin formation, VASP activity is inhibited.

Figure 3: Schematic of zyxin mutant constructs



Full length zyxin contains all 4 wildtype ActA repeat binding domains and 3 LIM domains. The LIM domains have been deleted from the constitutive active zyxinΔLIM construct, removing the inhibitory effects of LIM binding while retaining VASP binding capability. The dominant negative zyxin4AΔLIM construct also has deleted LIM domains. The ActA repeat domain has been altered as well, by substituting phenylalanines with alanines, abolishing VASP binding. All zyxin constructs are functional for α-actinin binding and for localization to cell-cell junctions.

Figure 4: Junctional actin rearrangement during EMT

(A-B) Phase contrast time lapse imaging of MDCK colonies. (A) Control colonies, without HGF, remain unchanged. (B) After adding HGF, cells first spread and then scatter. Brackets highlight spreading of colonies in first two hours. Arrowheads denote cell shape changes as EMT is initiated. Dotted lines show intracolony space formation as cell-cell junctions rupture and arrows indicate retraction fibers forming as cells separate. Bars = 50 μm. (C-G, I-K) Fixed MDCK cells were stained with E-cadherin antibodies (red) to label cell-cell junctions and phalloidin (green) to visualize F-actin. Bars = 10 μm. Untreated cells (C) exhibit actin tightly apposed to junctions. HGF treated cells (D-G) reorganize junctional actin into medial actin networks. (D) Individual actin cables, parallel to the AJ, form a transcellular actin network (bracket) which is membrane anchored in E-cadherin rich puncta. Membrane extrusions (visualized by E-cadherin) into the cytoplasm indicate tension at points of actin-membrane anchoring (arrowhead). (E) Transcellular actin cables are linked into a multicellular actin network spanning multiple cells through E-cadherin rich puncta. (F) Individual actin cables, anchored at AJs and incorporated into a higher order medial actin structure (asterisk), which also links to focal adhesions (arrow), form a radial actin network. Membrane extrusion into the cytosol indicates tension (arrowheads). (G) Individual actin cables (bracket) alongside a rupturing junction, and converging puncta (arrowhead) between intracolony spaces. Contrast to undisrupted junction (arrow) in which actin is tightly juxtaposed to the junction. (H-L) AJ associated actin decreases with HGF treatment.

Figure 4: Junctional actin rearrangement during EMT

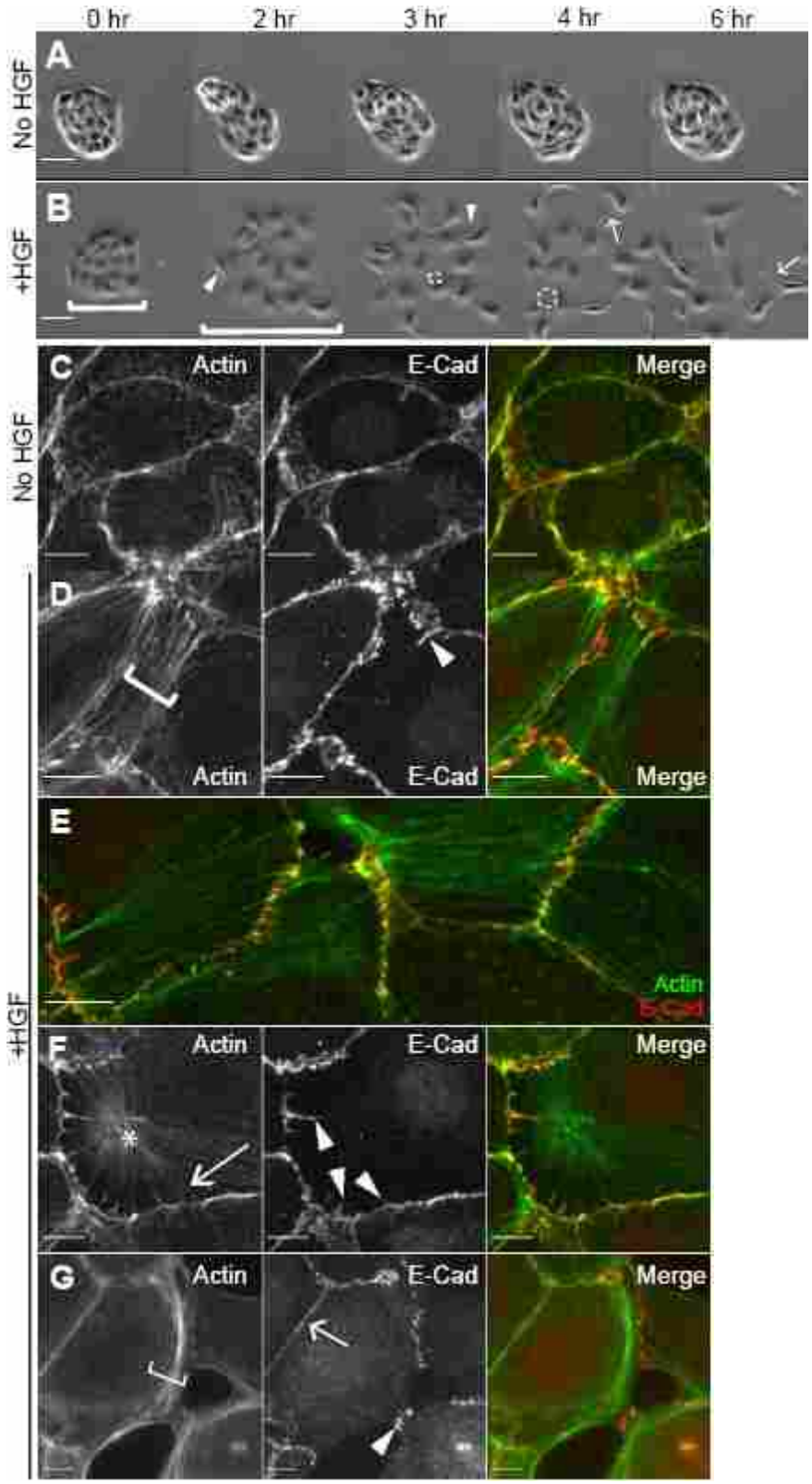


Figure 5: Junctional actin decreases during EMT

(A-E) Cell-cell junction associated actin decreases with HGF treatment. (A) Actin colocalization ratio: Average ratio of F-actin colocalized with E-cadherin / total F-actin, at different HGF treatment times. Error bars indicate SEM. (B) Representative image of junctional actin mask (actin colocalized with E-cadherin) and total actin mask, with and without HGF treatment. (C) Pseudocolor image (right) of original image (left) compares level of actin and E-cadherin co-localization along cell-cell junctions between spread (arrow) vs. unspread (arrowhead) cells after HGF treatment. (D-F) Line intensity profiles (E) showing actin intensity across cell-cell junctions in non-HGF treated control cells (D), and HGF treated cells (F). Dashed line in E shows phalloidin pixel intensity at each pixel position along the mask in control cells, and solid line shows pixel intensity in HGF-treated cells. Vertical lines indicate the position of the cell-cell junction in each profile. White lines in D and F denote masks used to generate line intensity profiles in E. Bars = 10 μm.

