MECHANOTRANSDUCTION AT CELL-CELL CONTACTS

BY

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DISSERTATION

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Abstract

For nearly 40 years, it was debated as to whether the differences in cadherin-dependent cell-cell adhesion energies could solely predict cell sorting. Although adhesion energies do influence cell sorting in vitro, recent studies suggested that that differences in cell adhesion, determined mainly by cadherin binding affinities and expression levels, were not sufficient to predict cell sorting. This result suggested that other factors contribute. Besides their adhesive function, cadherins are also signaling proteins. Naturally, the next question is whether differences in downstream signaling associated with differences in cadherin affinity might also contribute to cell segregation. Chapter 2 of this dissertation specifically therefore investigates the relationship between cadherin affinities and signaling by small Rho GTPases, which are cytoskeletal regulatory proteins that influence actin polymerization and myosin dependent contractility. Recent findings also suggested that adhesion energies and cell mechanics together influence cell sorting, but the link between the two parameters had not been established. Chapter 3 builds on recent findings that cadherin complexes are also force transducers, and addresses whether differences in cadherin affinity also differentially alter cell mechanics. Studies discovered a significant difference between force transduction triggered by homophilic versus heterophilic cadherin bonds that could differentially influence cell mechanics. I further investigated the mechanism of force transduction at cadherin junctions, as described in Chapter 4 of this thesis. Chapter 4 specifically focused on the role of α-catenin—a key component in cadherin complexes—as a critical force transducer at cell-cell adhesions.

To my Mom and to my Dad

Acknowledgments

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Chapter 1

Introduction

Cell surface adhesion receptors, or cell adhesion molecules (CAM) are proteins embedded in the cell membrane, which enable cells to bind to neighboring cells or to the extracellular matrix (ECM). Cells use these surface receptors to communicate with their environment. This communication is essential to cell health, development, and cell differentiation (Hynes, 2002). Cell adhesion proteins are also vital for the formation, organization and structure of all multicellular organisms (Gumbiner, 2005; Yap, Brieher, & Gumbiner, 1997). Cell surface adhesion proteins fall into four major families: cadherins, integrins, immunoglobulins and selectins (Halbleib & Nelson, 2006). Of these adhesion receptor families, two major classes of adhesion proteins are integrins, which mediate cell-ECM adhesion (Burridge & Chrzanowska-Wodnicka, 1996) and cadherins, which are the cell-cell cohesive proteins at intercellular contacts (Gumbiner, 1996; Takeichi, Inuzuka, Shimamura, Fujimori, & Nagafuchi, 1990).

Cell adhesion molecules are essential for morphogenesis—that is, the process of biological development and the evolution of an organism's physical shape. It concerns how biological structures form, and it involves a broad range of biological processes, including the formation, organization, maintenance, and remodeling of tissues and organs. Many cell-cell adhesion proteins, including cadherins play key roles in establishing and maintaining tissue organization (Takeichi, 1988, 1991). On the other hand, integrins contribute to cell adhesion, migration, and signaling events that also

govern cell differentiation and tissue morphogenesis. They facilitate cell migration by supporting tension at cell-ECM adhesions by migrating cells. Integrins also participate in signaling events that govern tissue differentiation and morphogenesis. Also in order to regulate tissue integrity, integrin-mediated adhesive contacts are required to withstand mechanical loads exerted on cells and tissue surfaces (Bökel & Brown, 2002).

Cadherins are a calcium-dependent superfamily of transmembrane adhesion proteins that are essential for the formation and maintenance of cell-cell contact. The cadherin superfamily comprises six major subgroups, including: classical cadherins, atypical cadherins, desmosomal cadherins, and flamin (Suzuki, 1996; Yap, Brieher, Pruschy, & Gumbiner, 1997). The classical type I cadherins are the most studied members of the superfamily, and their role in facilitating cell-cell adhesion is well established. So far, more than 20 cadherin subtypes have been identified. The type I classical cadherins are named based on the tissues from which they were first isolated from. For example C-cadherin is associated with cleavage stage in frog embryonic development, while E-cadherin was from epithelial tissues and N-cadherin was isolated from neural tissues (Gumbiner, 1996; Takeichi, 1991; Takeichi, 1995).

Cadherins are essential for normal biological development, and their abnormal expression is also linked with diseases (Geng, Shi, Yuan, & Wu, 2004; Gumbiner, 2005). Cadherin mutation and signaling downregulation had been reported to be associated with variety of skin and heart disorders (Resink, Philippova, Joshi, Kyriakakis, & Erne, 2009; Waschke, 2008). In epithelial cancers, it has been reported that malignant transformation is accompanied by an increase in N-cadherin expression along with a decrease in E-cadherin expression (Gumbiner, 2005; Pećina-Slaus, 2003). Pemphigus vulgaris (PV) is a

skin disease caused by autoantibodies that target desmosomal cadherins, and results in the loss of epidermal cell adhesion (Amagai, Klaus-Kovtun, & Stanley, 1991). Therefore it is important to thoroughly study cadherins at cell-cell contact and the mechanisms that regulate their adhesive, signaling and mechanical functions (Gumbiner, 2005; Jeanes, Gottardi, & Yap, 2008).

1.1 Cadherin Structure and Signaling at Cell-Cell Adhesions

The structure of classical cadherins consists of an extracellular domain, a transmembrane segment and a cytoplasmic region. The extracellular region of cadherins consists of five extracellular (EC) cadherin subtype domains (Figure 1.1), which play a major role in formation of adhesive contacts between cells (Takeichi, 1995; Yap, Brieher, & Gumbiner, 1997). These five domains, named EC1 through EC5 starting from the N-terminus, bind calcium ions at the interdomain junctions (Figure 1.1). More specifically, the binding of three calcium ions at each of the junctions between EC domains is necessary to form the rigid, protease resistant structure that is necessary for adhesion (Gumbiner, 1996; Nagafuchi & Takeichi, 1989; Yap, Brieher, & Gumbiner, 1997). Cadherins bind through their extracellular domains, while their intracellular domain is required for binding-dependent intracellular signaling and for mechanically linking cadherins to the actin cytoskeleton (Nagafuchi & Takeichi, 1988)

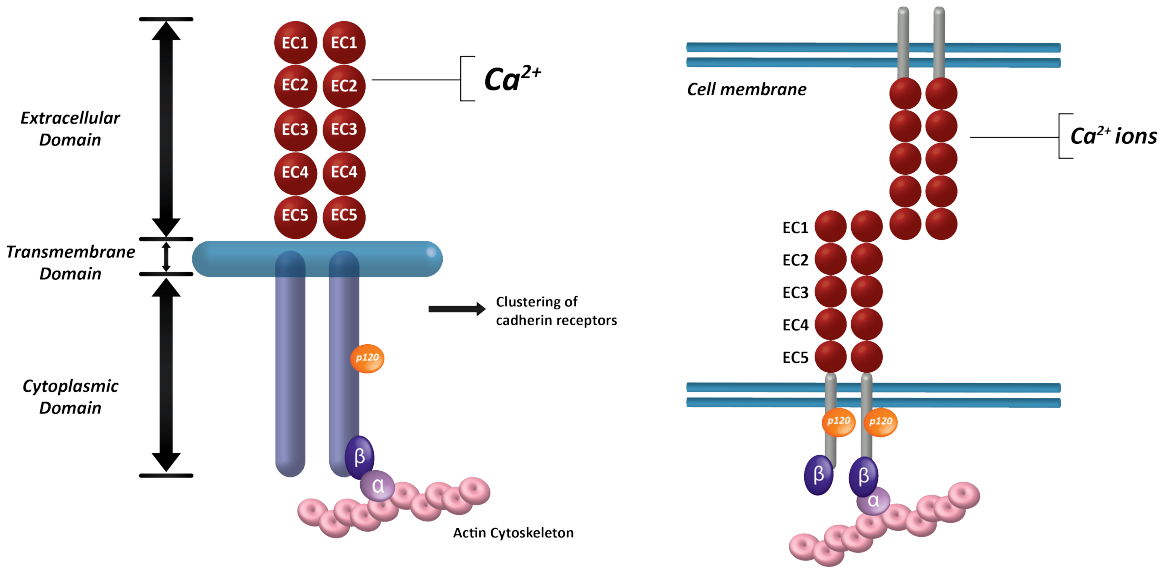

Figure 1.1 Schematic of the structure of classical cadherins and binding partners. The extracellular region consists of five extracelluar domains named EC1 to EC5. The extracellular region requires 3 calcium ions (Ca2+) to maintain rigid like structure. The cytoplasmic domain interacts with the actin cytoskeleton through its binding partners α-catenin and β-catenin. Juxtamembrane region of Cytoplasmic tail binds to p120 and distal catenin binding domain binds to catenins.

The cytoplasmic domain binds catenins, which link cadherin to actin, and are required for the maintenance of cell-cell contacts. This domain consists of two main regions: the distal region and the juxtamembrane region. The cytoplasmic domain is also a key player in intracellular signaling because it interacts with proteins that activate different signaling pathways that in turn dynamically regulate cell-cell adhesive contacts through actin cytoskeleton rearrangements (Fukata & Kaibuchi, 2001). Cadherins also indirectly regulate actin organization at cell-cell contacts through effectors that regulate the Rho GTPases, which are cytoskeletal regulatory proteins (Fukata & Kaibuchi, 2001; Kaibuchi, Kuroda, Fukata, & Nakagawa, 1999). It has been shown E-Cadherin adhesion activates c-Src signaling at cell–cell contacts (McLachlan, Kraemer, Helwani, Kovacs, & Yap, 2007). Homophilic ligation of E-cadherin also results in the activation of Rac and phosphoinositide 3-kinase (PI3K) to regulate cytoskeletal organization and the integrity of cadherin adhesions (Kovacs, Ali, McCormack, & Yap, 2002).

The interaction between the cadherin cytoplasmic domain and the catenin family of proteins is required for cadherin junction assembly, cadherin-mediated signaling, cytoskeletal anchoring, and the stability of cell-cell adhesions (Gumbiner, 2005; Takeichi, 1990). In addition to its potential involvement in signaling, the cytoplasmic region is also important for linking cadherin to the actin cytoskeleton through its intracellular binding partners, α-catenin and β-catenin (Gottardi & Gumbiner, 2001; Nagafuchi, Ishihara, & Tsukita, 1994). The distal cytoplasmic region binds β-catenin, which in turn binds to the actin-binding protein α-catenin (Shapiro & Weis, 2009). On the other hand, p120 catenin binds directly to the juxamembrane region of the cadherin cytoplasmic tail (Figure. 1.1) and has been implicated in cadherin-dependent regulation of Rho GTPase signaling (Reynolds, 2007; Reynolds & Roczniak-Ferguson, 2004). P120

catenin also regulates cadherin internalization (Oas et al., 2013; Xiao et al., 2005; Xiao, Oas, Chiasson, & Kowalczyk, 2007).

The cytoskeletal interaction is essential for the normal function of cadherins. The actin cytoskeleton is responsible for force transmission across the cell membrane, as well as for maintaining the cell structure. Cadherin coupling to the cytoskeleton is required for cadherin clustering, the maintenance of stable cell-cell contacts, and cell sorting during morphogenesis (Halbleib & Nelson, 2006; Yap, Brieher, Pruschy, et al., 1997).

1.2 Cadherins in Cell Sorting

Cadherins also play critical roles in regulating cell sorting and cell migration during tissue morphogenesis (Takeichi, 1988, 1991). Segregation of the cells into particular tissue layers and tissue boundaries formation is defined as a cell sorting process.

The dynamic regulation of cadherin adhesion and cell surface expression levels in response to various intercellular and intracellular signals is thought to underlie cell recognition and cell sorting, the formation and maintenance of tissue boundaries, and the coordinated cell movements and cell rearrangements in tissues (Nose, Nagafuchi, & Takeichi, 1988; Tepass, Godt, & Winklbauer, 2002). Cadherins are essential for the formation of highly polarized sheets of epithelial cells (Epithelial sheet) that line the cavities and surfaces of many tissues such as skin and the intestine (Halbleib & Nelson, 2006; Takeichi, 1995; Tepass et al., 2002).

Cadherin expression is regulated during tissue development, and cadherins have been shown to play an important role in cell sorting and tissue organization (Nose et al.,1988; Tepass et al., 2002). In developmental processes, cells that express different cadherin subtypes sort out into separate tissues. This observation of sorting out and the

selective aggregation of cells that express identical cadherin subtypes suggested that cadherins only bind to identical cadherins on adjacent cells—that is, they were assumed to be homophilic binding molecules (Nose et al., 1988). Homophilic interactions refer to the binding between identical proteins (cadherins), while in heterophilic binding, different proteins (cadherin subtypes) bind each other. The specificity of binding between cadherin extracellular domains was thought to be a fundamental requirement of the cell sorting process.

To test whether homophilic cadherin binding could influence cell sorting, cell aggregation studies were done with L-cells that were transfected with E- or P- cadherins (Nose et al., 1988). In this study, the different cell lines were stained with either green or red fluorescent dyes. A mixed suspension of both cell lines was stirred on shaker for 45 minutes, and images of the aggregates revealed that the cadherin expressing cells primarily formed clusters of E-cadherin or P-cadherin expressing cells. The specificity determining region of the proteins in these in vitro sorting assays was linked to the EC1 domain, as swapping the EC1 domain of P-cadherin with E-cadherin abolished this aggregation specificity (Nose, Tsuji, & Takeichi, 1990). When the EC1 domain of E-cadherin was exchanged with the P-cadherin EC1 domain, the E-cadherin expressing cells intermixed with P-cadherin expressing cells. These findings supported the view of that cadherins are homophilic binding molecules, and that the ectodomain has the ability to select among different types of cadherins in order to preferentially bind to a similar cadherin on neighboring cells (Gumbiner, 1996; Nose et al., 1988; Nose et al., 1990).

To assess the roles of cell adhesion molecules in tissue segregation, investigators used in vitro studies of the behavior of cell populations selected or designed to express

adhesion molecules in measured amounts. To explore the mechanism of cadherin-mediated cell sorting, two different in vitro assays, including the cell aggregation assay and the hanging drop assay were used to mimic cell sorting *in vivo*. In the former method, two cell populations expressing different cadherins are mixed together and stirred continuously for an hour to test whether the cells form separate aggregates or not (Foty & Steinberg, 2004; Niessen & Gumbiner, 2002). In the hanging drop method, cells are mixed in a droplet that supported on the lower surface of the lid of a Petri dish and after 24 to 48 hours of incubation, cell sorting patterns are analyzed (Foty & Steinberg, 2004; Niessen & Gumbiner, 2002).

Early studies of L-cells transfected with E-cadherins and P-cadherins showed cells are sorting out and forming aggregates indicated that E-cadherins and P-cadherins do not cross react (Nose et al., 1990). However, there is extensive evidence that cadherins do cross react and form heterophilic bonds. For example, cells expressing chicken B-cadherin and liver cell adhesion molecule (the E-cadherin homolog in chicken) completely formed mixed aggregates in aggregation assays (Murphy-Erdosh, Yoshida, Paradies, & Reichardt, 1995). Other studies also showed cross-reactivity between several of different cadherin subtypes (Duguay, 2003; Foty & Steinberg, 2005).

However, more recent cell sorting studies demonstrated that differences in the cell conditions used in the cell aggregation assay could lead to different results. It has been shown that slow shaking resulted in the formation of mixed aggregates while more vigorous shaking caused the same cells to sort into homophilic aggregates (Duguay et al., 2003). Another key factor, which appears to play major role in the cell sorting process is the cadherin expression levels. Researchers found that 25% differences in the expression

level of the identical cadherin subtype was quite enough to cause two cells populations that both expressed P-cadherin to sort out from each other (Steinberg & Takeichi, 1994). It was also shown that cells expressing the same levels of E-cadherins and P-cadherins intermixed in a different, long-term cell sorting assay. However, by changing difference in the expression levels on the two cell types by >25%, the cells sorted out into homophilic aggregates of cells expressing the same cadherin subtype (Steinberg & Takeichi, 1994).

The above-mentioned studies suggested that cell-surface intercellular adhesion energies drive cell segregation. Specifically, in vitro studies suggested that cell sorting depends on the identities of expressed cadherin subtypes as well on the surface densities of those cadherins. These two parameters would contribute to the adhesion energy between adjacent cells that is also referred to as the tissue surface tension.

These observations led to the proposal of the Differential Adhesion Hypothesis (DAH) by Steinberg as a model to predict tissue sorting behavior (Steinberg & Takeichi, 1994; Steinberg, 2007). The DAH postulates that the differences in the adhesion energies between cells cause cells to sort out, in order to reduce their interfacial free energy. This behavior is analogous to liquid-liquid phase separation. Differences in the cadherin identity as well as cadherin expression levels can result in differential adhesion energies, which could influence cell segregation (Steinberg, 1963). The DAH was supported by several in vitro studies, many of which are described in the above paragraphs (Gumbiner, 1996; Nose et al., 1988; Steinberg & Takeichi, 1994; Steinberg, 2007; Takeichi, 1995).

In contrast to the postulate that differences in cadherin adhesion drive cell sorting, studies showed that cadherins do bind and form heterophilic bonds and found that

measured binding affinities and adhesive energies of heterophilic cadherin bonds were not substantially different from homophilic cadherin interactions (Katsamba et al., 2009; Niessen & Gumbiner, 2002; Prakasam, 2006b). Biophysical studies with a surface force apparatus (SFA) quantified adhesion energies of homophilic and heterohphilic cadherin extracellular domain interactions, in order to test whether homophilic adhesion is thermodynamically preferred over heterophilic interactions. Those biophysical measurements showed that homophilic and heterophilic cadherin adhesion energies are quite similar to each other (Prakasam, 2006a; Prakasam, 2006b).