Figure 5: Junctional actin decreases during EMT

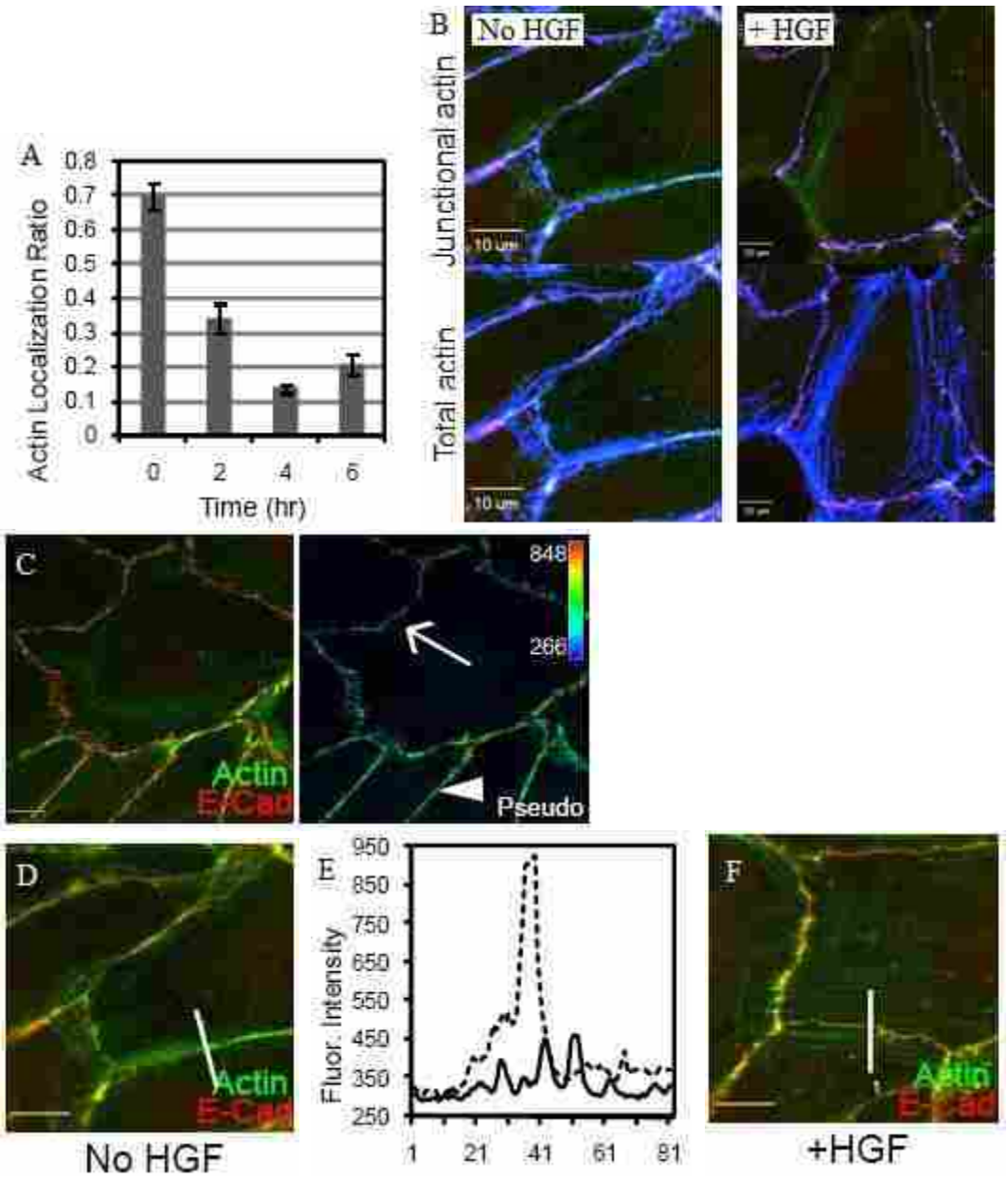
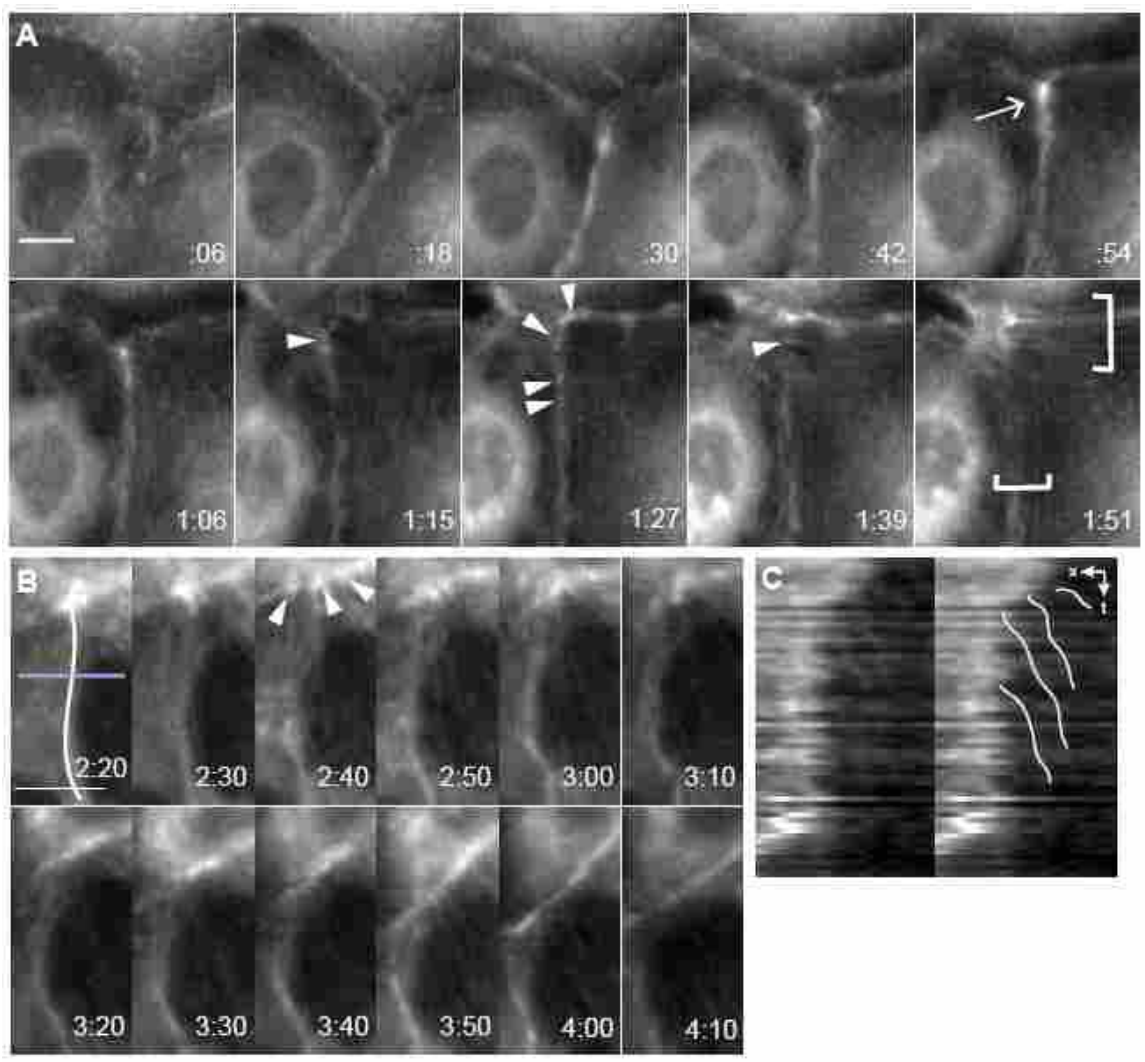


Figure 6: Actin cables detach from AJs early in EMT



(A-B) Time lapse imaging of stable GFP-actin expressing MDCK cells following HGF treatment. Time stamp hr:min. Bars = 10 μm. (A) Actin intensity increases at cell vertices (arrow) prior to dissociation of the vertex into discrete puncta (arrowheads). Dissociation of individual actin cables from the AJ is highlighted (bracket). (B) The vertex between cells dissociates into multiple, smaller actin puncta (arrowheads) as actin cables detach from the AJ. The white line indicates the initial position of the AJ and the gray line marks the mask analyzed in C. (C) Kymograph analysis of pixel intensity along the gray line, marked in the first panel of B, showing individual cables originating at and moving away from the AJ. The duplicate image traces cable movement for ease of reading.

Figure 7: Dynamic actin rearrangement during EMT

Dynamic actin networks form in cells undergoing EMT. (A-C) Time lapse imaging of stable GFP-actin expressing MDCK cells following HGF treatment. Time stamp hr:min. Bars = 10 μm. Individual actin cables detach from the junctional actin ring while maintaining membrane linkage at puncta to form medial actin networks. (A) Dashed lines indicate cell-cell contacts at the start of imaging. Cell vertex (arrowhead) brightens and disperses into puncta (bracket), where individual cables are anchored to form a multicellular actin network. (B) Formation of a radial actin network (asterisk), linked to actin-membrane anchor points prior to rupture of cell-cell junctions. (C) Formation and persistence of retraction fibers (arrow) anchored into puncta of actin fluorescence along AJs (bracket). A second vertex (arrowhead) also disperses.