Another study exploited a capillary flow assay to assess the effect of cadherin adhesion specificity on the cell sorting process. This approach determines the initial adhesion to cadherin extracellular domains on the walls of a capillary tube, as well as strengthening of adhesion over time. This study showed that CHO cells that expressed different cadherins bound equally well to substrata that coated with different purified cadherin extracellular domains (Niessen & Gumbiner, 2002). The results of this study also suggested that cadherins are not exclusively homophilic binding proteins. Together, the force measurements, solution binding studies, and flow assay results are important as they showed that cadherins cross react to form heterophilic bonds and did not show significant heterophilic specificity in the extracellular domains.

These findings raise questions regarding the molecular mechanism underlying correlations between cadherin specificity and cell sorting in vivo. In addition, if cadherin extracellular domains do not exhibit substantial binding selectivity, then what other cadherin-dependent parameters might contribute to cadherin-mediated cell sorting?

1.3 Cell Mechanics Contribute to Cell Sorting

The surprising results of a study investigating the role of mechanical properties of cells in cell sorting and tissue organization suggested that cortical tension, which is defined by the stiffness of cell cortex may also be a key factor that directs progenitor-cell sorting in zebrafish (Krieg et al., 2008). They used atomic force microscopy (AFM) to quantify the adhesive and mechanical properties of ectoderm, mesoderm and endoderm cells from gastrulating zebrafish embryos. They reported that differences in the adhesion exhibited by germ-layer progenitors alone is not enough to explain cell sorting behavior in vivo. Instead, differences in cortical tensions were critical for cell sorting behavior. These findings shed new light on additional parameters influencing cadherin-mediated cell sorting, and suggest that cell–cell adhesion and the contractility of cell interfaces both contribute to the tissue surface tension.

More recently Manning et al (Manning, 2010) proposed a model that explicitly accounts for the ratio of adhesion to cortical tension in determining tissue surface tension and cell sorting. Their model showed that as the ratio between these two quantities change, there is a transition from adhesion dominated to cortical-tension dominated cell sorting behavior (Manning et al., 2010). Given the cadherin requirement for cell segregation in vivo and in vitro, an essential question yet to be answered is whether cell sorting is governed solely by the biophysical properties of cadherin bonds or possibly by other cadherin-dependent factors, such as intracellular signaling and/or cell mechanics. Studies that address the former question are the focus of Chapter 2 in this thesis, and cadherin mechanotransduction and the underlying mechanism, which is discussed in following section, is focus of Chapters 3 and 4 of this work.

1.4 Mechanotransduction

Mechanotransduction is one possible mechanism that could link the roles of cadherin adhesion and cortical tension in cell sorting. Mechanotransduction is any of various mechanisms by which cells convert mechanical stimulus into electrochemical activity. Different forms of mechanical stresses are present in cellular environments (Figure 1.3). These mechanical stresses can broadly be categorized into external forces applied to tissue and internal forces, generated through the cytoskeleton network. Cells respond to these external and internal forces through a variety of feedback mechanisms, one of which is mechanotransduction. The most distinct types of forces exerted on cells are from adjacent cells during cell migration in morphogenesis and wound healing as a result of shear stress in the vascular tissue. Biochemical responses to mechanical forces lie behind many critical cell functions including cell migration, bone regeneration, and the formation of the vascular endothelium (Lecuit & Le Goff, 2007; Paluch & Heisenberg, 2009a). In recent years, the importance of mechanical forces in development, homeostasis and disease progression is increasingly appreciated (Hahn & Schwartz, 2009; Jaalouk & Lammerding, 2009; Schwartz & DeSimone, 2008).

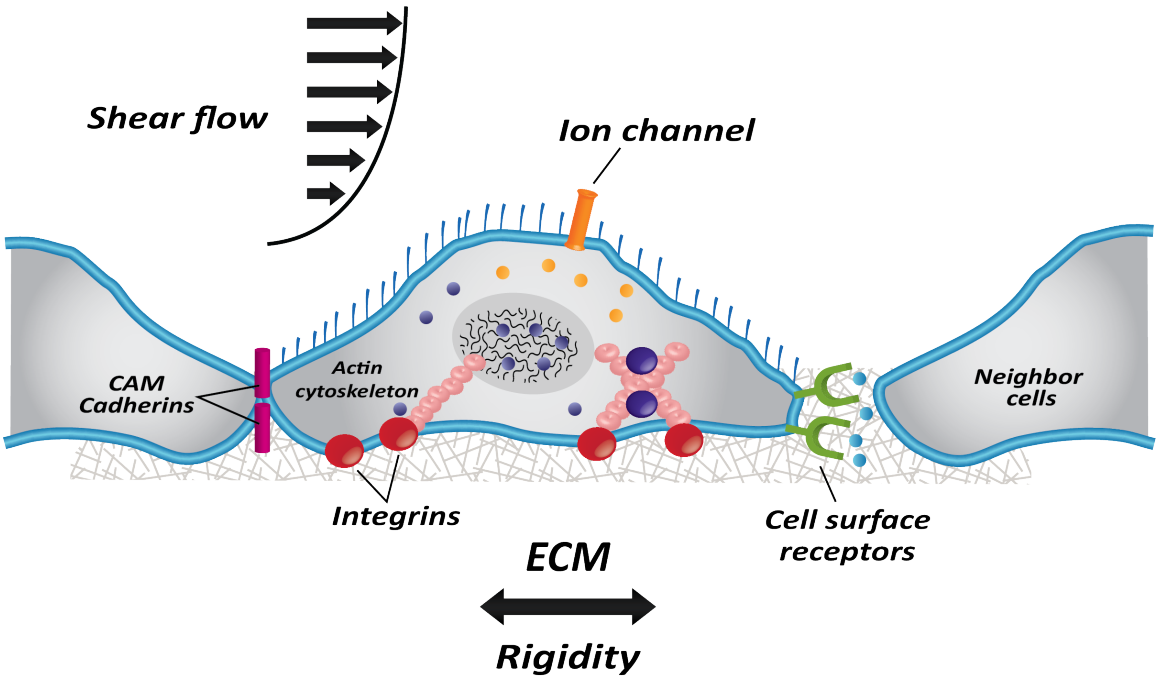

Figure 1.2 Cells use cell surface receptors to communicate with their environment. Cadherins bind adjacent cells together while integrins bind cells to the ECM (extracellular matrix). Cells use these receptors to sense mechanical perturbations in the environment and to transduce them into electro-chemical signals inside the cells to regulate cell functions.

There are many diseases associated with malfunctions in mechanotransduction system, such as loss of hearing, cardiac hypertrophy, atherosclerosis, and cancer (Butcher, Alliston, & Weaver, 2009; Garcia-Cardena, Comander, Anderson, Blackman, & Gimbrone, 2001; Kumar & Weaver, 2009; Palmer, 2005; Vollrath, Kwan, & Corey, 2007). The common underlying reason among most of these diseases is disruption of the elaborate force transmission mechanism manifested within interfaces between the extracellular matrix, cytoskeleton, and the nuclear interior (Jaalouk & Lammerding, 2009). The defects that affect cellular structure and cellular mechanosensing together with mutant proteins that are involved in downstream signaling pathways could lead to defective mechanotransduction.

Over the last decade, several studies have focused on identifying molecular components involved in cellular mechanotransduction (Katsumi, Orr, Tzima, & Schwartz, 2004; Wang, Dembo, Hanks, & Wang, 2001; Wang, Butler, & Ingber, 1993). Researchers proposed a few biological components as major cellular mechanosensor candidates that allow cells to probe their environment. Adhesion receptors at cell-cell contacts and focal adhesions at cell-ECM contacts are considered among those (Jaalouk & Lammerding, 2009; Schwartz & DeSimone, 2008; Wang et al., 2001). However, the key mechanosensor components and the underlying mechanisms remain to be elucidated.

There are three main categories of mechanical forces that affect the cells: (1) externally applied forces; (2) forces resulting from matrix mechanical properties (substrate rigidity); and (3) contractile forces generated by components in the cell (Chen, 2008; Chien, Li, & Shyy, 1998). Proteins that transduce force include membrane proteins (e.g., ion channels), extracellular matrix proteins (e.g., integrin and fibronectin), and cytoskeletal components (e.g., actin and intermediate filaments) (Humphrey, Dufresne, & Schwartz, 2014). Mechanosensors respond to tension fluctuations by inducing different types of biochemical changes such as signaling and conformational changes that cause the activation or deactivation of proteins of interest, protein reorganization, and complex formation (Collins et al., 2012; Conway et al., 2013; Kim et al., 2015).

Adhesion proteins such as integrins or cadherins form force-responsive adhesion complexes. These proteins receive mechanical cues from their environment and proteins within the adhesion complexes respond to forces transmitted either through an extracellular matrix or neighboring cell(s) (Jaalouk & Lammerding, 2009). It has already been shown that integrins couple cytoskeletal networks to the extracellular matrix and

sense substrate rigidity to modulate cell mechanics, in a myosin II dependent manner (Elosegui-Artola et al., 2014). For instance, there is ample evidence to support the view that the cells can sense the elastic modulus of the underlying substrate to modulate substantially distinct cellular functions. Gieger et al showed that in cells seeded on compliant substrate coated with fibronectin, focal adhesion size, stress fiber formation and traction forces increased as substrate stiffness increased (Geiger & Bershadsky, 2002; Geiger, Spatz, & Bershadsky, 2009).

Focal adhesions (FA) are integrin-based, multiprotein complexes that form a mechanical link between the actin cytoskeletal and the extracellular matrix. There is extensive evidence that focal adhesions are force sensitive and exhibit mechanosensitive properties (Cavalcanti-Adam et al., 2007; Katsumi et al., 2004; Shemesh, Geiger, Bershadsky, & Kozlov, 2005; Wang et al., 2001). For example, cells on hard, extracellular matrix-coated surfaces spread more compared to cells on softer substrates, in an integrin-dependent manner (Chou, Cheng, & LeDuc, 2009; Geiger et al., 2009).

Another interesting study showed that matrix elasticity dictates stem cell lineage fate (Engler, Sen, Sweeney, & Discher, 2006). Mesenchymal stem cells (MSC) were seeded on compliant substrates with elastic moduli ranging from around 1 kPa to 100 kPa. Results showed MSC seeded on the soft substrate differentiated into softer tissues with greater expression of neurogenic transcripts on softer substrates (0.1-1 kPa). MSCs culture on the hardest surface (34 kPa) developed into tissues with increased expression of osteogenic markers (Engler et al., 2006). On soft extracellular matrix, cytoskeletal tension at adhesion contacts do not develop and focal adhesions fail to mature, while an increase in mechanical load at sites of cell-matrix adhesions results in focal adhesion

growth. Integrin adhesions were also strengthened by increasing the amount of force on integrin-ligand bonds or on focal adhesions (Galbraith, Yamada, & Sheetz, 2002; Lo, Wang, Dembo, & Wang, 2000; Pelham Jr. & Wang, 1997).

Among many cell–cell adhesion molecules linked to the cytoskeleton, classical cadherins are perhaps the most important for dynamically regulating the transmission of forces between cells (Niessen, Leckband, & Yap, 2011). Although cadherins were previously considered to be passive points of force transmission between adjacent cells, recent findings showed that cadherin complexes actively sense mechanical changes in the cell environment and excite biochemical responses that direct cell behavior (Müller, 2008; Tzima et al., 2005).

Mechanosensing is not only limited to response to external forces, but cells can also use internally generated stresses generated by actomyosin contractility, for example, to probe the mechanical properties of their environment and display a wide range of responses. In other studies, Chopra et al (Chopra, Tabdanov, Patel, Janmey, & Kresh, 2011) used traction force microscopy to measure traction forces generated by myocytes plated on N-cadherin-coated substrata. Results demonstrated that N-cadherin-mediated traction forces are responsive to substrate stiffness. By increasing the elastic modulus of the gels, cells exerted more forces and cause more deformation on underlying substrate. Cell spreading on N-cadherin coated substrata was responsive to the substrate stiffness. It was reported that on soft cadherin-coated gels, cells were unable to form actin bundles. Also, cells on soft substrata could not form large cadherin-catenin complexes. Compared to cells on soft substrata, on hard surfaces cell generated firm actin cables and developed large cadherin-catenin complex (Chopra et al., 2011).

In pioneering research, Le Duc et al (le Duc et al., 2010) used magnetic beads coated with E-cadherin ectodomains to apply force to E-cadherin receptors expressed on F9 cells. This magnetic twisting cytometry approach was used to apply an oscillatory magnetic field perpendicular to the beads' magnetic moment, in order to generate shear force on receptors at the cell surface. Interestingly, it has been observed that cells become stiffer in very short period of time (within a minute), when cadherin receptors were mechanically perturbed (le Duc et al., 2010). This finding demonstrated that cadherin complexes are force transducers—a finding that may link cadherin complexes to changes in cortical tension.

The cadherin-mediated stiffening response was abolished in cells treated with blebbistatin, suggesting that the cell stiffening-response is myosin-dependent. Treating cells with latrunculin B or cytochalasin D abrogated cadherin-mediated stiffening, and indicated that cadherin force transduction required intact actin. In the absence of vinculin cadherin-mediated stiffening was only partially reduced. There was no paxillin or talin accumulation at cell-cell contact. Paxillin and talin are both involved in vinculin recruitment to integrin adhesions (del Rio et al., 2009; Lee, Kamm, & Mofrad, 2007; Pasapera, Schneider, Rericha, Schlaepfer, & Waterman, 2010; Tang, Mehta, & Gunst, 1999). The vinculin is recruited to stretched talin, such that removing vinculin would fully inhibit integrin-mediated force transduction (del Rio et al., 2009; le Duc et al., 2010). These findings imply that there are differences between cadherin-mediated mechanotransduction and integrin-induced force transduction (le Duc et al., 2010).

Although several studies tested whether cells could sense substrate rigidity through cadherin complexes, Yonemura el al (Yonemura, Wada, Watanabe, Nagafuchi,

& Shibata, 2010) focused on mechanism of mechanotransduction at cadherin-mediated cell-cell junctions. In an important finding, they identified α-catenin as tension transducer. α-Catenin is key factor in cadherin junction formation. Yonemura et al. (Yonemura et al., 2010) proposed a model where α-Catenin goes through conformational change under tension, causing vinculin binding site to be exposed (Figure 1.3). Consequently, the model predicts that vinculin recruitment is followed by actin and myosin accumulation through binding to vinculin. This local actin remodeling would account for the increase in measured cell stiffness in response to cadherin perturbations (le Duc et al., 2010).

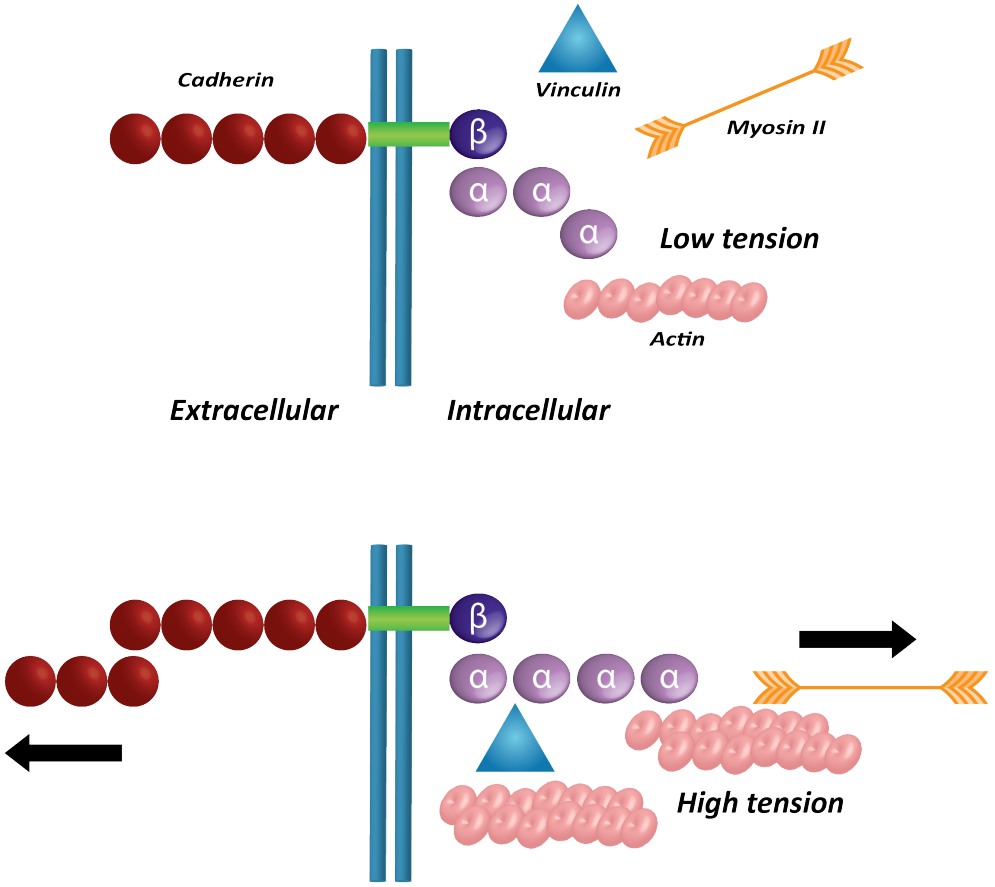

Figure 1.3 Suggested mechanism of force mediated vinculin recruitment to cadherin complexes. In non stretched mode (low tension) vinculin binding domain has been masked and α-Catenin cannot bind to actin. Under tension in the stretched mode (high tension), the α-Catenin domain is stretched and the vinculin binding domain is exposed, in order to enable vinculin to bind to the complex and recruit actin.

Mechanotransduction could result in cadherin-dependent differences in cortical tension that contribute to cell sorting. How cadherin binding-selectivity might contribute to cell mechanics has not been explored. Chapter 3 of this thesis further explores how the identity of cadherin ligands impacts mechanotransduction at cadherin-based cell-cell junctions. The goal is to test whether cadherin binding differences might contribute to differences in force transduction and altered cortical tension, in ways that could

contribute to cell sorting. Chapter 4 further considers the molecular mechanisms underlying cadherin-based mechanotransduction. In particular, I focus on the role of α-Catenin in cadherin-based mechanotransduction. I used different biophysical methods described below and genetically modified cells to explore the role of α-Catenin in mechanotransduction and substrate rigidity sensing.