Figure 7: Dynamic actin rearrangement during EMT

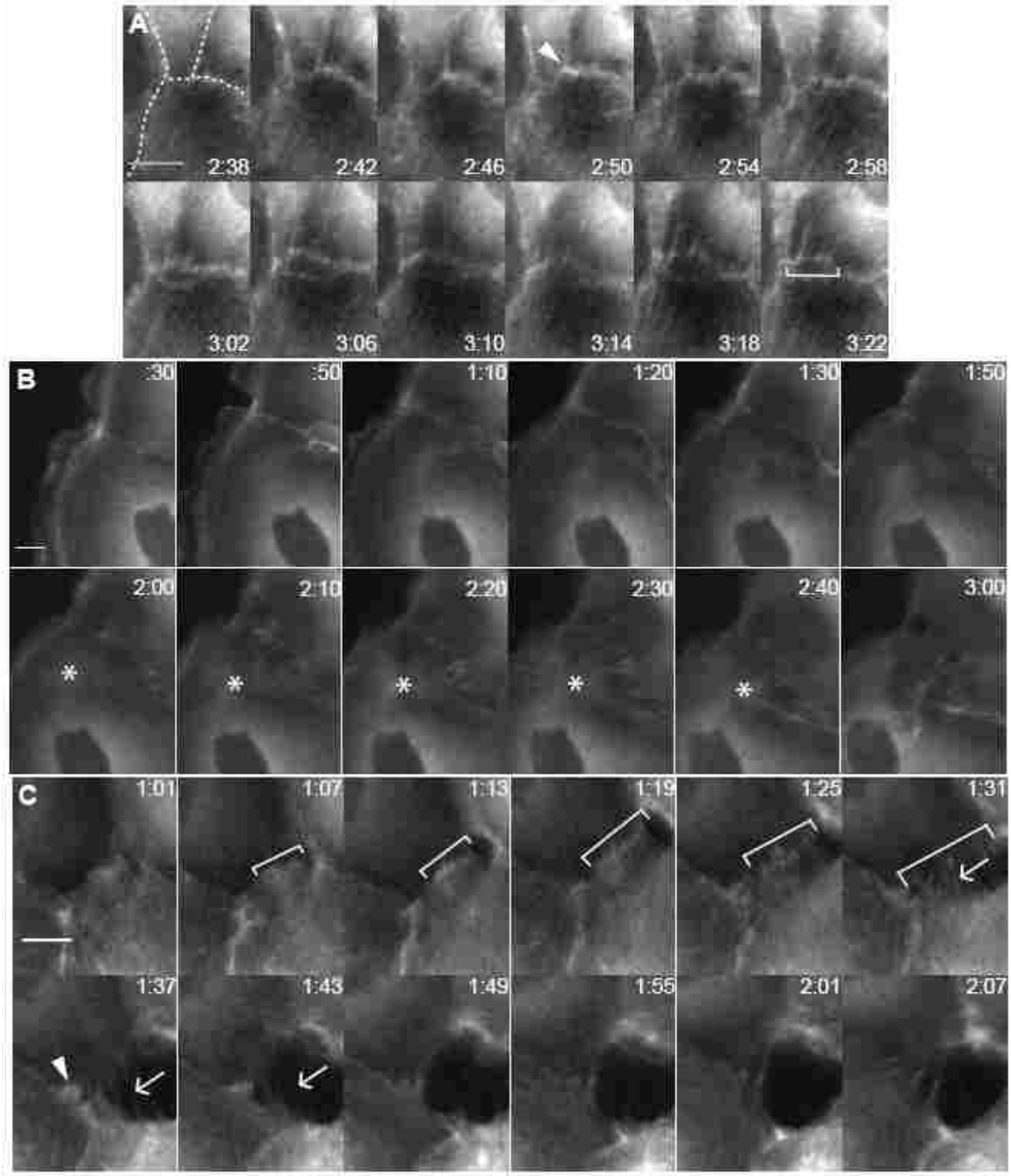
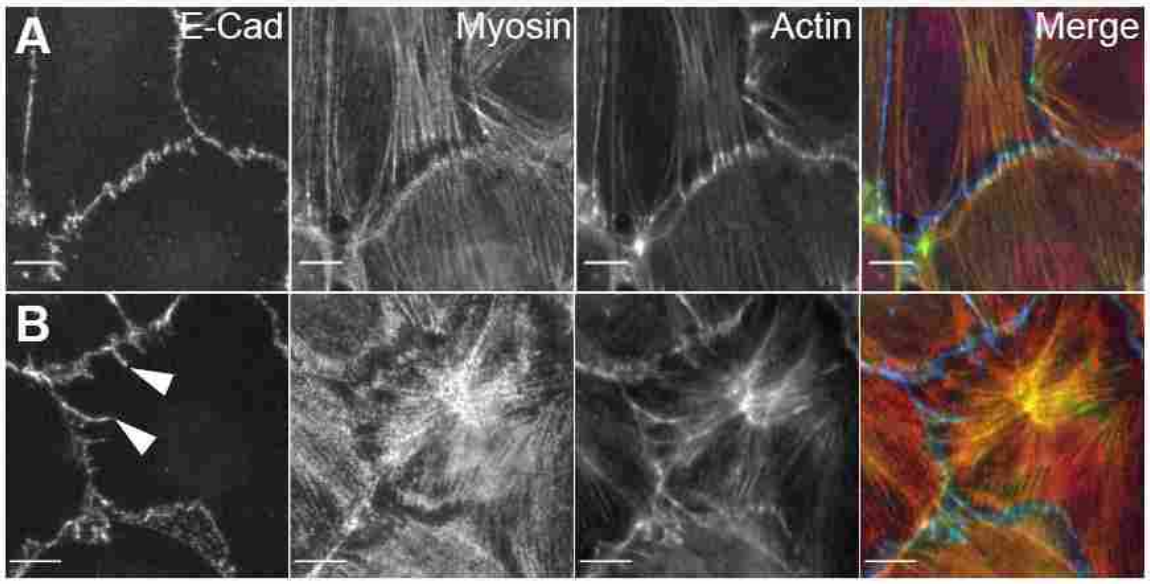
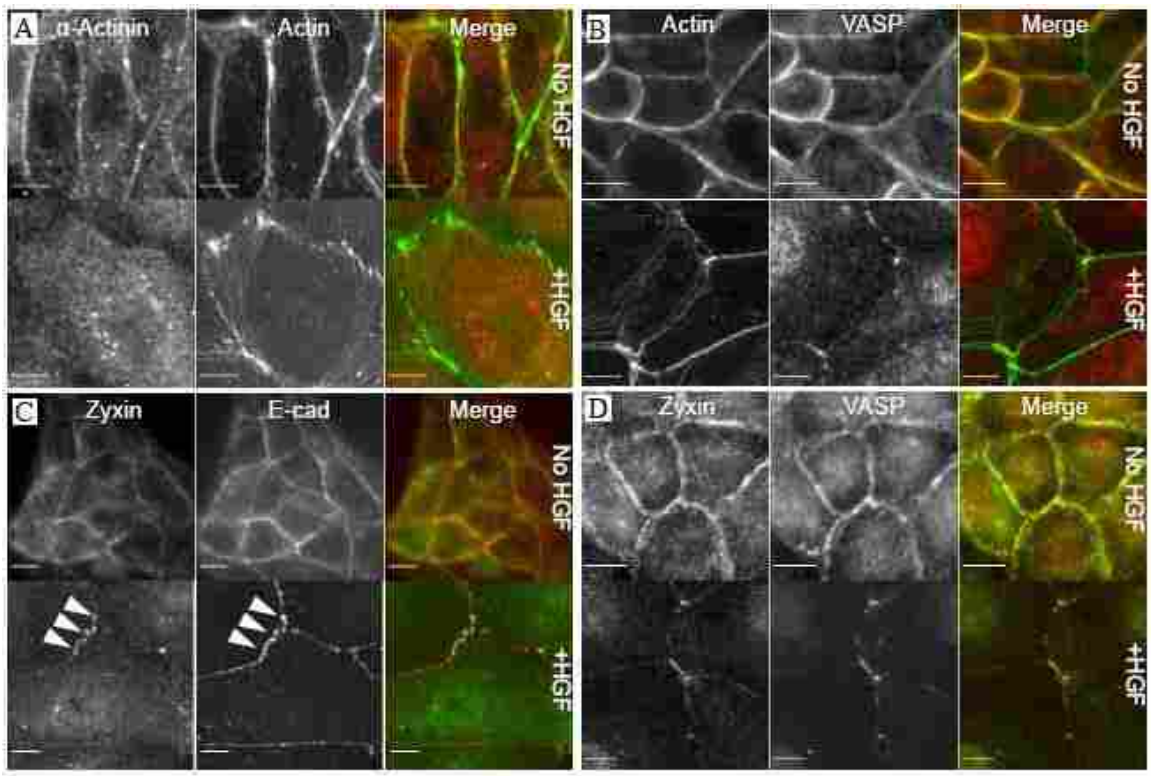


Figure 8: Individual actin cables are contractile



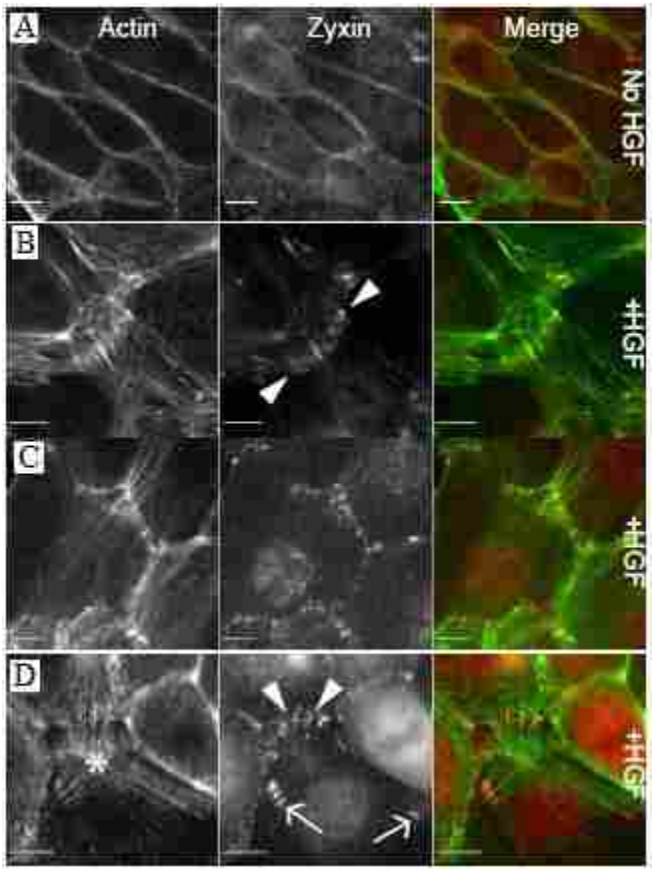
HGF treated MDCK cells were fixed and stained for myosin IIA (red), E-cadherin (blue) and phalloidin (green). Bars = 10 μm. Myosin IIA localizes to actin cables in both transcellular (A) and medial actin networks (B). Membrane extrusions (visualized by E-cadherin) into the cytoplasm indicate tension at points of actin-membrane anchoring (arrowheads).