1.5 Methods

In this thesis, two principal methods were extensively used throughout this research as major tools to study cadherin mechanotransduction: magnetic twisting cytometry (MTC) and traction force microscopy (TFM). MTC enables us to mechanically probe cadherin receptors on the cell surface by dynamically applying controlled shear and measure biochemical response in the form of changes in mechanical properties (cell stiffening) and subcellular distributions of proteins (Beningo & Wang, 2002a; Na & Wang, 2008; Wang et al., 1993) . Alternatively, in TFM force on the bonds is at steady state. Cells are seeded on an elastomeric substrate coated with protein of interest. We analyzed the amount of force exerted by the cells on the substrate based on the extent to which the cells deform elastic substrates with defined Young's moduli (Beningo, Lo, & Wang, 2002; Beningo & Wang, 2002b; Kandow, Georges, Janmey, & Beningo, 2007).

1.5.1 Magnetic twisting cytometry (MTC)

In magnetic twisting cytometry (MTC), ferromagnetic beads were modified with the extracellular domains of cadherins, or with other proteins. After allowing the beads to bind to the cell surface, a magnetic field is generated by electric coils around the sample (Figure 1.4). The ferromagnetic beads (Fe_3O_4, 4.9 μm in diameter) are horizontally

magnetized by applying a large magnetic field (1 Tesla). At this point, a sinusoidal twisting field is applied perpendicular to the magnetization axis, in order to generate a torque T on the beads and cause their sinusoidal displacement D relative to the cell surface.

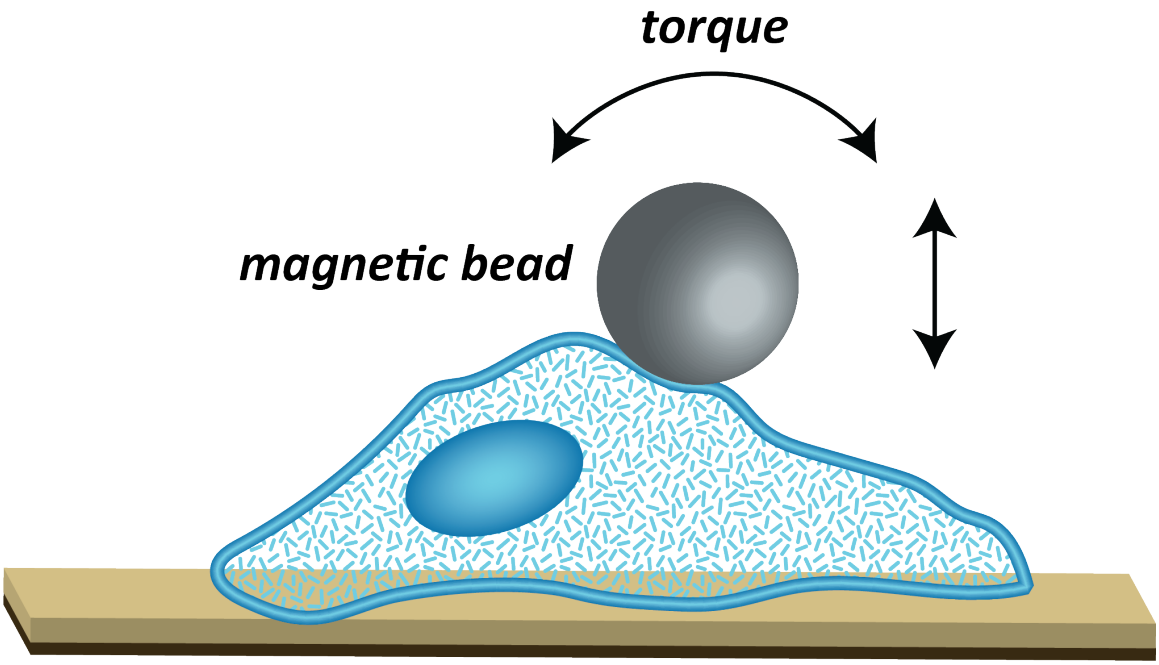

Figure 1.4 Schematic set up of Magnetic Twisting Cytometry. Twisting torques were applied to cadherin coated ferromagnetic beads (4.9 μm in diameter) beds bound to cells. The beads were magnetized by a strong (1,000 Gauss) magnetic field pulse for short period of time (1 ms) in the horizontal direction using the magnetizing coils. The magnetic twisting field of 60 Gauss with frequency of 0.3 Hz was applied continuously and orthogonal to the magnetization moment for 2 min.

Software records synchronized bead positions by the computer program through a CCD camera (Hamamatsu), which is integrated with a Leica inverted microscope. The bead displacement is converted to complex modulus including the real part of shear modulus (stiffness) and the imaginary part, which is a measure of dissipation. Changes in the displacement amplitudes depend on the modulus/stiffness of the bead-cell junction and corresponding cellular components. Stiffness (elastic modulus) is the term commonly

being used to describe mechanical properties of cells. Stiffness (λ) is defined by ratio of stress/strain. The Young's modulus E is defined as the ratio of tensile stress to tensile strain and is simply called the 'elastic modulus'. On the other hand, the shear modulus G is described by ratio of shear stress to the shear strain, and is applicable when material is under applied shear or torque.

In viscoelastic materials such as cells, we are measuring the dynamic modulus, which is ratio of stress to strain subject to oscillatory forces. In viscoelastic materials, stress and strain are not in phase and they exhibit a phase lag (0<phase lag<90). In viscoelastic materials, stored energy defined by elastic modulus and energy consumed as a heat representing viscous property of the material. For that reason, we only report elastic modulus changes. In magnetic twisting cytometry shear being applied directly at cadherin junction at cell surface. In MTC, the force induced changes cause biochemical changes that affect mechanical properties of the cells and are quantified from changes in bead displacements.

1.5.2 Traction force microscopy (TFM)

Traction Force Microscopy (TFM) was used to quantify the ability of cells to sense substrate rigidity through cadherin bonds. TFM is a widely used technique for measuring the endogenous forces that cells exert on surfaces on which they adhere. Forces at cadherin complexes can be applied indirectly through actomyosin versus direct method by applying external force at cadherin junction. Cells apply tugging forces (endogenous forces) to locally sense substrate stiffness (Liu et al., 2010). Myosin-dependent endogenous forces modulate integrin's and cadherin's role in rigidity sensing. (Maruthamuthu, Sabass, Schwarz, & Gardel, 2011; Plotnikov, Pasapera, Sabass, &

Waterman, 2012) In this method fluorescence beads are embedded in elastomeric hydrogels. By monitoring fluorescent marker beads, gel deformations due to the generation of contractile forces by cells are tracked. The traction forces are determined from the displacement field of beads in the gel relative to the bead positions in the absence of attached cells. The traction field is an image of cell traction force distributions, and is measured from the bead displacement field. The displacement field of the beads beneath each cell is resolved by imaging bead positions in two states of under stress (cells attached) and stress-free (cells detached).

The bead position field is calculated from differences in two images using a cross-correlation method. The traction field, which is an image of distribution of cell traction force, calculated from displacement field based on Boussinesq method. Using MATLAB software, the amount of force, that exerted by the cells on the substrate were analyzed and measured which defined as a cell tension (Butler, Tolic-Norrelykke, Fabry, & Fredberg, 2002; Tolic-Norrelykke, Butler, Chen, & Wang, 2002). The resolution is about 5 to 10 nm. The basic concept of TFM is illustrated in Figure 1.5.

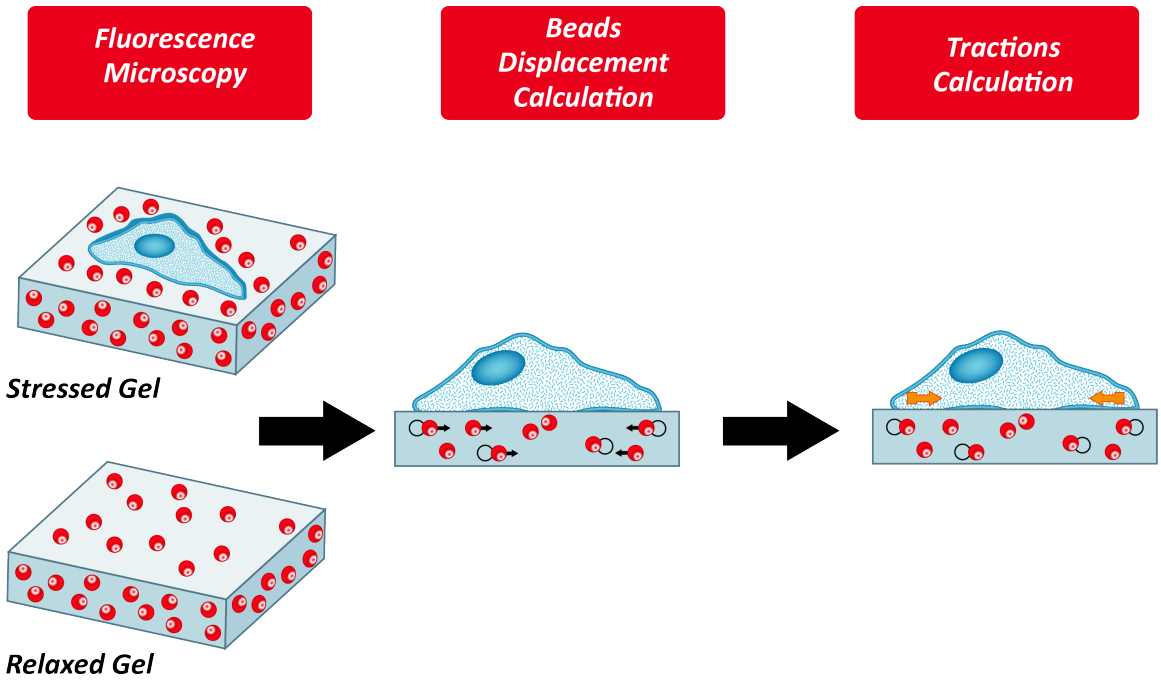

Figure 1.5 Schematic of Traction Force Microscopy. Cells were seeded on polyacrylamide gels with embedded red fluorescent beads (0.2 μm in diameter) embedded inside the hydrogel. Before and after single cell treatment, fluorescent images of the bead positions were taken in order to calculate the displacement field of the beads, by using digital image correlation (DIC) in a homebuilt MATLAB program. By using Boussinesq mathematical model (Butler, Tolic-Norrelykke, Fabry, & Fredberg, 2002) cell tractions were measured from the displacement field.

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Chapter 2

Cadherin Point Mutations Alter Cell Sorting and Modulate GTPase Signaling[1]

2.1 Introduction

Cadherins are intercellular adhesion proteins that are essential for maintaining the structural integrity of tissues. During morphogenesis, they are required for cell patterning, and, in mature tissues, they regulate crucial barrier functions (Gumbiner, 2005; Takeichi, 1995). The classical cadherins are the most extensively studied proteins in the cadherin superfamily. There are 20 known subtypes, which exhibit the same overall fold, but differ in their primary structure and tissue expression patterns. A central question is whether subtype-dependent sequence differences alter cadherin-mediated intercellular binding, and the implications of such differences for cadherin-dependent cell functions.

Investigations of differences between cadherin subtypes mainly focused on cadherin-dependent cell segregation. This is in part due to important in vitro studies suggesting that cell sorting depends on both the identities and surface densities of the expressed cadherin subtypes (Nose et al., 1988; Steinberg, 2007; Steinberg, 1963). Those findings suggested that that subtype dependent differences in intercellular adhesion energies direct cell sorting in vitro and possibly in vivo (Steinberg, 1963). This focused attention on cadherin affinities and their relationship to adhesion energies or the tissue

[1] Adapted, with permission, from H. Tabdili, A.K. Barry, M.D. Langer, Y.H. Chien, Q. Shi, K.J. Lee, S. Lu, D.E. Leckband, "Cadherin Point Mutations Alter Cell Sorting and Modulate GTPase Signaling," *Journal of Cell Science*, 2012, 125 (Pt 14): 3299-3309.

surface tension thought to influence cell segregation (Foty & Steinberg, 2005; Steinberg, 2007; Steinberg, 1963). The N-terminal cadherin domain is the main locus of cadherin binding differences that influence *in vitro* sorting. In the structures of complexes of the extracellular segment of Xenopus C-cadherin (Boggon et al., 2002) and truncated fragments of N-cadherin (Shan et al., 2000; L Shapiro, Kwong, Fannon, Colman, & Hendrickson, 1995) or E-cadherin (Haussinger et al., 2004; Pertz et al., 1999; Tomschy, Fauser, Landwehr, & Engel, 1996), the tryptophan at position 2 (W2) on the first extracellular domain (EC1, see Chapter 1, Figure 1.1) inserts into a hydrophobic pocket on the EC1 domain of the adjacent cadherin.

The high degree of sequence similarity among EC1 domains of type I classical cadherins begs the question of how this conserved binding motif supports cell binding selectivity. Yet, mutations in the W2 binding pocket alter cell-cell cohesion and sorting. Exchanging the N-terminal domain of E-cadherin with that of P-cadherin, or substituting residues 78 and 83 on mouse E-cadherin with the corresponding P-cadherin sequence altered the aggregation specificity of cells expressing the E-cadherin mutants (Nose et al., 1990). The A78M mutation abolished N-cadherin function (Tamura, Shan, Hendrickson, Colman, & Shapiro, 1998). Despite these qualitative observations, links between sequence differences, quantified affinities, and cadherin dependent functions have not been established. Solution binding affinities of recombinant, soluble fragments indicated that affinities differing by at least 5 fold correlated with in vitro cell sorting, assuming similar cadherin expression levels (Katsamba et al., 2009). However, semi-quantitative estimates of relative cell adhesion (Niessen & Gumbiner, 2002), quantified, protein-level adhesion energies (Prakasam et al., 2006a), strengths of single cadherin bonds (Shi,

Chien, & Leckband, 2008), or cohesive energies of cell aggregates (Duguay et al., 2003) do not always correlate with in vitro cell sorting outcomes. In vivo, the role of cadherin binding differences in cell sorting is less clear.

Differential cadherin expression correlates with retinal cell patterning in Drosophila, for example (Hilgenfeldt, Erisken, & Carthew, 2008) . Yet, cortical tension, rather than cell cohesion, appears to direct germ cell positioning in zebrafish embryos (Krieg et al., 2008). A possibility is that differential adhesion is unimportant in vivo, but correlations between mutations that impair cadherin adhesion and gastric cancer (Becker et al., 1999; Handschuh et al., 1999; Handschuh, Luber, Hutzler, Hofler, & Becker, 2001) suggest that altered cadherin adhesion may also modulate signaling. Differential cadherin adhesion could more broadly influence cell behavior through signaling. For example, affinity-dependent Rho GTPase signal amplitudes (Braga, 2002; Drees, Pokutta, Yamada, Nelson, & Weis, 2005; Noren, Arthur, & Burridge, 2003) could also modulate cortical tension, cell cycle progression, and differentiation (Fournier et al., 2008; Levenberg, Yarden, Kam, & Geiger, 1999). Studies of the broader impact of binding differences on in vitro cell sorting revealed that affinities between subtypes that differed by greater than ~ three fold could support cell segregation (Y.H. Chien, Ph.D thesis, 2009). However, the influence of these binding differences on signaling and its potential effect on cortical tension had not been considered.

This study investigated the impact of cadherin binding site mutations and their corresponding two-dimensional affinities on cadherin-ligation-dependent GTPase signaling. Comparisons of ectodomain mutants rather than cadherin subtypes enabled us to focus on affinity differences, independent of differences in cytoplasmic domain

interactions. Selected Xenopus C-cadherin mutants were based on sequence differences between amino acids near docked W2 in the hydrophobic pocket of N-cadherin. Prior micropipette measurements quantified the affinities of full length C-cadherin mutants in the native context of the cell membrane (Y.H. Chien, Ph.D thesis, 2009). These cadherin affinities were then compared with both in vitro cell sorting outcomes (Y.H. Chien, Ph.D thesis, 2009) and with ligation-dependent GTPase signaling, described in this thesis.

2.2 Design and Expression of C-cadherin Mutants

Chinese Hamster Ovary (CHO) cells that express the same densities of Xenopus C-cadherin (C-CHO) and chicken N-cadherin (N-CHO) sort out in agitated cell suspensions (Shi et al., 2008). Here we used these proteins as models to investigate the relationship between affinity differences due to binding site mutations and GTPase signaling. On the basis of sequence and structural comparisons of docked W2 at EC1-EC1 interfaces of Xenopus C-cadherin and mouse N-cadherin (Figures 2.1A, 2.1B), three sites in the EC1 domain of C-cadherin were mutated to the corresponding amino acid in chicken N-cadherin (Figure 2.1C). The EC1 domain of mouse N-cadherin (Figure 2.1B) is 98% identical to that of chicken N-cadherin. The K8NS10P double mutant potentially alters the docked W2 orientation. The other two mutations S78A and M92I involve more polar residues lining the W2 binding pocket that were postulated to play a greater role in modulating the affinity (Patel et al., 2003). Two other mutants Q23G and E83V did not express sufficiently well for these biophysical studies (Y.H. Chien, Ph.D thesis, 2009).