Figure 9: Dynamic localization of actin regulators during EMT



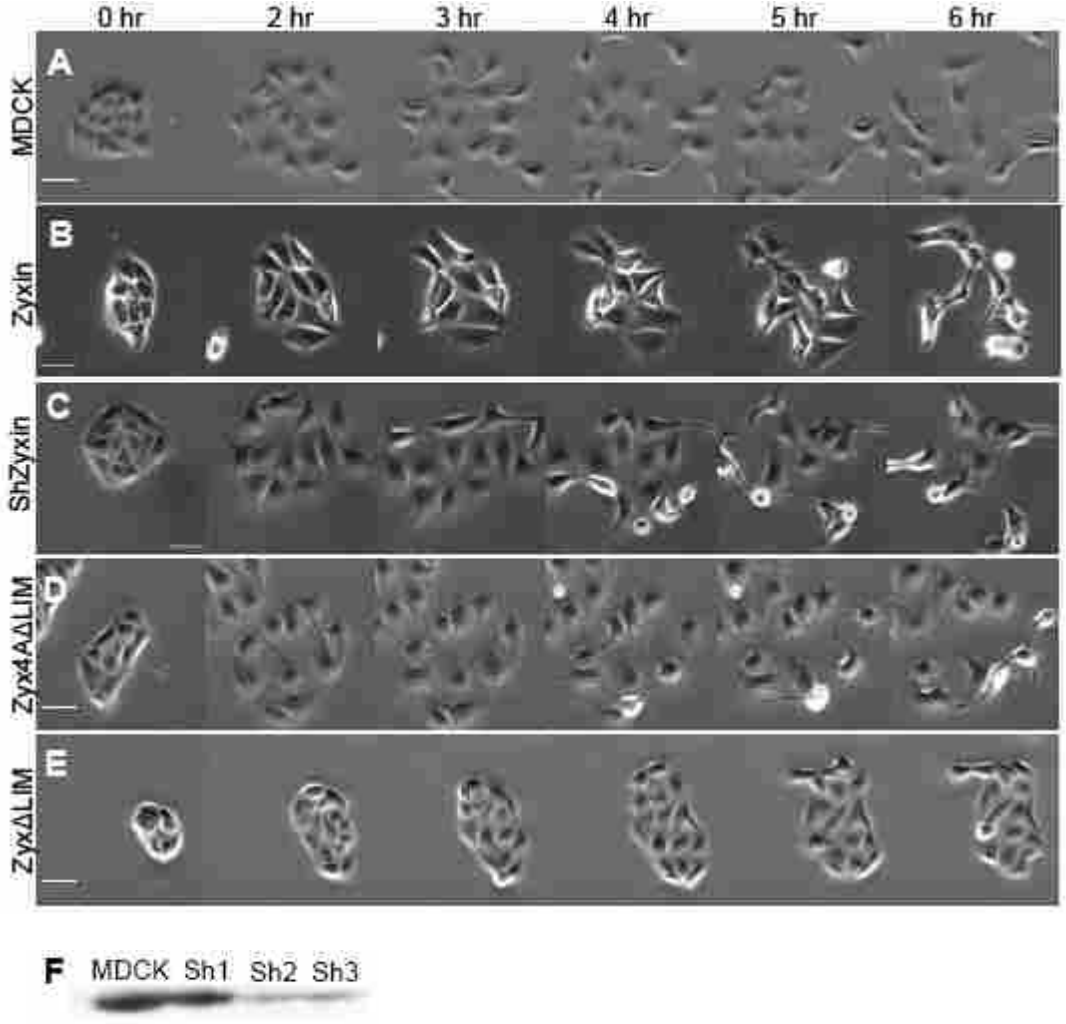
Actin regulatory proteins α-actinin, VASP and zyxin dynamically relocalize during HGF induced EMT. (A-D) Immuno- and phalloidin stained MDCK cells, with and without HGF treatment (2-6 hrs). Bars = 10 μm. (A) α-actinin (red) and phalloidin (green) depicts loss of α-actinin along cell-cell junctions, coincident with appearance of individual actin cables alongside junctions, after HGF treatment. (B) VASP (red) localizes with junctional actin (green) prior to HGF treatment. VASP is relocalized into puncta near cell vertices (arrowheads) to which ends of individual actin cables attach after HGF treatment. (C-D) Zyxin recapitulates VASP relocalization during EMT. (C) Zyxin (green) and E-cadherin (red). In the absence of HGF, zyxin lines adherens junctions. After HGF treatment, zyxin relocalizes to puncta. (D) Zyxin (green) and VASP (red) colocalize along cell-cell junction lengths prior to HGF, and to puncta after HGF treatment.

Figure 10: Zyxin puncta colocalize with individual actin cables



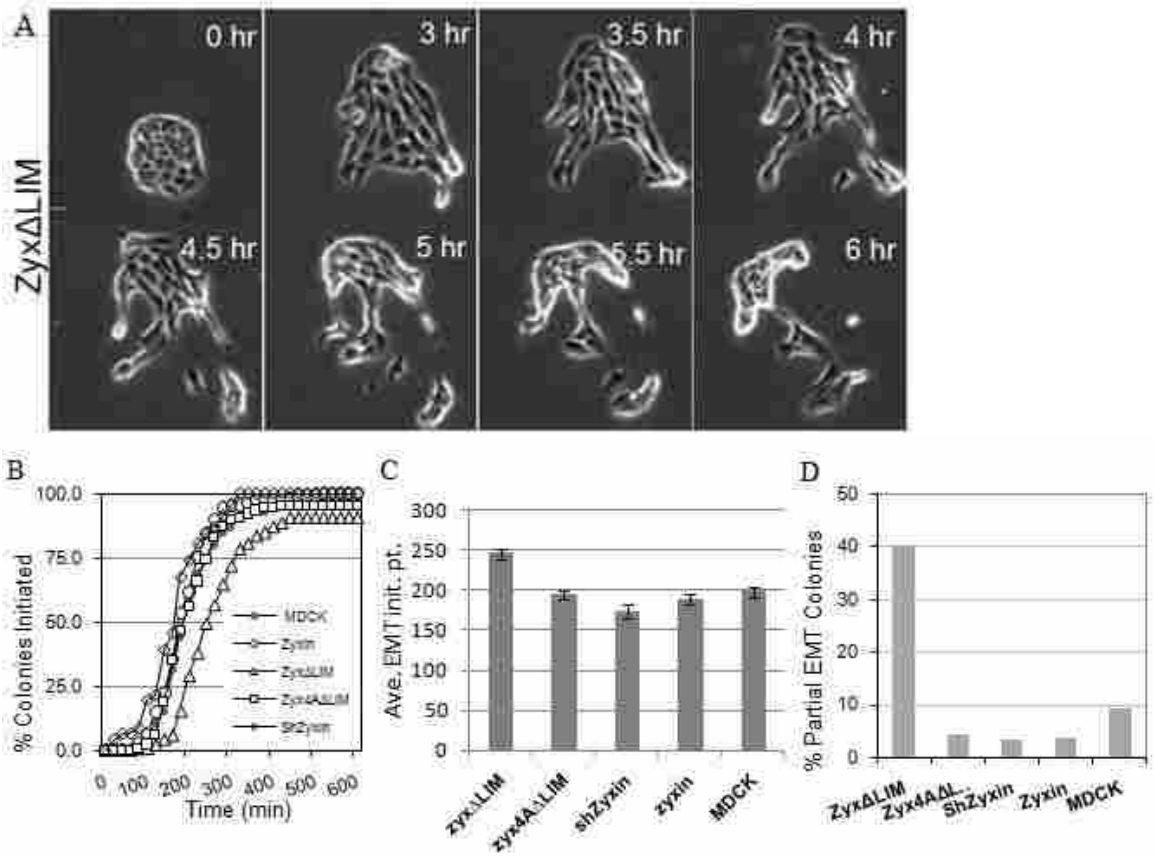
Zyxin puncta localize at ends of released actin cables. (A-D) Immuno- and phalloidin stained MDCK cells, with and without HGF treatment (2-6 hrs). Bars = 10 μm. Zyxin (red) and phalloidin (green) show dynamic zyxin and actin structures during EMT. (A) Control cells, no HGF. Zyxin colocalizes with junctional actin. (B) Perpendicular actin bundles attached to zyxin puncta (between arrowheads) associate with medial actin cables. (C) Individual actin cables, membrane anchored in zyxin puncta along cell-cell junctions at both ends, form a multicellular actin network. (D) Individual actin cables, membrane anchored in zyxin puncta at one end (arrowheads), and stress fibers anchored in focal adhesions (arrows) at one end, converge into a higher order hublike medial actin structure (asterisk), forming a radial actin network.

Figure 11: Cell line scattering behavior



Cells expressing constitutive active zyxinΔLIM exhibit altered cell scattering behavior. (A-D) Phase contrast live cell imaging showing HGF induced scattering response of (A) parental MDCK cells, and MDCK cells stably expressing (B) full length zyxin, (C) zyxin shRNA (shZyxin), (D) dominant-negative zyxin (zyxin4AΔLIM), or (E) constitutively active zyxin (zyxinΔLIM), during HGF treatment. Bars = 50μm. (F) Western blot showing zyxin expression in MDCK and shZyxin lines. Expression levels, determined by densitometry, were knocked down to 15.1 and 14.1% of parental zyxin expression in sh lines 2 and 3. ShZyxin results reported below are an average of data from lines 2 and 3.

Figure 12: Constitutive active zyxin generates a cell scattering phenotype

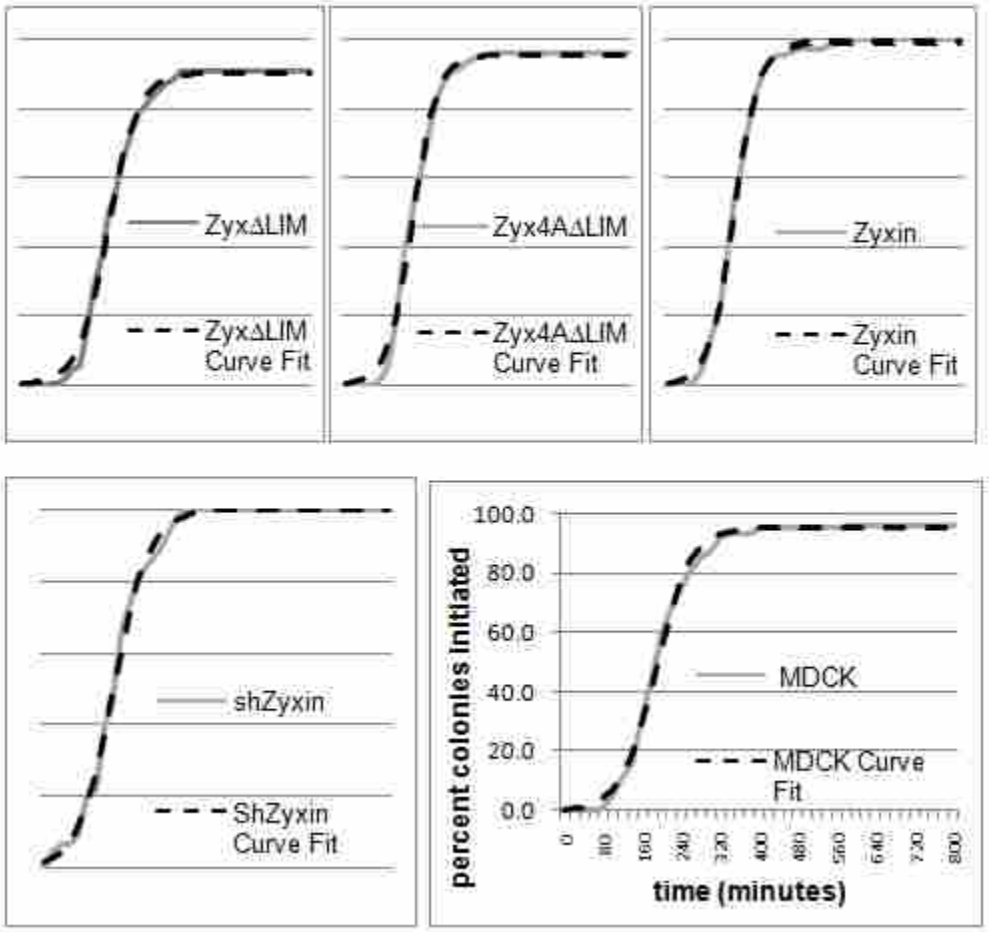


E	EMT initiation $t_{1/2}$	p value	% partial EMT	p value
MDCK	192 min	-	9.2	-
zyxin	182 min	0.82	3.7	0.53
zyxin4AΔLIM	191 min	0.83	5	0.65
zyxinΔLIM	248 min	0.007	40	0.001
shZyxin	162 min	0.023	3.3	0.54

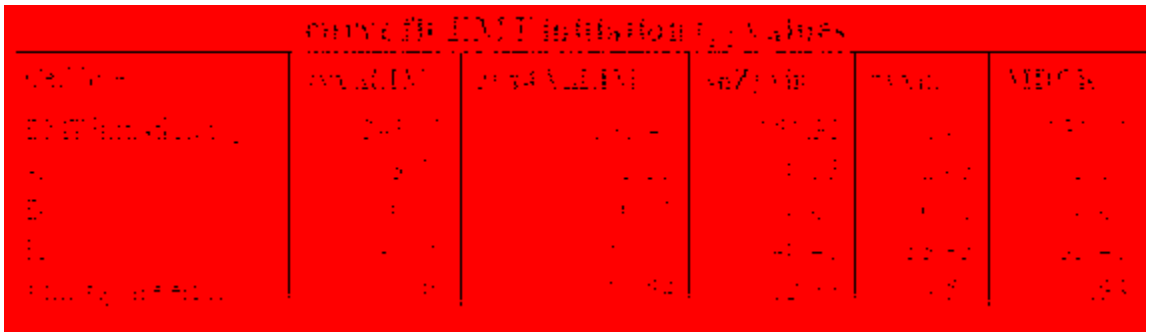
Constitutive active zyxinΔLIM expressing MDCK cells exhibit a delay in EMT initiation and abnormal scattering behavior. (A) Bright light phase contrast time series of zyxinΔLIM cells exhibiting the partial EMT phenotype, in which cells become migratory but show decreased cell separation and long, persistent retraction fiber formation. (B-C) EMT initiation point was determined for each colony analyzed in each cell line. (B) EMT initiation curves for indicated cell lines, showing percent colonies initiated over time. (C) Average EMT initiation point for each cell line. (D) Percent of colonies analyzed exhibiting a partial-EMT scattering phenotype. (E) EMT initiation $t_{1/2}$ calculated from curves in A, (p-values compare curve shape by Weibull regression and Yates chi square analysis) and % colonies showing partial EMT (p-values from z-test for proportions).

Figure 13: EMT initiation halftime calculation

A

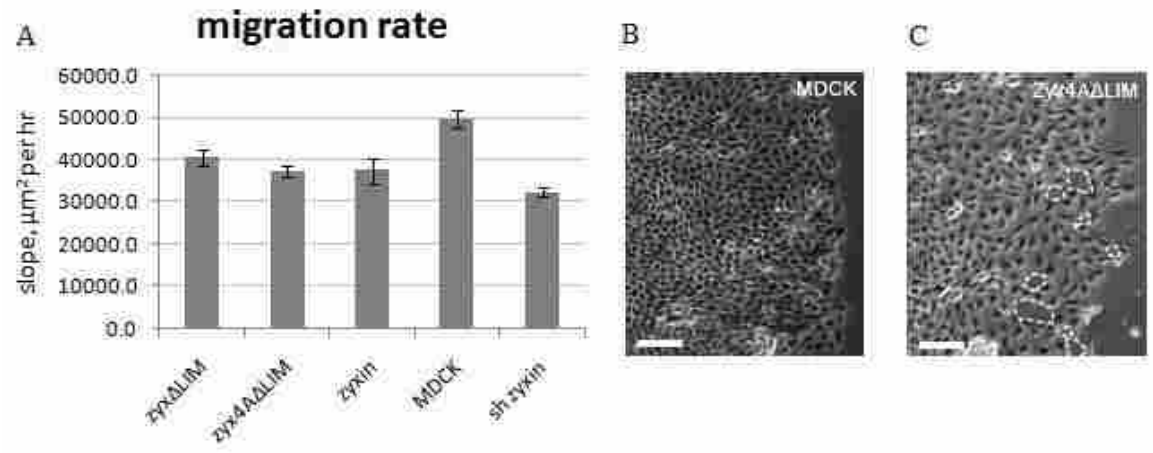


B



Raw data of EMT initiation point (minutes after HGF treatment) for each cell line were curve fitted minimizing total sum square error, using Microsoft Excel to determine the constant values, A, B and K. EMT initiation $t_{1/2}$ values were then calculated using the following formula: EMT initiation $t_{1/2}$ = -ln (((1/50)-B)/A) K. (A) Dashed line shows curve fit to raw data (solid line) of EMT initiation point for colonies in each cell line over time. All axes are the same as shown for MDCK. (B) Table shows curve fit error and formula constant values used to calculate EMT initiation $t_{1/2}$ values.