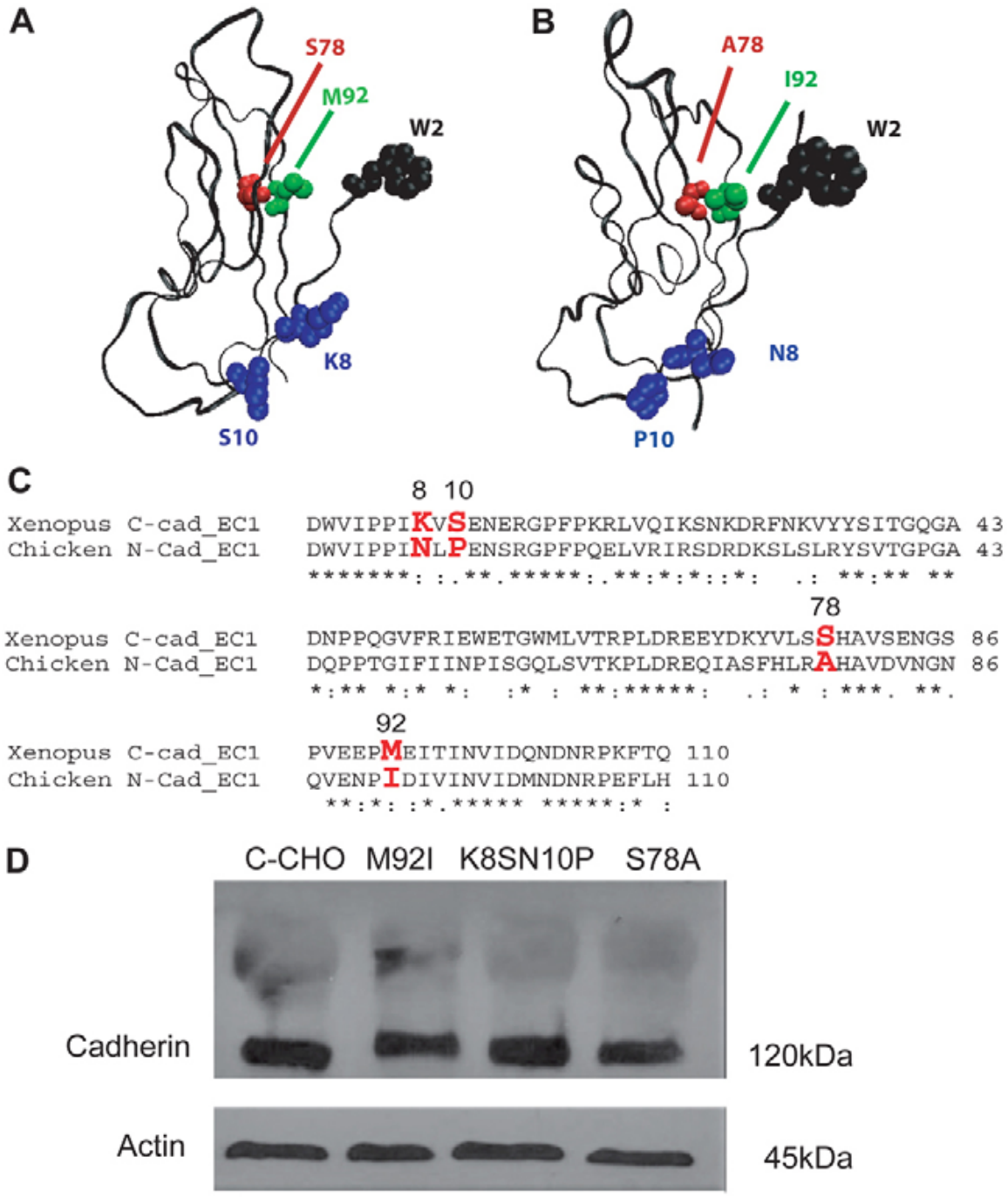

Figure 2.1 Crystal structure of the EC1-EC1 complex. (A) Xenopus C-cadherin (Protein Data Bank access code 1L3W). (B) Murine N-cadherin (Protein Data Bank access code 1NCG). Both structures were generated with Visual Molecule Dynamics (VMD) (W. Humphrey, Dalke, & Schulten, 1996). The positions of the amino acids mutated in this study are labeled blue, red and green. (C) Sequence alignment between C-cadherin and N-cadherin EC1 domains. Red letters indicate the loci of amino acid mutations. (D) Western blots of CHO-K1 cells expressing C-cadherin mutants and WT C-cadherin. The biotinylated cell surface cadherin was detected with an antibody against the cytoplasmic domain of C-cadherin.

Clones that express the C-cadherin mutants were selected according to expression level, by quantitative FACS and by Western blots of cell surface proteins. Comparisons of in vitro cell sorting and quantitative GTPase activation measurements require cell populations that express similar cadherin surface densities. The following clones (cadherin surface densities in parentheses) were selected for these studies: WT C-cadherin (18/μm^2), K8NS10P (20/μm^2), S78A (22/μm^2), M92I (19/μm^2), and WT N-Cadherin (16/μm^2). The expression levels of WT and mutant C-cadherins were compared to each other and to an actin loading control by Western blots of the biotinylated surface proteins (Figure 2.1D). The Western blots agreed with FACS measurements, which used antibodies against the ectodomains (Y.H. Chien, Ph.D thesis, 2009).

2.3 Ligation-dependent Rac1 Activation

Quantitative measurements of Rac1-GTP levels in cells seeded on either CEC1-5-Fc or NEC1-5-Fc substrata demonstrated the correlation between binding affinities and signaling. In these comparisons between signaling amplitudes, the immobilized protein (ligand) densities, the cell type, the overall protein scaffold, including the cytoplasmic domain, the cadherin expression levels, and the measurement time are the same. Therefore, the only known variable is the affinity between the cadherins mediating cell attachment. Rac1-GTP in C-CHO cells increased up to 45 minutes after attachment to CEC1-5-Fc substrata (5 x 10^3 cadherin/μm^2) (Figure 2.2A), similar to prior reports (Noren et al., 2003). By contrast, N-CHO on either CEC1-5-Fc or NEC1-5-Fc (5 x 10^3 cadherin/μm^2) did not exhibit any change in Rac1-GTP levels. The difference between N-CHO and C-CHO was not due to differences in cadherin expression (Table 2.1). Due to the robust Rac1 activation in C-CHO at 45 minutes, we compared Rac1-GTP levels 45

minutes after seeding different cells onto either CEC1-5-Fc or NEC1-5-Fc substrata (Figure 2.2B). A key finding is that the Rac1-GTP levels increased with the two-dimensional cadherin affinity (Figure 2.2C).

In C-CHO bound to CEC1-5-Fc, the Rac1-GTP increased by a factor of 6.3±0.3 relative to the basal levels at t=0 minutes (Figure 2.2B). The quantitative data are summarized in Table 2. K8NS10P ligation to CEC1-5-Fc increased Rac1-GTP 7±2 fold over initial Rac1-GTP levels (Figure 2.2B; Table 2.2). The latter is statistically similar to WT C-cadherin ($p = 0.69$). The M92I mutant, with an intermediate affinity for WT CEC1-5-Fc between K8NS10P and S78A, triggered a 3.7 fold increase in Rac1-GTP (Figure 2.2B; Table 2.2). By contrast, in cells expressing S78A, the Rac1-GTP was 0.6±0.2. Rac1-GTP levels were unchanged in N-CHO seeded on CEC1-5-Fc. Conversely, on NEC1-5-Fc substrata, the Rac1-GTP levels in WT C-CHO and N-CHO were 0.9±0.1 and 0.8±0.3, respectively (Figure 2.2B; Table 2). The K8NS10P cell adhesion to NEC1-5-Fc similarly did not activate Rac1-GTP. Figure 2.2C shows the correlation between the measured affinities of cell-surface C-cadherin, determined by micropipette measurements described elsewhere (Y.H. Chien, Ph.D thesis, 2009) and Rac1-GTP activation.

To address possible differences in GTPase signaling at cell-cell junctions, global Rac1 activation was quantified in confluent cell monolayers, following a calcium switch. In these studies, cadherin junctions are disrupted, by removing the calcium required for cadherins' adhesive function, and junction recovery is reactivated by calcium re-addition. Although it was only possible to analyze Rac1-GTP levels at homophilic cell-cell junctions, the results were qualitatively similar to signaling triggered by adhesion to

immobilized, recombinant ectodomains (Figure 2.3).

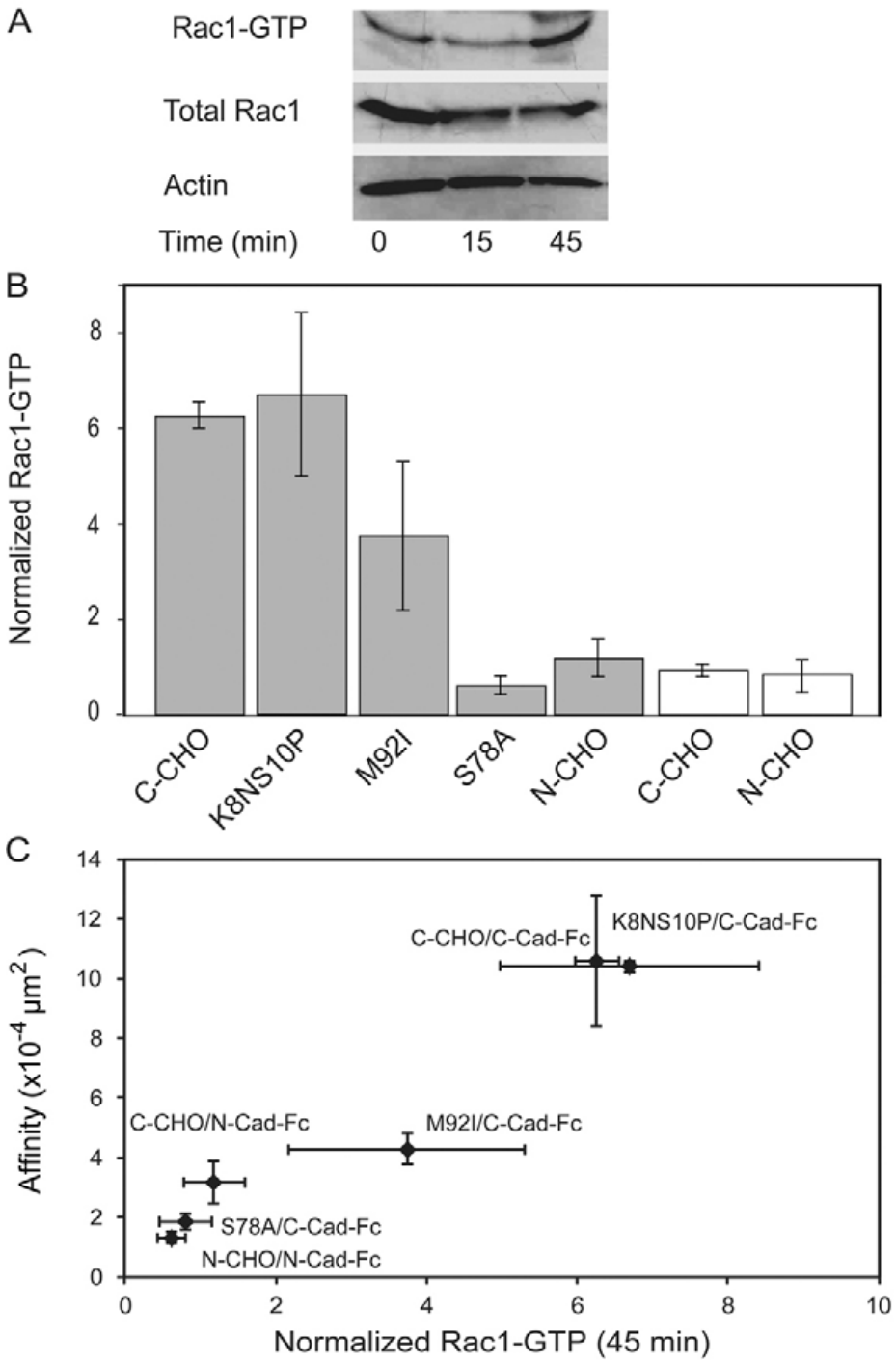

Figure 2.2 Rac1-GTP levels in cells cultured on either CEC1-5-Fc or NEC1- 5-Fc substrata. (A) Western blot analysis of the Rac1-GTP levels in C-CHO (21 cadherins/ μm^2) seeded on CEC1-5-Fc substrata (5x10^4/ μm^2) at t=0, 15 and 45 minutes. The top, middle, and bottom panels indicate the Rac1-GTP, the total Rac1, and the actin loading control, respectively. (B) Normalized Rac1-GTP 45 minutes

after attaching cells to the different cadherin-coated substrata. The cadherin subtype expressed on the CHO cell is indicated below each bar. Gray bars indicate the use of CEC1-5-Fc substrata, and white bars denote NEC1-5-Fc substrata. The cadherin surface densities are in Table 2.1. (C) Plot of the two-dimensional cadherin binding affinity versus Rac1-GTP levels 45 minutes after cell adhesion. Data are means ± s.d.

Table 2.1 Two-dimensional cadherin affinities and dissociation rates (from Tabdili et al., 2012)

Expressed cadherin	m_R (μm^{-2})	Cadherin-Fc on red blood cells	m_L (μm^{-2})	k_r ($second^{-1}$)	K_a ($\times 10^{-4}$ μm^2)	R^2
C-cadherin	18	C-cadherin	10	0.59±0.21	10.6±2.2	0.89
N-cadherin	15	N-cadherin	69	1.13±0.37	1.85±0.26	0.99
N-cadherin	15	C-cadherin	19	0.82±0.37	3.2±0.7	0.97
C-cadherin	14	N-cadherin	38	1.32±0.26	3.48±0.20	0.90
K8NS10P	41	C-cadherin	6	1.20±0.28	10.30±0.79	0.87
K8NS10P	41	N-cadherin	9	1.59±0.27	2.5±0.1	0.8
S78A	46	C-cadherin	16	1.44±0.60	1.65±0.25	0.94
S78A	46	N-cadherin	9	1.74±0.84	2.35±0.37	0.74
M92I	16	C-cadherin	16	2.30±0.72	4.29±0.54	0.95
M92I	16	N-cadherin	44	1.92±0.55	1.88±0.23	0.93

Table 2.2 Rac1-GTP triggered by cell adhesion to ectodomain-coated substrata

Cell Line	Substrate Protein	Rac1-GTP Change
N-CHO	NEC1-5-Fc	0.8±0.3
C-CHO	CEC1-5-Fc	6.3±0.3
N-CHO	CEC1-5-Fc	1.2±0.4
S78A	CEC1-5-Fc	0.6±0.2
K8NS10P	CEC1-5-Fc	7±2
M92I	CEC1-5-Fc	4±2

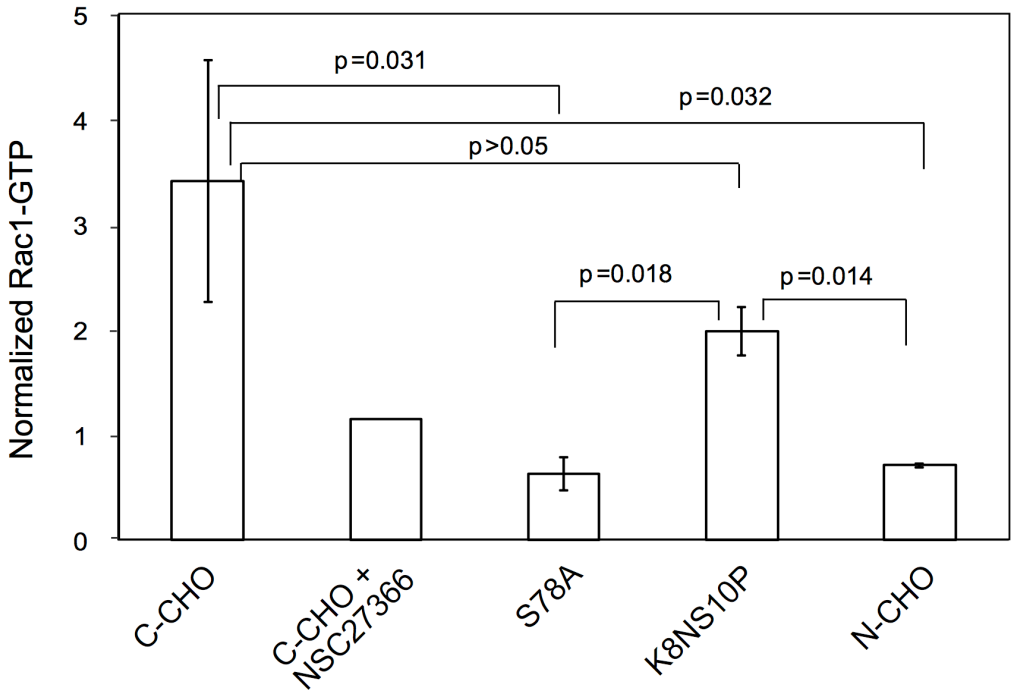

Figure 2.3 Normalized Rac1-GTP in a confluent cell monolayer. Rac activity measured 45 minutes after activating intercellular junctions with 1.8 mM Ca^{2+}. The cadherin subtype expressed on the CHO cell is indicated below each bar. The control was obtained by measuring Rac1-GTP levels, in the presence of the Rac inhibitor NSC23766. The cadherin expression levels on the different cells are similar.

2.4 Ligation-dependent RhoA Activation

The measured RhoA-GTP levels in confluent cell monolayers, following a calcium switch, were compared with Rac1-GTP activated under similar conditions Figure 2.4. Neither WT C-cadherin nor K8NS10P, which have similar affinities for CEC1-5-Fc, activated significant levels RhoA-GTP (Figure 2.4). By contrast, nascent N-CHO junctions increased RhoA-GTP 2.5-fold, 90 minutes after calcium addition. Interestingly, the S78A mutant activated similar RhoA-GTP levels to N-CHO, but with slower kinetics. This inverse correlation between Rac1 and RhoA activation levels agrees with reports suggesting that RhoA and Rac1 have antagonistic effects (Comunale et al., 2007;

Wildenberg, Dohn, Carnahan, Davis, Lobdell, Settleman, & Reynolds, 2006).

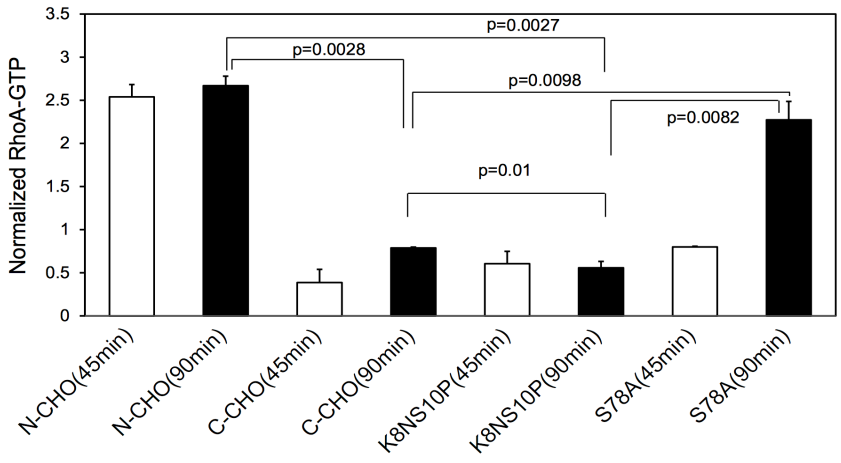

Figure 2.4 Normalized RhoA-GTP. RhoA activity measured in cells 45 and 90 minutes after activating intercellular junctions with 1.8 mM Ca^{2+}. The cadherin subtype expressed on the cells is indicated below each bar. The cadherin expression levels on the different cells are similar.

2.5 Cortical Tension

Magnetic twisting cytometry (MTC) measurements tested whether cadherin ligation might affect the global stiffness of these cells. Probing N-CHO and C-CHO with fibronectin modified beads interrogated global changes in cytoskeletal tension through integrin bonds (Potard, Butler, & Wang, 1997). The results were mixed. The cadherin subtypes altered cell rigidity to different extents when expressed in CHO-K1 cells (Figure 2.5). The substratum ligand also influenced cell stiffening, but not in a way that compares simply to ligation-dependent GTPase activity. This suggests a more complex relationship between cadherin ligation and global cell mechanics.

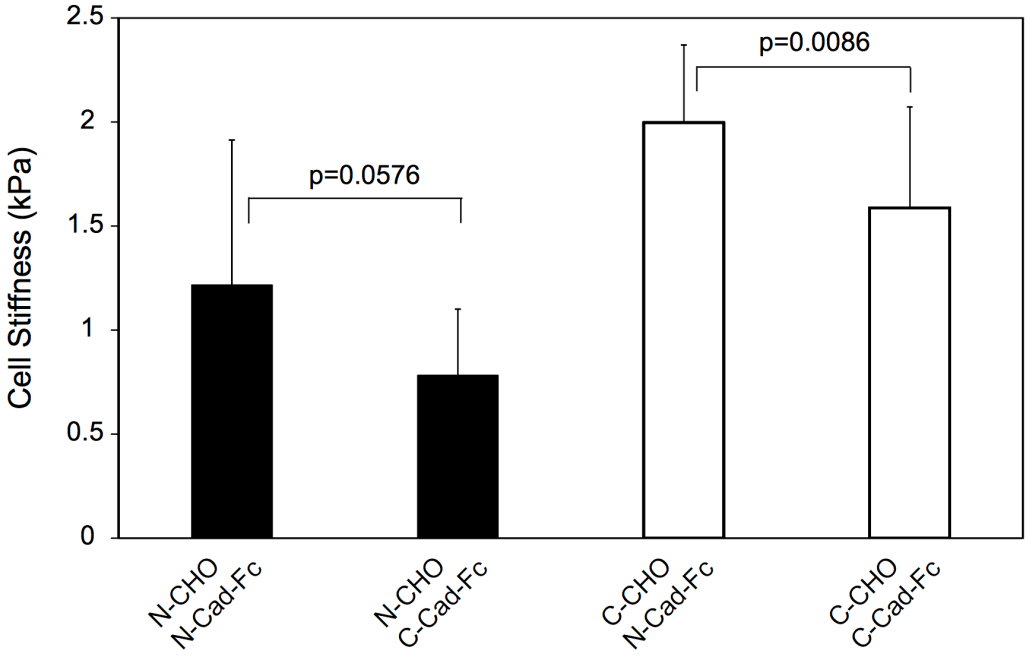

Figure 2.5 Cortical Tension Measurements. Measured stiffness of single cells cultured on either CEC1-5-Fc or NEC1-5-Fc coated substrata in GMEM containing 0.05 v/v% FBS. Cell stiffness was determined by magnetic twisting cytometry, using beads coated with fibronectin. The cadherin pairs supporting cell adhesion are indicated below the bars.

2.6 Discussion

The main finding of this study is that relative cadherin affinities correlate with differences in GTPase signaling, but not with the cortical stiffness. Prior measurements of cadherin binding affinities demonstrated that small sequence differences in the binding pocket of the EC1 domain of classical cadherins could generate substantial affinity differences between cadherin subtypes. The point mutation S78A in C-cadherin correlated with a six-fold decrease in the two-dimensional affinity for WT C-cadherin and switched the cell sorting specificity of C-cadherin so that cells expressing the S78A mutant intermixed with N-cadherin expressing cells. By contrast, a modest 2.5-fold

decrease in the M92I affinity caused cells to intermix with both WT C-CHO and WT N-CHO (Y.H. Chien, Ph.D thesis, 2009). These results suggest that relatively large differences in binding affinities for the same overall protein scaffold, e.g. C-cadherin, correlate with CHO cell sorting in vitro. The affinity appears to be particularly sensitive to sequence variations at positions 78 and 83 in EC1; namely, S78A substantially reduced the C-cadherin affinity, A78M ablated N-cadherin adhesive function (Tamura et al., 1998). Similarly, mutations at positions 78 and 83 in human E-cadherin altered the in vitro sorting patterns of L1 cells expressing the proteins (Nose et al., 1990). The A78M mutation in E-cadherin also allosterically altered epitope accessibility on EC1 (Harrison, Corps, Berge, & Kilshaw, 2005). The quantified affinity differences of different C-cadherin mutants and their impact on cell sorting enabled us to further investigate possible signaling differences underlying observed sorting behavior.

The K8NS10P mutation did not significantly alter the two-dimensional affinity for WT CEC1-5-Fc, Rac1 activation at 45 minutes, or the RhoA-GTP levels at 45 and 90 minutes. Prior biophysical measurements of the W2A mutant showed that adhesion between WT C-cadherin and the W2A mutant is intermediate between homophilic WT C-cadherin and homophilic W2A adhesion (Prakasam et al., 2006a). We would thus expect the two-dimensional affinities between CEC1-5-Fc and the mutants to be somewhat higher than between identical mutants. The Rac1 assay results presented in this thesis are consistent with this trend, and suggest that, although K8NS10P does not detectably alter the affinity for CEC1-5-Fc, it may lower the affinity between identical mutants and correspondingly alter Rac1 activation.

Prior biophysical studies identified multiple bonds between cadherin ectodomains

that require different domains (Bayas, Leung, Evans, & Leckband, 2006; Bibert et al., 2002; Chappuis-Flament, Wong, Hicks, Kay, & Gumbiner, 2001; Chien et al., 2008; Perret, Leung, Feracci, & Evans, 2004; Shi et al., 2008; Sivasankar, Gumbiner, & Leckband, 2001; Tsukasaki et al., 2007; Zhu et al., 2003). All five EC domains are needed to recapitulate the kinetic signature of the wild type protein (Chien et al., 2008). A fast, initial binding step requires EC1, but a second, slower step requires EC3 (Y. H. Chien et al., 2008) and is modulated by N-glycosylation on domains EC2 and EC3 (Langer, Guo, Shashikanth, Pierce, & Leckband, 2012). The results presented in this thesis show that the two-dimensional affinity differences associated with EC1-dependent binding correlate with ligation-dependent Rac1 activation.

The logarithm of these two-dimensional, EC1-mediated binding affinities are proportional to the protein adhesion energies (Prakasam et al., 2006b). However, in micropipette measurements (1) the affinities and kinetic rates are not determined by mechanically breaking cadherin bonds, and (2) micropipette measurements probe the full-length cadherins in the native environment of the cell membrane. Previously published micropipette data (Tabdili et al., 2012) demonstrate that cadherins with similar two-dimensional affinities (< ~3-fold differences) and correspondingly similar adhesion energies do not induce cell segregation, at the cadherin expression levels considered. This agrees with prior studies (Prakasam et al., 2006b; Shi et al., 2008) and could explain the absence of correlations between adhesion and sorting in some cases (Duguay, 2003; Niessen & Gumbiner, 2002; Prakasam et al., 2006b). Importantly, a 3-fold difference in the three-dimensional (solution) affinity corresponds to a difference in bond energies of 1 kcal/mole at 37°C that could be readily offset by cadherin expression levels or cortical

tension (Duguay, 2003; Kalantarian et al., 2009; Krieg et al., 2008; Manning et al., 2010; Steinberg & Takeichi, 1994; Winklbauer, 2009). The capacity of cadherin affinities to regulate actin organization through GTPase signaling introduces an additional mechanism that could augment or offset differential cadherin adhesion. The two-dimensional C-cadherin affinities correlate with ligation-dependent Rac1 activation. Thus, affinity differences due to mutations or binding to heterophilic ligands could similarly modulate signaling. Because we compared proteins with the same overall backbone and cytoplasmic domain, this correlation might not apply for general comparisons across cadherin subtypes due to possible differences in their interactions with GTPases (Anastasiadis et al., 2000; Boulter et al., 2010). For example, C-cadherin and E-cadherin activate Rac1 (Noren et al., 2003; Yap & Kovacs, 2003), but N-cadherin ligation triggers RhoA activation (Charrasse, Meriane, Comunale, Blangy, & Gauthier-Rouviere, 2002; Comunale et al., 2007; Marrs, Theisen, & Brusés, 2009; Taulet et al., 2009). Whether the latter is due to low N-cadherin affinity and Rac1/RhoA antagonism (Boulter et al., 2010; Burridge & Doughman, 2006; Comunale et al., 2007; Wildenberg, Dohn, Carnahan, Davis, Lobdell, Settleman, & Reynolds, 2006) or to differences in GTPase interactions with N-cadherin complexes (Anastasiadis et al., 2000) remains to be determined.

Given the role of cadherin-dependent GTPase activation in cytoskeletal regulation (Takaishi, Sasaki, Kotani, Nishioka, & Takai, 1997) and cell cycle control (Liu, Nelson, Pirone, & Chen, 2006), this potential link between cadherin affinities and GTPase signaling could have broader consequences for cadherin-dependent cell functions. Compromised cadherin adhesion is associated with cancer. For example, the E-cadherin

exon 8 deletion is associated with human gastric and breast cancers, and it reduces Rac1 activation, with a corresponding increase in RhoA activity (Deplazes et al., 2009). The findings presented in this thesis suggest that differential cadherin binding affinities may have more diverse physiological and mechanical roles than merely modulating cell adhesion.

2.7 Materials and Methods

2.7.1 Plasmids and cell lines

The cDNAs for the full length Xenopus C-cadherin and the C-cadherin W2A mutant in pEE14 plasmids were gifts from B. Gumbiner (University of Virginia, Charlottesville, VA). The cDNA encoding the full-length chicken N-cadherin in the pEGFP-N1 plasmid was from Andre Sobel (Institut du Fer a Moulin, Gif-sur-Yvette, France). These plasmids were transfected into Chinese Hamster Ovary (CHO-K1) cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA). CHO-K1 cells stably expressing the full length C-cadherin were selected as described (Brieher, Yap, & Gumbiner, 1996). CHO-K1 cells expressing the full-length chicken N-cadherin were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 v/v% FBS and 400 mg/ml G418 (Sigma-Aldrich, St Louis, MO).

2.7.2 FACS quantification of cadherin surface expression levels

Cadherin surface expression levels were quantified by flow cytometry (Chesla, Selvaraj, & Zhu, 1998; Chien et al., 2008). Cells were labeled with protein-specific antibodies against the ectodomains. C-cadherin expressing cells were labeled with anti-C-cadherin antibody (C/EP/B-Cadherin (clone xC-12), Santa Cruz Biotechnology, Santa Cruz, CA)

followed by the secondary fluorescein isothiocyanate (FITC)-conjugated antigoat IgG (whole molecule; Sigma). Chicken N-cadherin was detected with monoclonal mouse anti-N-cadherin (Clone GC-4, Sigma) and then fluoresceinisothiocyanate (FITC)-conjugated anti-mouse IgG (whole molecule; Sigma). The antibody labeling was in phosphate buffered saline (PBS) containing 1 w/v% bovine serum albumin (BSA) at pH 7.4. The fluorescence intensities of labeled cells were measured with an LSR II flow cytometer (BD Biosciences) (Zhang et al., 2005). The fluorescence intensity calibration curve was obtained with calibrated FITC-labeled standard beads (Bangs Laboratories, Fishers, IN) (Zhang et al., 2005).

2.7.3 Rac1 activation assay

Rac1-GTP was determined with a published Rac1-GTP immuno pull-down assay (Benard & Bokoch, 2002; Noren et al., 2003). Cells expressing cadherins at ~20/ μm^2 were seeded onto substrata coated with cadherin ectodomains. CEC1-5-Fc and NEC1-5-Fc surface densities, determined by isotope labeling (Yeung et al., 1999), were 5 x 10^3 molecules/ μm^2 and 4 x 10^3 molecules/ μm^2, respectively. To prepare these substrata, 10 cm, non tissue culture polystyrene plates (Fisher Scientific, Pittsburgh, PA) were incubated with 5 ml of 30 μg/ml cadherin EC1-5- Fc in HEPES buffer (20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.5) for 1 hour at 23°C, and stored overnight at 4°C before use. Controls used plates coated with poly-L-lysine (PLL). At least 30 minutes prior to use, the coated plates were washed with HEPES buffer, and equilibrated with serum-free, phenol red-free DMEM at 37°C. Cells were maintained at confluence for 2 days before the experiment, detached from tissue culture plates with 0.01% trypsin in 1**x** HBSS, supplemented with 1 mM $CaCl_2$ (Nose et al., 1988), and then

collected by centrifugation. Cells were then re-suspended in serum-free DMEM prior to seeding at 3-4 x 10^6 cells on the coated dishes. At defined intervals, the plates were washed twice with ice-cold HEPES buffer and lysed with 750 ml ice-cold lysis buffer per plate. Cells were removed with a cell scraper, and the lysate was clarified by centrifugation at 14,000 g for 2 minutes at 4°C. Then 20 ml of the clarified supernatant was analyzed for total Rac1 by Western blot, with anti-Rac1 antibody (Cytoskeleton, Denver, CO).

The remaining lysate was added to 30 μl of GST-PBD beads. Just prior to use, the beads were washed with ice-cold lysis buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 200 mM NaCl, 1% v/v Nonidet P-40, 5% v/v glycerol, and Roche complete protease inhibitors at pH 7.5) for 10 minutes with gentle shaking at 4°C. The beads were collected by centrifugation and stored on ice. Each time point required 30 μl beads. After mixing the lysis buffer and beads, the GST-PBD bead slurry was centrifuged and washed three times with ice-cold lysis buffer. After the final wash, the beads were collected by centrifugation, and boiled in SDS-PAGE buffer. Western blots with anti-Rac1 antibody (Cytoskeleton Inc., Denver, CO) determined the amount of Rac1-GTP in the lysate. The Rac1-GTP was normalized by the Rac1-GTP at t=0 minutes, defined by cells in suspension, and compared to the total Rac1 in the cells and to the actin loading control. Rac1-GTP levels showed a robust increase at 45 minutes (Figure 2.5), so Rac1-GTP was determined 45 minutes after seeding cells on different substrata.

2.7.4 Cortical tension measurements

Differences in cortical tension were determined by quantifying the global cell stiffness with Magnetic Twisting Cytometry (MTC) and use of magnetic beads covalently

modified with fibronectin (Wang et al., 1993). Glass-bottom Petri dishes were incubated overnight with an anti immunoglobulin Fc antibody, followed by rinsing and incubation with 0.5 mg of either CEC1-5-Fc or NEC1-5-Fc for 4 hours at 4°C. After rinsing with HEPES buffer, the surfaces were incubated with 1 w/v% BSA at room temperature for 30 minutes. Ferromagnetic beads (4.9 μm; Spherotech), chemically activated with ethyl-3-(dimethylaminopropyl)-carbodiimide and Nhydroxysuccinimide (Prakasam et al., 2006a; Prakasam et al., 2006b), were covalently modified with fibronectin. Cells stably expressing different cadherins were grown to confluence, detached with PBS containing 3.5 mM EDTA and 1 w/v% BSA, collected by centrifugation, and seeded at low density on the cadherin-Fc coated substrata, in medium supplemented with 0.05 v/v% FBS. These measurements focused on isolated cells to eliminate interference from cell-cell adhesion. After 4 hours at 37°C, fibronectin-coated beads were incubated with the cells for 20 minutes. All MTC measurements were performed on an inverted microscope (Leica) using a 20x objective and a cooled charge-coupled device camera (Orca2; Hamamatsu Photonics). After initial bead magnetization, an oscillating magnetic field perpendicular to the bead magnetic moment was applied for a defined period. The bead magnetic moment constant of 0.12 Pa/Gauss was calibrated as described (Wang et al., 1993). The bead displacements were measured and converted to the complex modulus (Wang et al., 1993).

2.7.5 RhoA activation assay

Ligation-activated RhoA-GTP was quantified following cadherin activation with a calcium switch with a commercial G-LISA kit (BK 124; Cytoskeleton, Denver, CO). Confluent cells in 10 mm Petri dishes were serum starved for 24 hours, before addition of

4 mM EGTA, which disrupts cell-cell junctions (Noren et al., 2003). After a one-hour incubation with EGTA, the medium was exchanged with medium containing 1.8 mM calcium, but lacking serum. Duplicate samples were analyzed at t=0, 45 and 90 minutes. After specific time periods, plates were immediately treated with ice-cold lysis buffer, as described for Rac1 assays. Cell lysates were scraped into tubes and clarified by centrifugation, before snap-freezing in liquid nitrogen. Lysates at t=0 minutes were prepared by re-suspending cells in lysis buffer. The protein concentration in the cell lysates was normalized to the t=0 minute sample, and equal amounts of total protein were incubated in duplicate in a 96-well GLISA assay plate. RhoA-GTP levels were determined with a microplate spectrophotometer (Molecular Devices SpectraMax M2). The change in RhoA-GTP was normalized relative to the corresponding zero time-point samples.