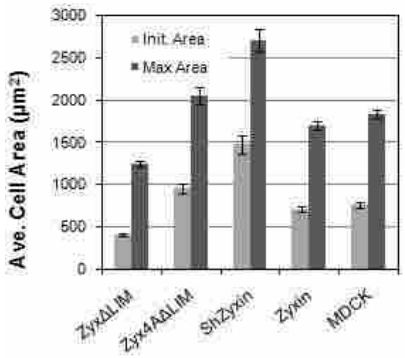
Figure 14: Zyxin effects on migration rate



Zyxin mutant expressing MDCK cells migrate at the same rate. (A) Migration rate, determined by wound healing, does not differ between mutant zyxin cell lines. Error bars denote SEM. (B-C) Representative images of wound edge after 6 hrs of MDCK (B) or zyxin4AΔLIM cells (C) during wound healing. Intracolony spaces indicated by dashed lines. Note increased intracolony space formation in leading edge and increased cell spreading of zyxin4AΔLIM cells compared to MDCK. Bars = 100 um.

Figure 15: Cell spreading analysis

A

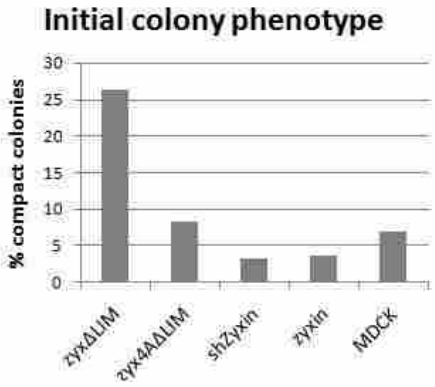


B

Area incr. ratio

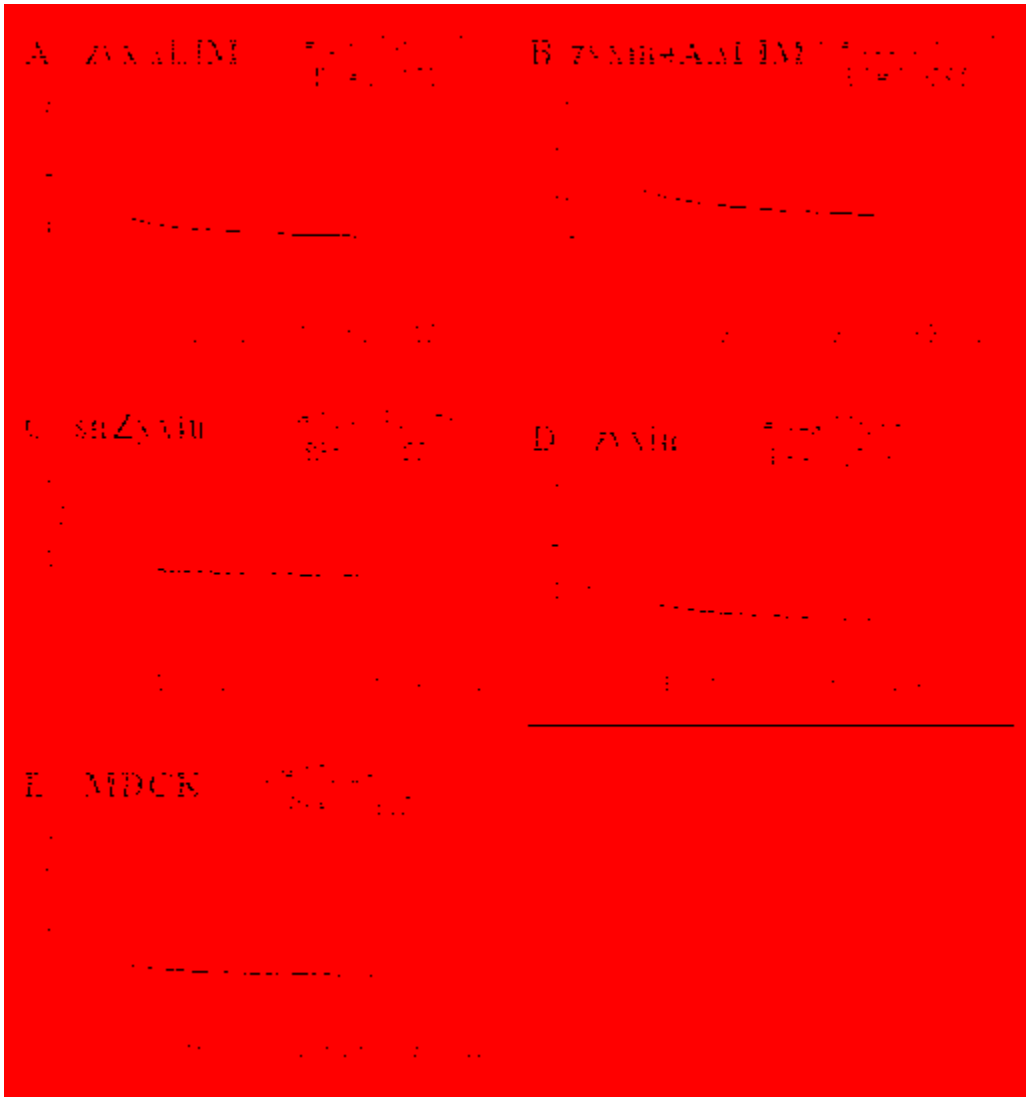
ZyxΔLIM =	3.7
Zyx4AΔLIM=	2.5
Zyxin =	2.2
MDCK =	2.8
shZyxin =	2.0
(SEM =	0.1)

C



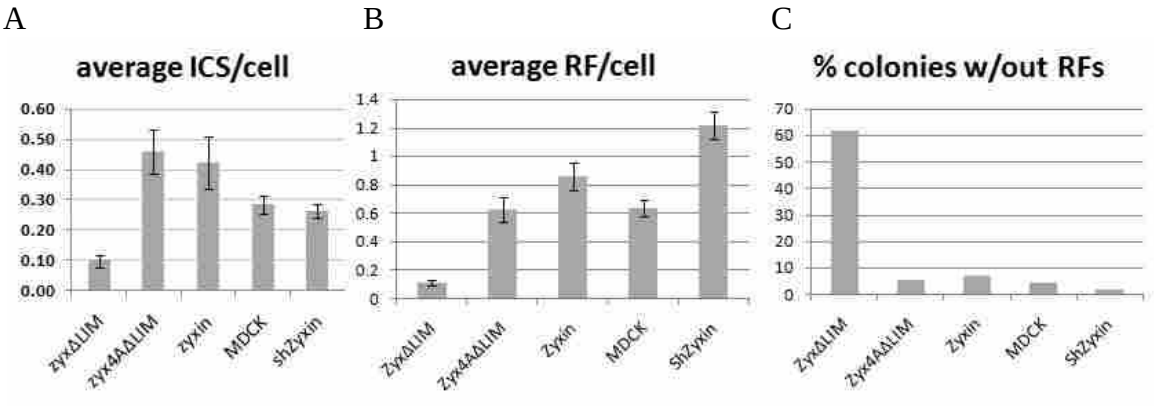
Zyxin depleted cells are less compact, whereas constitutive active zyxin cells are more compact, both initially and after spreading in response to HGF. Average initial and maximal cell areas reached after spreading in response to HGF treatment were calculated by measuring colony area, dividing by the number of cells, and averaging for each cell line. (A) Average initial and maximal cell area. Error bars depict SEM. (B) Area increase ratio determined by dividing average maximal area by initial cell area. SEM = 0.1 for all cell lines. (C) Percent colonies in each cell line exhibiting a compact phenotype initially.

Figure 16: Initial cell area - EMT initiation point correlation analysis



Initial compactness does not affect when colonies initiate EMT. (A-E) Correlation between initial cell area and time of EMT initiation after addition of HGF was measured by curve fitting data of average initial cell area vs. time of EMT initiation for each cell line. R^2 values demonstrate no correlation between EMT initiation point and initial colony compactness.

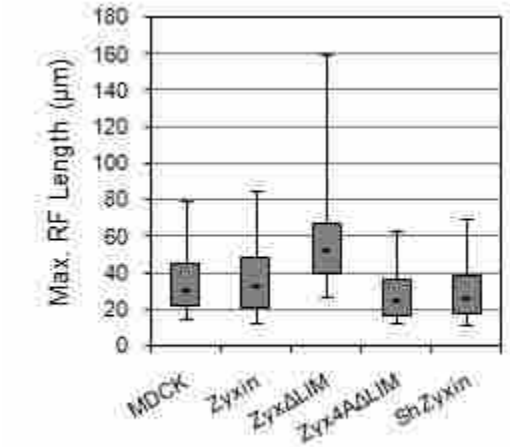
Figure 17: Cell-cell junction rupture events



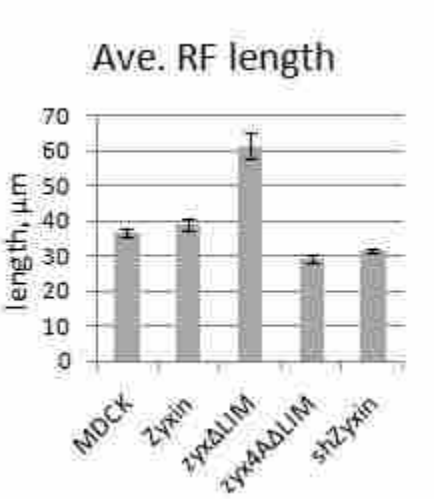
Constitutive active zyxin expression reduces cell-cell junction rupture events while zyxin depletion increases junction rupture. (A) Intracolony space (ICS) formation was counted in each colony, divided by the number of cells in the colony, and averaged. (B) Retraction fibers (RF) formed in each colony, divided by the colony cell number, and averaged. (C) Percent of colonies which form no RFs in each cell line. Error bars depict standard error of the mean (SEM).