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Chapter 3

Cadherin-Dependent Mechanotransduction Depends On Ligand Identity But Not Affinity[2]

3.1 Introduction

Cadherins are essential adhesion proteins at intercellular junctions in all cohesive tissues. In mature tissues, they maintain the mechanical integrity of cell-cell junctions, and regulate the barrier properties of tissues such as the vascular endothelium and intestinal epithelia. In development, cadherins are essential for morphogenesis (Gumbiner, 2005). Following the first demonstrations that dissociated embryonic cells re-aggregated with cells from the same germ layer in vitro (Steinberg & Gilbert, 2004; Townes & Holtfreter, 1955), in vitro assays demonstrated that different cadherins induce cells to segregate away from each other (Friedlander, Mege, Cunningham, & Edelman, 1989; Nose et al., 1988). In vitro assays, which visualized cell aggregate compositions either in hanging droplets or in agitated cell suspensions, suggested that cell-sorting might be driven by differential adhesion (Friedlander et al., 1989; Steinberg, 1962, 1963; Townes & Holtfreter, 1955) and the minimization of surface free energies, attributed to differences in cadherin binding affinity or surface expression (Foty, Pfleger, Forgacs, & Steinberg, 1996; Foty & Steinberg, 2005; Steinberg, 2007; Steinberg, 1962, 1963).

Observations support the hypothesis that surface free energy minimization determines cell organization *in vitro* and *in vivo*. Cells in the ommatidium of the

[2] Adapted, with permission, from Tabdili H, Langer M, Shi Q, Poh YC, Wang N, Leckband D. Cadherin-dependent mechanotransduction depends on ligand identity but not affinity. J Cell Sci. 2012 Sep 15; 125(18): 4362-4371.

Drosophila retina adopt patterns that are similar to surface-tension-dependent shapes of soap bubbles (Hayashi & Carthew, 2004; Hilgenfeldt et al., 2008; Kafer, Hayashi, Maree, Carthew, & Graner, 2007). In Drosophilia, oocyte positioning in the egg chamber requires DE-cadherin expression and appears to correlate with the local DE-cadherin expression levels (Godt & Tepass, 1998). Genetic switches control the programmed down regulation and expression of different cadherins during neural crest cell emergence and migration out of the neurepithelium (Niessen, Leckband, & Yap, 2011; Takeichi et al., 1990).

Cadherins were initially thought to mainly form homophilic bonds, based on the tendency of cells expressing the same cadherin to co-aggregate in vitro (Nose et al., 1988). However, semi-quantitative estimates of interfacial tension and cell adhesion (Duguay, 2003; Niessen & Gumbiner, 2002), as well as quantitative measurements of cadherin adhesion energies and solution binding affinities (Chien et al., 2008; Harrison et al., 2010; Katsamba et al., 2009; Prakasam et al., 2006; Shi et al., 2008; Vendome et al., 2011), contributed to a more nuanced view that cadherin subtypes cross-react, but that their relative adhesion energies determine cell segregation patterns. Comparisons of cadherin-dependent, *in vitro* cell sorting with solution binding affinities suggest that affinities differing by > 5 fold support cell segregation (Katsamba et al., 2009; Tabdili et al., 2012b) . However, in other cases, smaller adhesive differences did not predict cell-sorting outcomes (Niessen & Gumbiner, 2002; Prakasam et al., 2006; Shi et al., 2008).

Despite the appeal of the surface-free-energy-minimization arguments, cell surface adhesion energies do not account for the effect of cortical tension on intercellular interactions. Increasing evidence suggests that myosin II dependent contractile forces are

central to determining cell shape, intercellular extension, and the maintenance of tissue boundaries (Bertet, Sulak, & Lecuit, 2004; Lecuit, 2005; Lecuit & Lenne, 2007; Lecuit, Lenne, & Munro, 2011; Lecuit and Le Goff, L., 2007). Studies increasingly also show that mechanical forces exerted during development alter signaling and actomyosin dynamics (Kasza & Zallen, 2011). Cell elongation and intercalation are driven by anisotropic tension in cells that originates from asymmetric intracellular myosin II and actin distributions (Bertet et al., 2004; Cavey, Rauzi, Lenne, & Lecuit, 2008; Rauzi, Lenne, & Lecuit, 2010; Tepass et al., 2002). During cell division, the strict maintenance of some cell compartments appears to be regulated largely by cortical actomyosin bundles adjacent to the membrane (Monier, Pelissier-Monier, Brand, & Sanson, 2010). The elastic-like properties of tissues also appear to influence cell organization in Xenopus embryos (Luu, David, Ninomiya, & Winklbauer, 2011). In a comparison of the relative influence of adhesion versus cortical tension, single cell force spectroscopy measurements demonstrated that cohesive forces between Zebrafish germ line progenitor cells did not specify cell localization in the embryo (Krieg et al., 2008). Instead, cortical tension appeared to determine cell positioning. Theoretical analysis predicts that cortical tension and adhesion energies coordinately influence sorting (Manning et al., 2010).

The seemingly overriding role of cell mechanics in directing some cell movements in vivo was puzzling in light of the cadherin requirement for morphogenesis and cell segregation in vitro. The connection between cadherin-dependent sorting and cortical tension was not obvious. However, the recent discovery that cadherin complexes are intercellular force sensors suggests a cadherin-dependent mechanism that could bridge both the cadherin requirement for cell sorting and cadherin-mediated changes in

cortical tension (Ladoux et al., 2010; le Duc et al., 2010; Liu et al., 2010; Yonemura et al., 2010). Cadherins are both adhesion proteins and cytoskeletal regulatory proteins (Niessen et al., 2011). Although cadherin ligation alone activates changes in cytoskeletal organization through Src and GTPases, cadherin complexes actively respond to applied force to alter cell mechanics (Ladoux et al., 2010; le Duc et al., 2010; Yonemura et al., 2010). An unanswered question has been whether cadherin-binding specificity could also modulate cell mechanics.

This study demonstrates that mechanotransduction at cadherin complexes is ligand dependent, but that ligand-selective force sensation is not determined by the affinities of the cadherin bonds. Magnetic twisting cytometry and traction force microscopy assessed mechanotransduction in response to acute, bond shear and to endogenous contractile forces on cadherin bonds, respectively. Micropipette measurements of cadherin-mediated intercellular binding kinetics determined the two-dimensional (2D) binding affinities and dissociation rates of the identical cadherin pairs as probed in mechanical measurements. Comparisons of cadherin binding affinities with mechanotransduction responses show that homophilic, but not heterophilic cadherin ligands trigger junction reinforcement, independent of the cadherin affinities. Qualitatively similar results were obtained with five different cell lines and three different classical cadherins. They suggest that, although classical cadherin binding affinities differ, the ligand-dependent modulation of cell mechanics may play a greater role in regulating intercellular boundaries.

3.2 Results

3.2.1 Cadherin-dependent mechanotransduction is ligand-dependent

To determine how reported differences in cadherin affinities (Katsamba et al., 2009; Tabdili et al., 2012b) (Table 3.1) affect force transduction through cadherin adhesions, magnetic twisting cytometry measurements (Figure 3.1D) were carried out with different cells and different cadherin ligands.

Table 3.1 Best fit parameters from nonlinear least squares fits of EC1-dependent cell–cell binding kinetics to Eqn 1 (from Tabdili et al., 2012a)

Test cell	Cadherin	Density (#/μm^2)	Red cell	Density (#/μm^2)	2D affinity ($\times 10^{-4}$ μm^2)	Off rate (s^{-1})
MDCK	k9 E-cadherin	17	k9 E-cad.Fc	7	3.6±0.2	1.7±0.3
MDCK	k9 E-cadherin	17	ck N-cad.Fc	20	2.5±0.2	1.5±0.3
MDA-MB-435	hN-cadherin	93	ck N-cad.Fc	32	0.70±0.06	1.9±0.3
MDA-MB-435	hN-cadherin	93	k9 E-cad.Fc	7	1.2±0.2	1.5±0.3
MCF7	hE-cadherin	7	k9 E-cad.Fc	18	4.2±0.2	2.4±0.4
MCF7	hE-cadherin	7	ck N-cad.Fc	37	2.7±0.1	2.2±0.4

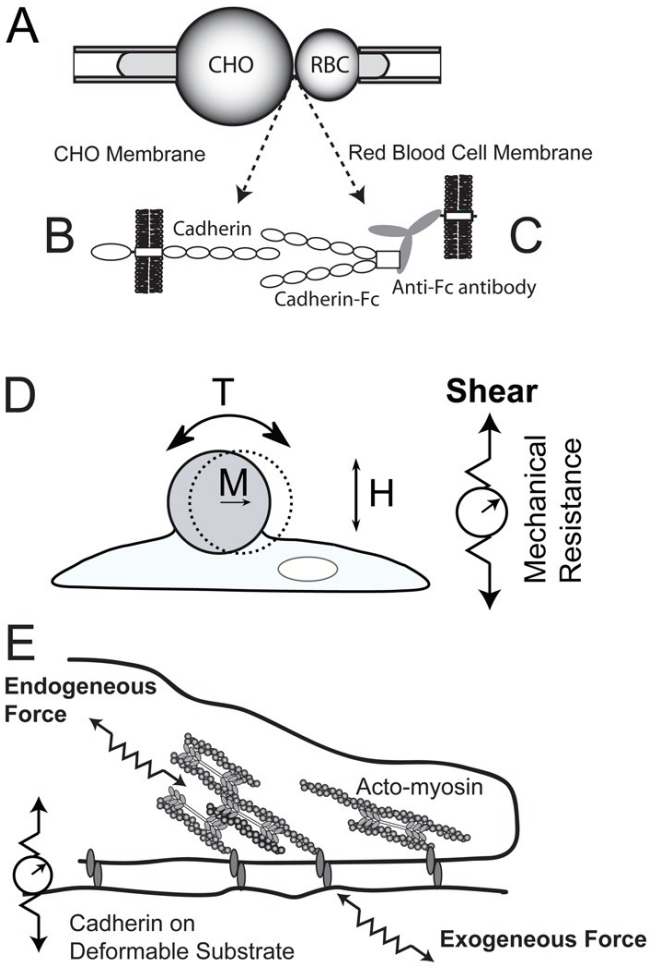

Figure 3.1 Schematic of micropipette manipulation, magnetic twisting cytometry, and traction force experiments. (A) In micropipette manipulation measurements (MP), CHO cells expressing the full cadherin (B) are brought into contact with red blood cells modified with oriented cadherin extracellular domains (C). The cadherin on the CHO cells (left) contacts Fc-tagged cadherin extracellular domains bound to the red cell membrane by immobilized, anti-Fc antibodies. (D) In Magnetic Twisting Cytometry (MTC), cadherin-modified magnetic beads attached to cell membranes are subject to an oscillating field *H*, which generates torque *T* on cell surface proteins. Changes in the bead displacement amplitudes reflect changes in the junction stiffness (right) due to junction remodeling and/or global cell contractility. (E) Traction force measurements quantify the contractile forces exerted at cadherin adhesions between cell surface proteins and cadherin ectodomains immobilized on compliant polyacrylamide gels.

These measurements quantify changes in cadherin junction mechanics in response to shear forces applied to cadherins the cell surface by ligand-modified beads. These studies used ferromagnetic beads that were modified with recombinant, extracellular domains of either the same cadherin subtype as expressed on the cell (homophilic ligand) or a different subtype (heterophilic ligand). In these measurements, the bead is

magnetized parallel to plane of the cell, and an orthogonal oscillatory magnetic field induces a torque on the bead, causing it to twist (Figure 3.1D). The resultant bead displacement reflects the viscoelasticity of the bead-receptor-cytoskeletal junction, such that changes in the bead displacement reflect junction remodeling and cell contractility.

Figure 3.2 compares results from MTC measurements conducted with four different cell lines: MDCK, C2C12, MCF7, and MDA-MB-435 cells. The probe beads were modified with Fc-tagged ectodomains of chicken N-cadherin, canine E-cadherin, or Xenopus C-cadherin, as in the micropipette measurements described elsewhere (Tabdili et al., 2012b). Figure 3.2A shows the percent change in the stiffness of the bead-cell junction, relative to unperturbed bonds. Here, the adhesive junction was between N-cad-Fc coated beads and N-cadherin on C2C12 cells. As reported previously with F9 cells (le Duc et al., 2010), the cadherin junction stiffens in response to acute, applied bond shear. This stiffening response is ablated by treatment with EGTA (Figure 3.2A), which chelates Ca^{2+} ions required for cadherin function. It is also abolished following F-actin depolymerization by treatment with latrunculin B (Figure 3.2B).

The mechanotransduction is therefore cadherin and F-actin dependent, in agreement with previous findings (le Duc et al., 2010). By contrast, when the beads were modified with a different cadherin subtype, e.g. C-cad-Fc or E-cad-Fc, there was no change in junction stiffness relative to controls. Bead pulls with an anti-N-cadherin antibody, which recognizes the N-terminal EC1 domain, also failed to induce junction remodeling (Figure 3.2B). The results of measurements with CHO cells stably transfected with N-cadherin shown in Figure 3.6.

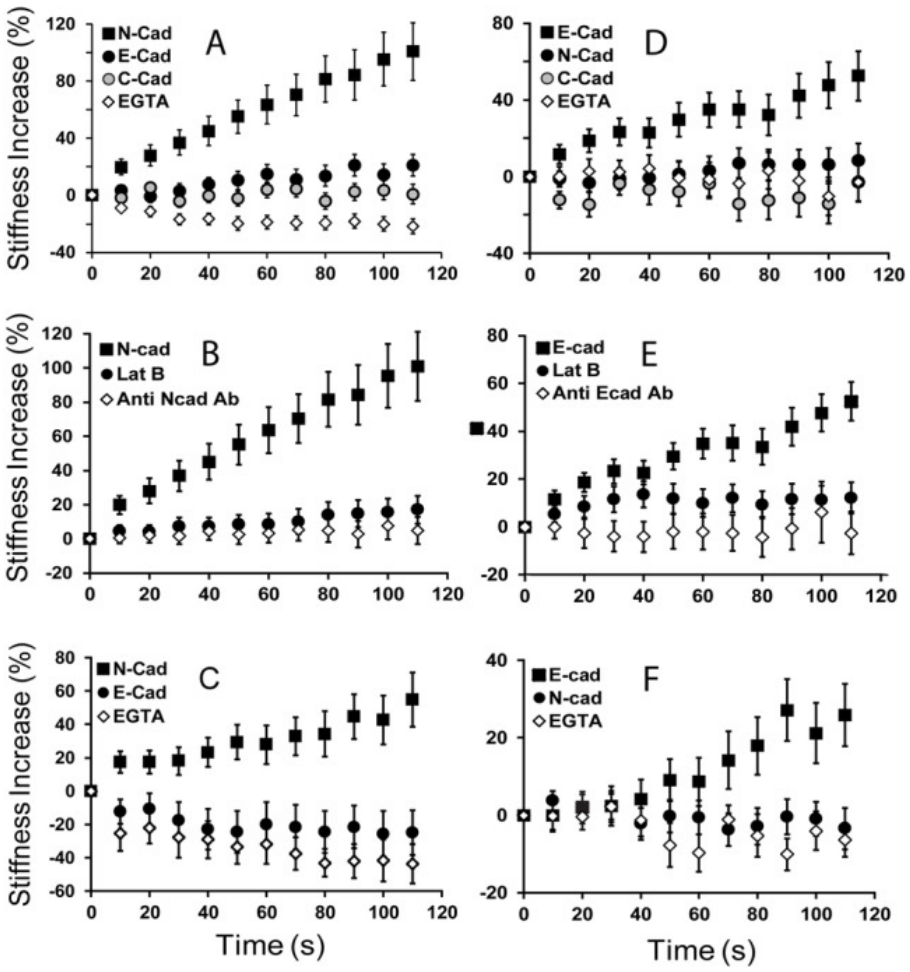

Figure 3.2 Cadherin-dependent mechano-transduction is ligand-selective. (A) MTC measurements of the force-induced stiffening response of C2C12 cells probed with beads coated with N-cad-Fc (black squares), E-cad-Fc (black circles) or C-cad-Fc (gray circles). Controls were with N-cad-Fc beads and 4mM EGTA (white diamonds). (B) Control measurements with C2C12 cells probed with beads coated with anti-N-cadherin antibody (white diamonds), or with N-cad-Fc in the absence (black squares) and presence (black circles) of latrunculin B. (C) MTC measurements of the force-induced stiffening response of MDA-MB-435 cells probed with beads coated with N-cad-Fc (black squares), E-cad-Fc (black circles) or C-cad-Fc (gray circles). Controls were with N-cad-Fc in the presence of 4mM EGTA (white diamonds). (D) MTC measurements of MCF7 cells probed with beads coated with E-cad-Fc (black squares), N-cad-Fc (black circles) or C-cad-Fc (gray circles). Controls were with E-cad-Fc, in the presence of EGTA (white diamonds). (E) MCF7 controls with E-cad-Fc coated beads in the presence of Lat B (black circles) or with beads coated with anti-E-cadherin antibody. (F) The stiffening response of MDCK cells probed with E-cad-Fc (black squares) or N-cad-Fc (black circles). Control measurements were with EGTA (white diamonds). In A-F, each time point represents >200 beads**.**

In all cases investigated (Figure 3.2A-F), only homophilic ligands induced junction stiffening during the first 120s of shear modulation, and heterophilic ligands consistently failed to induce any response (Figure 3.2). C2C12 and MDA-MB-435 cells

both express endogenous N-cadherin (Figure 3.2A-C), and shear applied only to beads coated with N-cad-Fc, but not E-cad-Fc or antibody, triggered force-activated junction remodeling. Similarly, MCF7 and MDCK cells (Figure 3.2D-F), which express endogenous E-cadherin, required E-cad-Fc coated beads to induce junction stiffening.