Figure 18: Retraction fiber length analysis

A

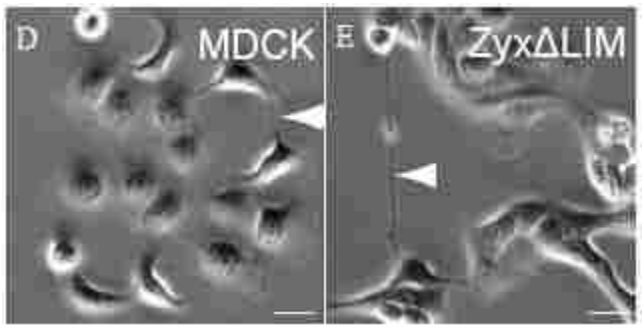


B



C

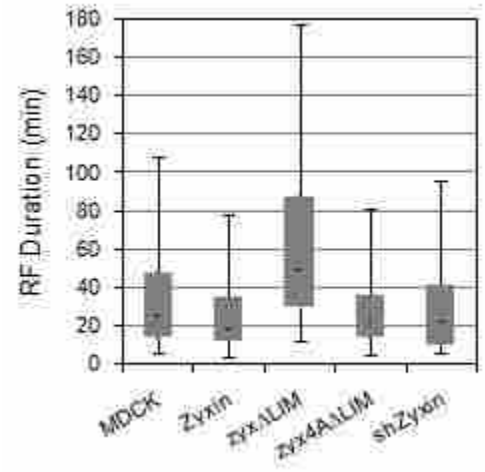
	MDCK	zyxin	zyxin4AΔLIM	zyxinΔLIM	shZyxin
Maximum RF length, µm	37	39	29	62	27
p-value (2 sided)	-	0.64	0.074	2.04 E-07	0.0049



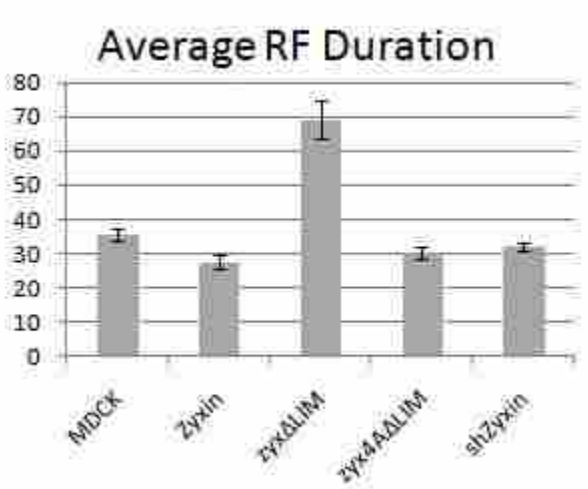
Retraction fiber (RF) length is longer in constitutive active zyxinΔLIM cells. (A) Frequency distribution of maximum lengths of RFs formed during HGF-induced scattering. Box shows 25th and 50th percentiles. Whiskers show 5th and 95th percentiles. (B) Average maximal RF length reached for each cell line. Error bars depict SEM. (C) Average maximal RF length values. p-Values determined compared to MDCK by 2 tailed t-test. (D-E) Phase contrast images of (D) an RF (arrowhead) of representative maximum length, in control MDCK cells and, (E) a long RF in constitutively active zyxinΔLIM cells. Bars = 30 um.

Figure 19: Retraction fiber duration analysis

A



B

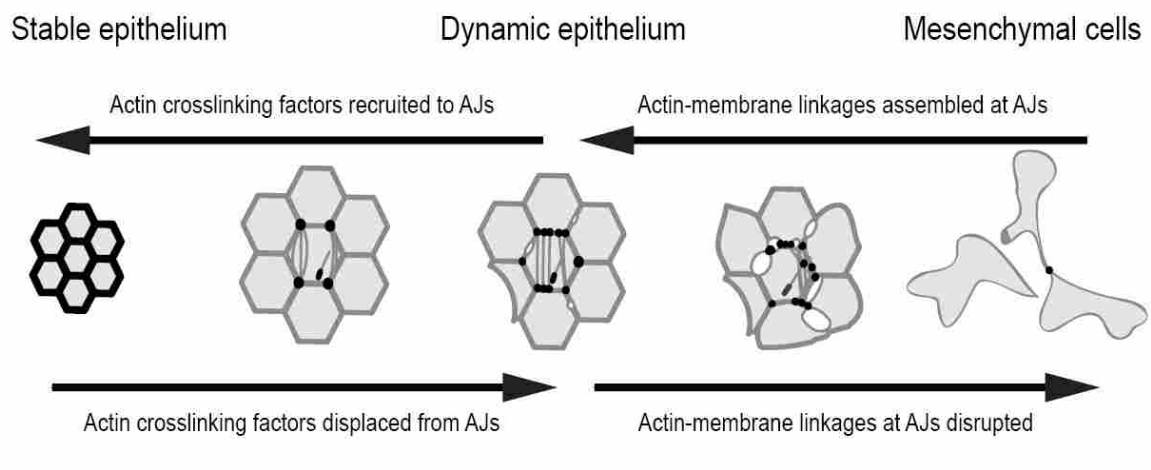


C

	MDCK	zyxin	zyxin4AΔLIM	zyxinΔLIM	shZyxin
RF duration, min	36	28	30	69	31
p-value (2 sided)	-	0.22	0.39	4.92 E-06	0.34

Retraction fibers (RFs) persist longer in constitutive active zyxinΔLIM cells. (A) Frequency distribution of maximum duration of RFs formed during HGF-induced scattering. Box shows 25th and 50th percentiles. Whiskers show 5th and 95th percentiles. (B) Average maximal RF duration reached for each cell line. Error bars depict SEM. (C) Average maximal RF duration. p-Values determined compared to MDCK by 2 tailed t-test.

Figure 20: Model of actin reorganization during EMT



Displacement of actin filament bundling proteins, α-actinin and VASP, from AJ lengths results in release of individual actin cables. These cables are reorganized into medial contractile actin networks. Actin-membrane linkage of individual cables is mediated by zyxin-VASP complexes, which relocalize to punctate points along AJs. Contractility of medial actin networks enables cell shape and position changes without loss of cell-cell contact, as occurs in partial EMT. Disruption of actin-membrane linkages results in detachment of individual cells, as seen in full EMT. The reverse of these processes is observed in cell-cell junction formation. (Model shows actin structure only in center cell of colony).

CURRICULA VITA

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Phone: (801)785-6646

Current Position: PhD candidate, Dept. of Physiology and Developmental Biology, Brigham Young University

Research Experience

Continuing research, Brigham Young University, under Drs. Marc Hansen and Michael Stark, Dept. of Physiology and Developmental Biology, April 2009 – current. Studying the role of zyxin in trigeminal placode morphogenesis in an embryonic chick model.

PhD research, Brigham Young University, under Dr. Marc Hansen, Dept. of Physiology and Developmental Biology, Nov. 2005 – current. Studying the role of zyxin-VASP complexes in regulating actin reorganization in cells undergoing epithelial mesenchymal transition using an MDCK tissue culture model.

PhD first year lab rotation, Brigham Young University, under Dr. Jeff Barrow, Dept. of Physiology and Developmental Biology, Sept. – Nov. 2005. Studying the role of non-canonical wnt signaling in limb development using a transgenic mouse model.

PhD first year lab rotations, University of Rochester, under Drs. Rick Borch and Patricia Hinkle, Dept. of Pharmacology, Oct. 1991- Mar. 1992. Gated Ca++ ion channel studies.

Botanical Research Assistant, Brigham Young University, under Dr. Paul A. Cox, Dept. of Botany and Range Science, 1988, 1991. Field and laboratory investigations of *Zannichellia spp.*

Pharmacognosy Research Assistant, University of Uppsala, Sweden, under Dr. Lasse Bohlin, Dept. of Pharmacognosy for Dr. Paul Cox, BYU. Sept.- Dec. 1987. Preliminary pharmacological screening of Samoan medicinal plant extracts.

Genetics laboratory assistant, under Drs. Duane Jeffery and James Farmer, Dept. of Zoology, BYU. 1983-1985. *In situ* DNA hybridization studies of *Drosophila spp.*

Work Experience

PhD candidate, research assistant, Dept. of Physiology and Developmental Biology, Brigham Young University, 2005 – current.