The finding that only homophilic cadherin ligation induces the mechanoresponse was unexpected, in light of the binding affinities quantified with the identical cells and cadherin ligands (Table 3.1). Both protein adhesion measurements and solution binding affinities show that cadherin subtypes cross-react, often with heterophilic affinities that are intermediate between those of the homophilic bonds (Katsamba et al., 2009; Prakasam et al., 2006; Steinberg, 2007). Here, although the heterophilic ligands bind cell-surface cadherins, as demonstrated by micropipette manipulation measurements, they do not trigger force transduction.

The ligand requirement for mechanotransduction is further demonstrated by measurements with beads modified with anti-N-cadherin or with anti-E-cadherin antibodies. Both antibodies recognize the N-terminal domains of the respective target cadherins. Although the antibody-modified beads firmly bound to cadherins on C2C12 and MCF7 cells, neither triggered an active response to applied bond shear (Figure 3.2B, 3.2E). This agrees with a similar result obtained with beads modified with DECMA-1 (E-cadherin blocking antibody) and F9 cells (le Duc et al., 2010).

3.2.2 Cadherin complexes are rigidity sensors

To address the possibility that bead pulls may not reflect physiologically relevant stress, complementary traction force measurements tested the ability of cadherin complexes to sense substrate rigidity, and to proportionally alter endogenous contractile stress. Prior

studies of force sensation at focal adhesions demonstrated that mechanoresponses to exogenous force parallel substrate rigidity sensing (Geiger et al., 2009). In these studies, endogenous contractile forces exert physiological forces at cadherin adhesions to elastomeric substrata coated with cadherin ectodomains (Figure 3.1E). The cadherin pairs mediating these cell-substratum adhesions are identical to those probed by micropipette manipulation and in magnetic twisting cytometry.

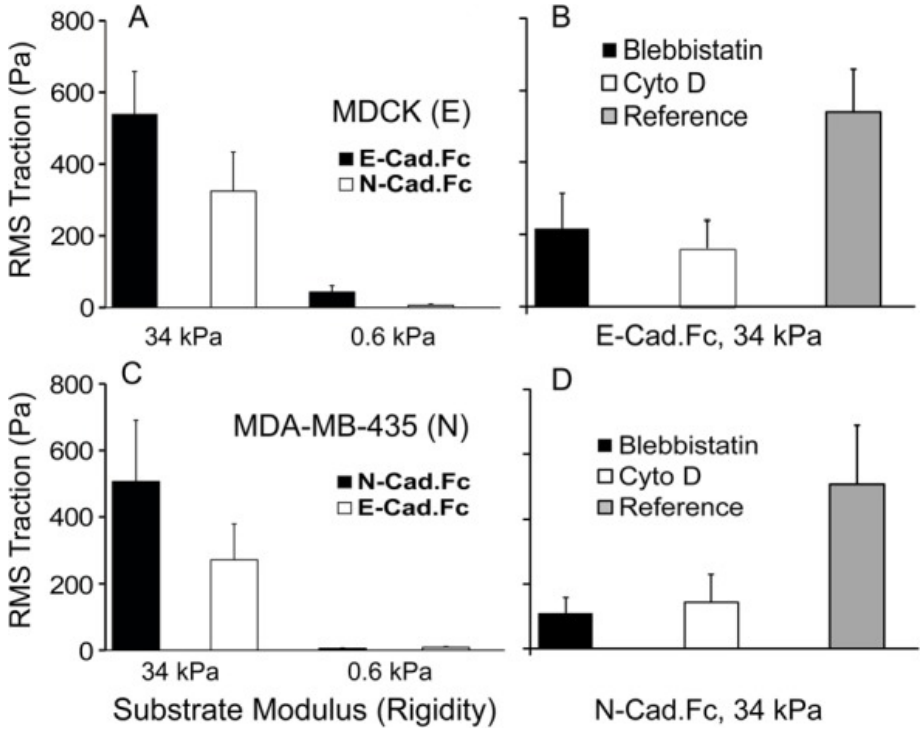

Figure 3.3 Cadherin-based traction forces are rigidity and ligand-dependent. (A) RMS (root-mean-square) traction forces (Pa) exerted by MDCK cells on soft (0.6 kPa) and rigid (34 kPa) gels coated with E-cadh.Fc (homophilic) or N-cad-Fc (herophilic) ligand. (B) Traction force exerted by MDCK cells seeded on E-cad-Fc-coated, rigid (34 kPa) gels, after treatment with blebbistatin or cytochalasin. (C) RMS traction forces exerted by MDA-MB-435 cells on soft (0.6 kPa) and rigid (34 kPa) gels coated with N-cad-Fc (homophilic) or E-cad-Fc (heterophilic) ligand. (D) Control RMS traction force exerted by MDA-MB-435 cell on N-cad-Fc-coated, rigid (34 kPa) gel, after treatment with blebbistatin or cytochalasin D (Cyto D).

Figure 3.3A compares traction forces generated by MDCK cells on polyacrylamide gels with elastic moduli of 34 kPa and 0.6 kPa, when coated with E-cad-Fc or with N-cad-Fc ligand. At ~160 ng/ μm^2, the immobilized protein densities were

similar for both cadherin ectodomains on both hydrogels. MDCK cells generated greater traction on rigid gels coated with E-cad-Fc than on soft gels (Figure 3.3A), confirming that E-cadherin complexes also sense substrate rigidity. MDA-MB-435 cells similarly exhibited rigidity-dependent traction forces on N-cad-Fc-coated hydrogels (Figure 3.3C). This demonstrated rigidity sensing via homophilic cadherin ligation agrees with a prior report of myoblast traction forces on elastomeric pillars coated with N-cadherin (Ladoux et al., 2010). Blebbistatin (50 μM) and cytochalasin D (4 μM) substantially reduced the traction forces generated on rigid substrata (Figures 3.3B, 3.3D), in agreement with prior findings (Ladoux et al., 2010).

By contrast, on the stiffer gels (34 kPa) coated with heterophilic cadherin ligands, both MDCK cells and MDA-MB-435 cells generated substantially lower traction forces (Figure 3.3A, 3.3C). The lower traction forces exerted by MDCK cells on N-cad-Fc coated gels might be explained by the lower heterophilic bond affinity relative to the homophilic E-cad-Fc affinity (Table 2.1). However, this would not explain the behavior of MDA-MB-435 cells because the measured homophilic N-cadherin affinity is lower than the heterophilic affinity (Table 3.1). Yet the cells exert greater traction forces on N-cad-Fc coated gels. On soft hydrogels (0.6 kPa), both cell types were more rounded, and traction forces were low and ligand-independent, within experimental error.

To further test the role of cell contractility in traction force generation MDA-MB-435 cells treated with nocodazole (20 μM) on gels with elastic moduli of 0.6 kPa and 34 kPa. Microtubule depolymerization increases cell contractility via a Rho-GTPase dependent pathway (Danowski, 1989). As expected, nocodazole treatment increased the traction forces. As shown in Figure 3.7, the magnitude of the increase in traction forces

were almost the same in cells adhering to homophilic versus heterophilic cadherin ligand, on the gels with the same elastic modulus.

Different from the MTC measurements, heterophilic ligation reduced the traction forces by only ~50% relative to controls with blebbistatin or cytochalasin D treated cells. This could be due to compensatory mechanisms regulating cell contractility on these substrata. For example, cells also generated rigidity-dependent traction forces on poly (L-lysine) coated gels ~3 hr after seeding in serum free medium (not shown), suggesting that parallel mechanisms may regulate global cell contractility. This behavior is not due to integrin interference since immunofluorescence did not detect focal adhesions at the basal surface.

Consistent with a functional role of tension in stabilizing cadherin adhesions, more cells attached and spread on the more rigid substrata coated with homophilic cadherin ligands (Figure 3.4A-B). Again, the greater MDCK cell densities on E-cad-Fc than on N-cad-Fc-coated substrata might initially be attributed to the relative affinities of the homophilic versus heterophilic E-cadherin bonds. However, greater numbers of MDA-MB-435 cells adhered to rigid substrata coated with N-cad-Fc relative to E-cad-Fc, despite the lower affinity of the homophilic N-cadherin bond (Table 3.1). On the softer gels, there was no statistically significant difference in cell attachment densities to either homophilic or heterophilic ligands.

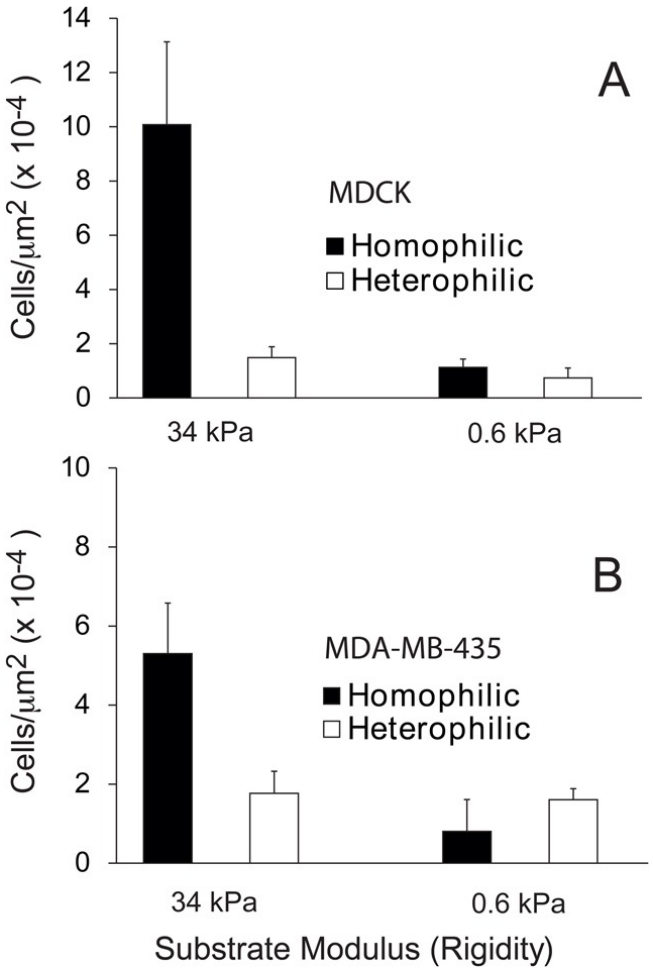

Figure 3.4 Cell attachment densities on rigid substrata are ligand dependent. (A) Density of MDA-MB-435 cells attached to substrata with Young's moduli of 34 and 0.6 kPa modified with N-cad-Fc (homophilic) and E-cad-Fc (heterophilic) ligands 4hr after cell seeding in serum free medium. (B) Density of MDCK cells on substrata with Young's moduli of 34 and 0.6 kPa coated with E-cad-Fc (homophilic) and N-cad-Fc (heterophilic) ligands, 4hr after cell seeding in 0.5 v/v% FBS.

In Figures 3.5A-C, well defined actin stress fibers and punctate paxillin staining are apparent in MDA-MB-435 cells spread on collagen-coated semi-rigid polyacrylamide (34 kPa) in the presence of serum (10 v/v% FBS). The absence of paxillin staining at the basal cell surface, on both soft and rigid gels (Figure 3.5D, 3.5E), ruled out integrin interference in either the traction force or cell attachment measurements. the same cells

were more rounded on soft (0.6 kPa), N-cad-Fc-coated gels, and there were no stress fibers or paxillin at the basal surface.

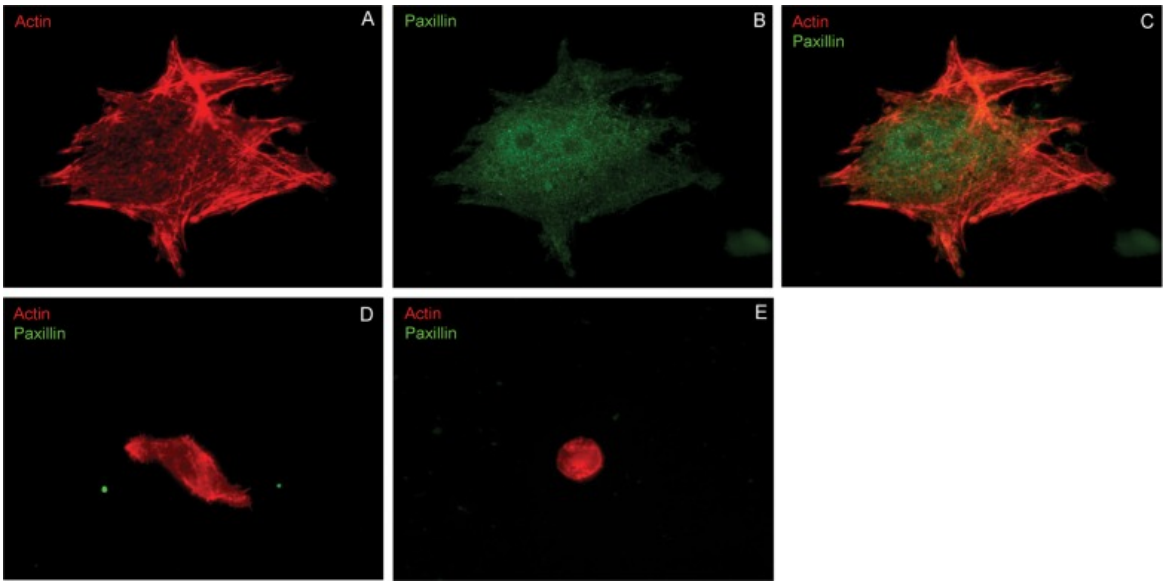

Figure 3.5 Traction forces and seeding densities on rigid hydrogels are cadherin-mediated. (A) Actin and (B) paxillin immuno-staining in MDA-MB-435 cells seeded on rigid (34 kPa), collagen-coated hydrogels, 6 hr after seeding in RPMI medium. (C) Merged fluorescence images of MDA-MB-435 cells seeded on rigid collagen-coated hydrogels (D) Paxillin and actin staining in MDA-MB-435 cells seeded on N-cad-Fc-coated hydrogel (34 kPa), 6 hr after seeding in serum free medium. (E) Paxillin and actin immune-staining of MDA-MB-435 cells seeded on N-cad-Fc-coated soft (0.6 kPa) hydrogel, 6hr after seeding in serum free medium.

3.3 Discussion

These findings demonstrate that mechanotransduction at cadherin adhesions requires homophilic ligation, and that force-activated junction remodeling appears to be insensitive to differences in the intrinsic binding affinities of the cadherin bonds. The binding affinity and cell surface adhesion energy do not determine the magnitude of force-dependent, cadherin-mediated changes in cell mechanics. This is somewhat analogous to findings that focal adhesion-mediated rigidity sensing and adhesion are de-

coupled (Engler et al., 2004). However, the mechanisms of cadherin adhesion, binding selectivity, and mechanotransduction are distinct from integrins.

The results suggest that differences in mechanical changes at stressed cadherin junctions could supersede more subtle differences in cadherin affinities. This could explain, in part, the finding that cortical tension better predicted cell positioning in Zebrafish embryos than the cohesiveness of the germ line progenitor cells (Krieg et al., 2008). It was unclear how to reconcile the latter result with the extensive literature suggesting that cadherin-dependent, differences in adhesion energies could direct cell sorting (Duguay , 2003; Foty & Steinberg, 2004; Foty & Steinberg, 2005; Katsamba et al., 2009; Niessen et al., 2011; Nose et al., 1990; Steinberg & Takeichi, 1994; Steinberg, 2007; Steinberg, 1963). Our findings suggest a cadherin-dependent mechanism that could both determine cohesive energies and regulate junctional or possibly global (Chopra et al., 2011) cell mechanics.

The two-dimensional affinities determined from micropipette studies (Table 3.1) represent the equilibrium, time-independent properties of EC1-EC1 bonds, but the MTC and traction force measurements are mechanical approaches that may reflect different properties of cadherin bonds. For example, single bond rupture forces depend on dissociation rates rather than affinities (Dudko, Mathé, Szabo, Meller, & Hummer, 2007; E. Evans & Ritchie, 1997). However, none of the bond parameters determined thus far generally favor homophilic over heterophilic bonds. The dissociation rates determined from micropipette measurements (Table 3.1), single bond rupture studies (Shi et al., 2008), or SPR studies (Katsamba et al., 2009) do not correlate with the mechanoselectivity. Neither do the single bond rupture forces (Shi et al., 2008). Some

studies suggested that the heterophilic binding frequency, which is related to the association rate, might be lower than for homophilic bonds (Berx & van Roy, 2009; Panorchan et al., 2006), but this is not the case for the protein pairs considered here. Thus, the cadherin binding properties alone do not appear to confer mechanical selectivity.

The ligand-dependent mechanical differences are manifest at stressed cadherin junctions, and are expected to exert greater influence at stressed intercellular adhesions. On soft hydrogels, for example, where cells exert low contractile stress, there was no distinguishable ligand-dependence of the traction forces (Figure 3.3A, 3.3C). Thus, interfacial energies might dominate cell-cell interactions in some cases, such as in soft tissue environments or in vitro cell sorting assays, where intercellular forces may be insufficient to effect significant differences in cortical tension. In this context, it is noteworthy that CHO cells, which are commonly used for in vitro sorting assays do not exert large contractile forces (Leader, Stopak, & Harris, 1983) (Figure 3.6). Conversely, large intercellular forces generated during convergence extension or cell intercalation movements may be sufficient to activate cadherin-dependent changes in intercellular mechanics (Kasza & Zallen, 2011).