PhD candidate, teaching assistant PDBio 482 (Developmental Biology), Dept. of Physiology and Developmental Biology, Brigham Young University, under Dr. Michael Stark. Jan – Apr 2007.

PhD candidate, teaching assistant PDBio 363 (Physiology lab), Dept. of Physiology and Developmental Biology, Brigham Young University, under Dr. William Winder. Jan – Apr 2006.

PhD candidate, teaching assistant PDBio 482 (Developmental Biology), Dept. of Physiology and Developmental Biology, Brigham Young University, under Dr. Jeff Barrow. Sept. – Dec. 2006.

Horseback riding instructor, Pleasant Grove, UT, part time. 1996 – current.

Editor, Professional Pre-Press, Pleasant Grove, UT, edited text books, part time. 1999-2005.

Veterinary technician, Bowman Animal Clinic, Raleigh, NC, 1993-94.

Teacher of AP Biology, Meridian School, 12th grade class. 931 E. 300 N. Provo, UT. Headmaster Lee Allan. 1990/91 school year.

Teaching Assistant, Biology 200 (general biology for majors), under Dr. Paul Cox, Dept. of Botany and Range Science, Brigham Young University. Jan –Apr. 1989 and 1991.

Teaching Assistant, General and Honors Biology 100 (general biology), under Dr. Sam Rushforth, Dept. of Botany and Range Science, Brigham Young University. Apr. – Dec. 1989 and Apr. – June 1990.

Teaching Assistant, Biology 100, under Drs. William Bradshaw and James Barnes, Dept. of Zoology, Brigham Young University. Sept. 1986- Apr 1987.

Research Assistant, Dept. of Zoology, BYU, under Dr. Duane Jeffery. Sept- Apr 1984-5.

Groom. Finger Lakes Race Track. Brandon Racing Stables. Farmington, NY. April – Aug., 1983 and 1984.

Memberships

American Society for Cell Biology (2006 - current)
Phi Kappa Phi Honor Society
Society for Ethnobiology
Society of Economic Botany
The American Scandinavian Foundation
National Honor Society

Honors and Awards

BYU Graduate ORCA Grant, 2007-2008, $6000
BYU Cancer Research Center Fellowship, Apr. – Aug. 2007, $6670
BYU Cancer Research Center Fellowship, Apr. –Aug. 2006, $6300
BYU Graduate Mentoring Awards, 2006, 2007 and 2008, $5000 each
Travel grant to San Francisco, CA, $1260, Sept. 2008
Travel grant to Washington, DC, $1075, Oct. 2007
Travel grant to San Diego, CA, $500, Oct. 2006
NSF Graduate Fellowship Honorable Mention, 1990
NSF Graduate Fellowship Honorable Mention, 1989
BYU Graduate Botanical Scholarships, 1988-1991
Travel grant to Cincinnati, $350, 1989
Travel grant to Mexico City, $450, 1988
Outstanding Chemistry Student award, BYU, 1988
Botanical Science award, BYU, $800, 1988
Graduated *Magna cum Laude*, BS, BYU, 1988
Inducted into *Phi Kappa Phi*, 1987
Grant for pharmacological research, University of Uppsala, Sweden, $1500, 1987
Travel grant to Sweden, $800, 1987
ASBYU research grant recipient, $150, 1986
BYU academic scholarship recipient, 1983-1988

Dissertation, thesis, publications:

L. Rebecca Bakkevig Sperry. 2009. Zyxin Regulates Epithelial-Mesenchymal transition by Mediating Actin-Membrane Linkages at Cell-Cell Junctions. Dissertation, BYU.

L. Rebecca Bakkevig Sperry. 1991. Three Studies in Ethnobotanical and Indigenous Plants: Pharmacological Activity of the Samoan Ethnopharmacopoeia, The Ethnobotanical Uses of the Genus *Equisetum* L. C. Rich, The Pollination Ecology of *Zannichellia palustris* L. (Zannichelliaceae). Master's Thesis, BYU.

You-Hao Guo, R. Sperry, C. D. K. Cook, and P. A. Cox. 1990. The pollination ecology of *Zannichellia palustris* L. (Zannichelliaceae). *Aquatic Botany* 38:341-356.

Cox, P. A., L. R. Sperry, M. Tuominen, and L. Bohlin. 1989. Pharmacological activity of the Samoan Ethnopharmacopoeia. *Economic Botany* 43:487-497.

Barton, R. J., K. R. Herrick, J. H. Whiting Jr., M. D. Pliley, L. A. Nelson, R. Bakkevig, J. L. Farmer, and D. E. Jeffery. 1988. Chromosome homologies in *Drosophila melanogaster, D. Virilis, and D. silvestris* resolved by *in situ* DNA hybridization. *Genome* 30(suppl. 1)L267. (abstr.).

Posters:

LR Sperry, B Fowler, N Bishop, R Vellinga, MDH Hansen. Actin Remodeling During Loss of Cell-cell Adhesion. Poster 335-B270: ASCB annual meeting, Washington DC, Dec 2, 2007.

LR Sperry, N Bishop, J Bramwell, M Brodeur, B Fowler,R Vellinga, MDH Hansen. Zyxin is a Regulator of HGF-induced Cell-cell Dissociation in MDCK Cells. Poster 337-B272: American Society for Cell Biology annual meeting, Washington DC, Dec 2, 2007.

L. Sperry, N. Bishop, S. Maxfield, Z. Lewis, D. Staley, R. Vellinga, M. Hansen. Zyxin Associates with Dynamic Actin Structures and Alters Cell Scattering Response in HGF-Treated MDCK Cells. Poster #2525/B234. American Society for Cell Biology annual meeting, San Francisco, CA, Dec. 13-17, 2008.

N. H. Bishop, R. Sperry, M. Carter, D. Staley, M. Hansen. Actin Rearrangements in Disassembly of Cadherin-Based Cell-Cell Contacts during EMT. Poster #995/B203. American Society for Cell Biology annual meeting, San Francisco, CA, Dec. 13-17, 2008.

Chromosome Homologies in *Drosophila melanogaster, D. virilis and D. silvestris* Resolved by *in situ* DNA hybridization. Whiting, J. H. Jr., M. D. Pliley, L. R. Bakkevig, R. J. Barton, L. A. Nelson, K. R. Herrick, J. L. Farmer, and D. E. Jeffery. XVIth International Congress of Genetics. August 20-27, 1988.

Presentations:

Dynamic actin remodeling and a role for zyxin/VASP in Epithelial-Mesenchymal Transition of MDCK cells. L. R. Sperry. Dept of Physiology and Developmental Biology graduate seminar presentation: Oct 2008.

Regulation of actin organization during EMT. L. R. Sperry. BYU Cancer Research Center summer presentations: Aug 2007.

Function of the actin regulator zyxin in Epithelial-Mesenchyme Transition. L. R. Sperry. Dept of Physiology and Developmental Biology graduate seminar presentation: Feb 2007.

Zyxin and actin dynamics during cell-cell junction formation. L. R. Sperry. BYU Cancer research Center summer presentations: Aug 2006.

Regulation of actin dynamics at cell-cell junctions: implications for cancer metastasis. L. R. Sperry. Dept of Physiology and Developmental Biology graduate seminar presentation: Mar 2006

Ethnobotanical Uses of *Equisetum.* L. R. Sperry. Society for Economic Botany meetings. Knoxville, TN. June 1989.

Preliminary Screening of Samoan Medicinal Plants. R. Bakkevig, P. A. Cox, and L. Bohlin. Society for Ethnobiology meetings. Mexico City, Mexico. March 1988.

Preliminary Screening of Samoan Medicinal Plants. R. Bakkevig, P. A. Cox, and L. Bohlin, American Society of Pharmacognosy and Japanese Society of Pharmacognosy joint meetings. Park City, UT. July 1988.

The Samoan Ethnopharmacopoeia. R. Bakkevig, P. A. Cox, and L. Bohlin. Presented at Brigham Young University Chemistry Seminar, June 1988.

Effective Teaching Techniques. R. Bakkevig. Presented at Biology 100 Teaching Seminar, Brigham Young University, August 1988.

Extra Curricular Activities:

I am an avid horsewoman and enjoy raising and training horses as well as giving riding lessons. I also enjoy gardening, hiking and camping. I love to be outdoors and to be active. Never the less, one of my favorite activities is to curl up warmly with a good book. I enjoy a good novel and also read a lot of nonfiction; history and philosophy being my favorites. Knitting, playing games and sports such as racquetball, tennis and basketball are other activities I enjoy.

I spend time in a variety of civic activities. I volunteer at my local elementary school where I assist in reading programs, coach knowledge bowl teams and help out in classes. I am active in my church and have served in positions with leadership responsibility in youth and adult groups. I am active in the Boy Scouts of America scouting program and am currently a Cub Scout leader. I volunteer my time and horses at local city celebrations. I am also an assistant 4H leader in a local horse club.