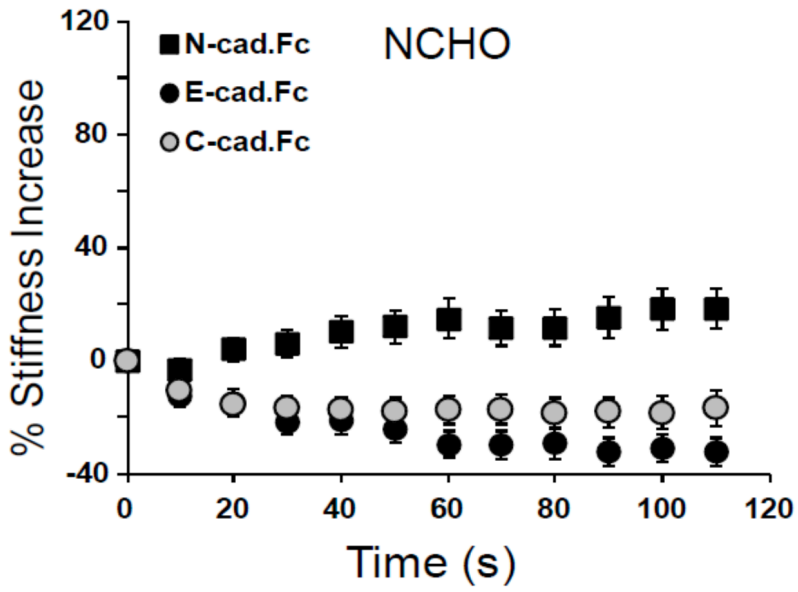

Figure 3.6 N-cadherin mechanotransduction is ligand selective. MTC measurements of the force-induced stiffening response of N-CHO cells probed with beads coated with N-cad-Fc (black squares), E-cad-Fc (black circles) or C-cad-Fc (gray circles).

These results are also intriguing, in light of the postulated mechanism of cadherin-dependent mechanotransduction. Cadherin bond stress is thought to induce a conformational change in α-catenin bound to the cadherin/β-catenin complex that exposes a cryptic site in α-catenin (Yonemura et al., 2010). The latter recruits actin-binding proteins such as vinculin to junctions. The finding that mechanical stimulation with anti-cadherin antibodies (le Duc et al., 2010) or heterotypic ligands fails to activate mechanotransduction indicates that tension on cadherin ectodomains alone is insufficient to trigger the requisite change in α-catenin.

The molecular basis for cadherin mechano-selectivity remains to be determined. The independence cadherin bond properties and mechanotransduction selectivity suggests that additional molecular factors may contribute to force transduction at intercellular

junctions. There is precedence for this. Anti-VE-cadherin antibody-coated beads failed to activate mechanotransduction via VE-cadherin junctions (Tzima et al., 2005). However, the VE-cadherin complex comprises the vascular endothelial growth factor receptor and PECAM, which appears to be the flow-sensitive mechano-transducer between endothelial cells subject to fluid shear stress (Hahn & Schwartz, 2009).

Whether other membrane proteins contribute to selective force transduction by classical cadherins remains to be determined. Possible candidates are protocadherins (Chen & Gumbiner, 2006; Deplazes et al., 2009; Taveau et al., 2008), receptor tyrosine kinases (Brady-Kalnay, Rimm, & Tonks, 1995; Hellberg, Burden-Gulley, Pietz, & Brady-Kalnay, 2002; McLachlan & Yap, 2011) or growth factor receptors (Perrais, Chen, Perez-Moreno, & Gumbiner, 2007; Shibamoto et al., 1994; Tzima et al., 2005; Williams et al., 2001). Recent findings demonstrated a role for protocadherin-19 in the regulation of N-cadherin dependent cell-cell adhesion and migration (Papusheva & Heisenberg, 2010; Taveau et al., 2008), and PAPC regulates the adhesive activity of C-cadherin during Xenopus morphogenesis (Chen & Gumbiner, 2006). Nevertheless, the mechanoselectivity demonstrated here with five cell lines and three classical cadherin subtypes demonstrates that cadherin selectivity can modulate both intersurface adhesion energies and cell mechanics, both of which instruct morphogenesis and maintain compartment barriers in mature tissues.

3.4 Materials and Methods

3.4.1 Materials

Blebbistatin, cytochalasin D (Cyto D), latrunculin B (Lat B), monoclonal anti-N-cadherin

antibody (clone GC-4), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), Poly-L-Lysine, 3-aminopropyl-triethoxysilane (APS), and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Nocodazole was from Santa Cruz Biotechnology (Santa Cruz,CA). Anti-E-cadherin and anti-paxillin antibodies were purchased from BD Bioscience (San Jose, CA). Acrylamide, N,N'-methylene-bis-acrylamide (Bis), TEMED, and ammonium persulfate (AP) were obtained from Bio-Rad (Hercules, CA). (NHS) and N-Succinimidyl-6-(4'-azido-2'-nitrophenyl-amino) hexanoate (Sulfo-SANPAH) were from Pierce Biotech (Rockford, IL).

3.4.2 Plasmids and cell lines

The cDNA encoding the full-length chicken N-cadherin in the pEGFP-N1 plasmid was provided by Dr. Andre Sobel (Institut du Fer a Moulin; Gif-sur-Yvette, France). Canine E-cadherin cDNA in pEGFP-N1 plasmid was a gift from Dr. James Nelson (Stanford University, Palo Alto, CA). These plasmids were transfected into Chinese hamster ovary (CHO-K1) cells, using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Clones expressing wild type N-cadherin (N-CHO) were selected in Dubelco's Modified Eagle Medium (DMEM) supplemented with 10 v/v% FBS and 400 μg/ml G418. The cadherin expression levels of all cells were determined by quantitative flow cytometry.

C2C12 mouse myoblasts were maintained in low glucose DMEM supplemented with 20 v/v% FBS and 2 mM L-glutamine. MCF7 human breast epithelial cells were cultured in DMEM, supplemented with 10 v/v% FBS and 1% non-essential amino acids (NEAA, Fisher, Pittsburgh, PA). Madin-Darby-Canine-Kidney (MDCK) cells were cultured in DMEM supplemented with 10 v/v% FBS. MDA-MB-435 cells were cultured

in RPMI 1640 supplemented with 10 v/v% FBS.

3.4.3 Protein production and purification

Recombinant, E- and N-cadherin ectodomains with C-terminal Fc-tags were stably expressed in human embryonic kidney cells (293HEK). Cells were cultured in DMEM containing 10 v/v% FBS. Fc-tagged canine E-cadherin and chicken N-cadherin ectodomains (E-cad-Fc and N-cad-Fc) were purified from the conditioned medium with a protein-A Affigel (Bio-Rad, Hercules, CA) affinity column followed by gel-filtration chromatography. The protein purity was assessed by SDS-PAGE.

3.4.4 Magnetic twisting cytometry (MTC)

In magnetic twisting cytometry experiments (Figure 3.1D), polystyrene-coated, 4.9 μm diameter, ferromagnetic beads with carboxyl surface groups (Spherotech, Lake Forest, IL) were covalently coated with specific Fc-Tagged protein, poly(L-lysine) (PLL), or blocking antibody, and then allowed to bind to the cell surface. The beads were first activated with EDC/NHS, by incubation with EDC (10 mg/ml) and NHS (10 mg/ml), in MES buffer (50 mM, 100 mM NaCl, pH 5.0) for 15 min at room temperature, on a shaker. The beads were centrifuged at 12000 x g for 15 min, and then incubated with 75 μ g of the ligand of interest (Fc-tagged cadherin ectodomains, PLL, or Blocking antibody) per mg beads for 2 hours at room temperature, in coupling buffer (20 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 8.0). In order to prevent bead aggregation, beads were sonicated for 3 seconds before they were added to the cells. The protein-coated beads were then allowed to settle on a confluent cell monolayer for 20 min, before applying torque. To

disrupt F-actin, cells were treated with 4 μM latrunculin B or 4 μM cytochalasin D for 10 min before twisting measurements.

All MTC studies were carried out with cells cultured in glass-bottomed dishes on a heated microscope stage at 37°C. Cells and beads were visualized with an inverted microscope (Leica) with a 20X, 0.6 numerical aperture objective and a cooled charge-coupled device camera (Orca2; Hamamatsu Photonics). First, beads were magnetized horizontally by applying a short, 1 Tesla field for ~1 ms. Next, an oscillating magnetic field of 60 Gauss at a frequency of 0.3 Hz was applied orthogonal to the magnetic moment of the beads, for up to 120 seconds. This generates a modulated shear stress on the bead-cell junctions. The bead displacements were recorded with a CCD camera, and displacements were converted to the complex modulus of the junction, using custom software. The specific modulus is the ratio of the applied torque to the bead displacement, G = T/D, which gives the complex modulus of the bead–cell junction. The thus determined viscoelastic moduli follow a log-normal distribution, from which the geometric mean and standard deviation of the elastic modulus of the junction are obtained.

3.4.5 Traction force microscopy (TFM)

Fourier Transform Traction Force Microscopy (FTTFM) was carried out with compliant polyacrylamide gels surface-modified with cadherin ectodomains. Polyacrylamide gels were prepared as described (Beningo et al., 2002). Red fluorescent microspheres (0.2 μm, Molecular Probes, Eugene, OR) were embedded in gels. The Young's moduli of the gels used in this study were 0.6 kPa and 34 kPa. The gels were covalently modified with 0.2 mg/ml of human or chicken anti-Fc antibody using Sulpho-SANPAH (Pierce Biotech,

Rockford, IL). The samples were illuminated after each of two successive 200 μL additions of fresh Sulpho-SANPAH stock solution (0.5 mg/ml, 100mM HEPES, pH 7.5). Samples were illuminated at 320 nm for 8 min, at 3-4 inches from two 15 W lamps. After each irradiation period, the samples were washed with 100 mM HEPES. After the second wash, anti-Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 0.2 mg/ml was added and incubated with the gel overnight at 4°C. The substrata were rinsed to remove unbound antibody, and then incubated with N-cad-Fc or with E-cad-Fc (0.2 mg/ml in 20 mM HEPES, pH 7) for 4 hours at 4°C. The substrata were rinsed and blocked with 1 w/v% BSA in PBS for 30 min at room temperature.

Prior to seeding cells on the cadherin-Fc-coated gels, cells were detached from tissue culture flasks using 3.5 mM EDTA and 1 w/v% BSA in PBS, and then 2000-4000 cells/cm^2 were seeded onto the hydrogels. MDA-MB-435 cells were cultured in serum-free medium, and MDCK cells were cultured in medium with 0.5 v/v% FBS. Measurements were conducted 6 hours after cell seeding. The bead displacement field was measured before and after cell detachment with 3.5 mM EDTA and 1 w/v% SDS in PBS. The constrained traction maps were calculated from the displacement field as described (Figure 3.7).

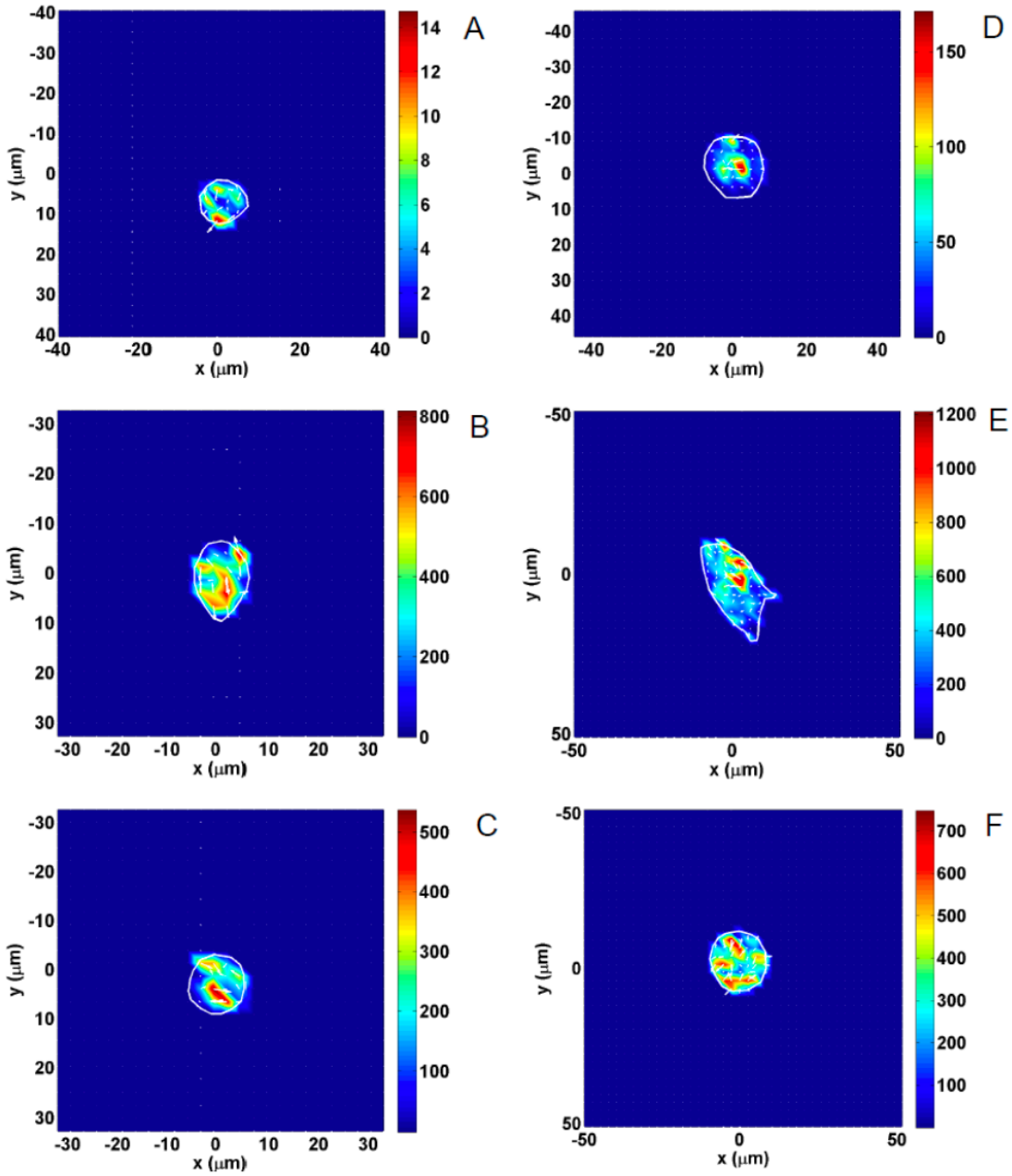

Figure 3.7 Figure S.2 Traction force "heat maps" of cells on "rigid" (34 kPa) or "soft" (0.6 kPa) hydrogels coated with E-Cad-Fc or with N-Cad-Fc ligand. (A-C) Traction force "heat maps" of single MDA-MB-435 cells on soft (A: 0.6 kPa) and rigid (B: 34 kPa) gels coated with N-Cad-Fc (homophilic ligand), and MDA-MB-435 cell on a rigid (C: 34 kPa) gel coated with N-Cad-Fc (heterophilic ligand). (D-F) Traction force "heat maps" of single MDKC cells on soft (D: 0.6 kPa) and rigid (E: 34 kPa) gels coated with E-Cad-Fc (homophilic ligand), and MDCK cell on a rigid (F: 34 kPa) gel coated with N-Cad-Fc (heterophilic ligand).

In traction force measurements following nocodazole treatment, MDA-MB-435 cells were seeded on soft (0.6 kPa) and semi-rigid (8.8 kPa, data not shown) and rigid (34 kPa) polyacrylamide gels coated with N-cad-Fc or E-cad-Fc for 6 hours. After obtaining phase contrast images of the single cells, a second fluorescence image of the beads near the surface was acquired. Bead displacements were again imaged after cells were treated with nocodazole (20 μM) for 30 min. The fluorescence images obtained after removing the cells gave the reference bead positions in the absence of traction force generation. In order to quantify the effect of nocodazole on the traction forces, we compared the reference bead positions to the bead displacements generated by cells before and after nocodazole treatment (Figure 3.8).

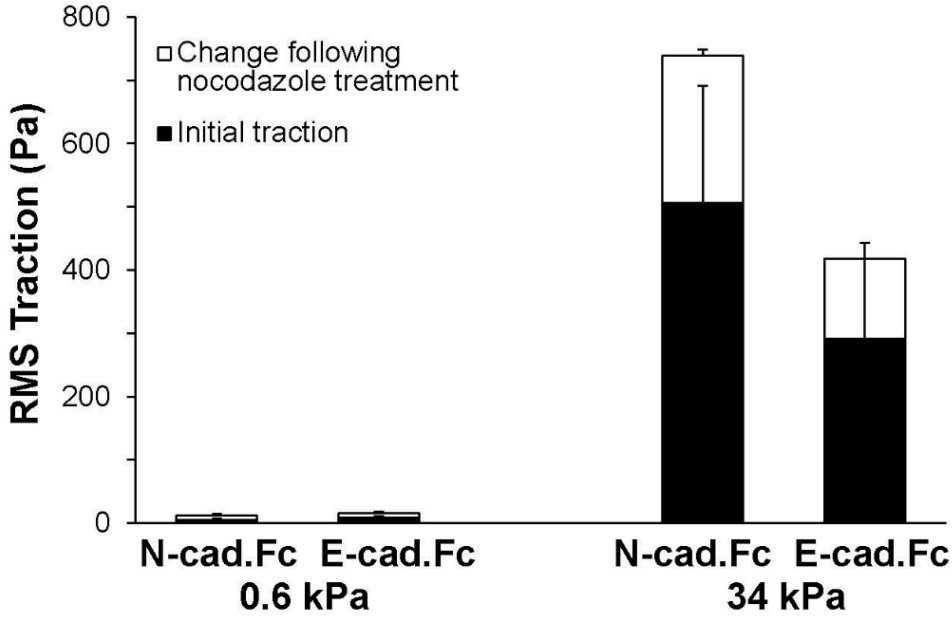

Figure 3.8 Nocodazole treatment increased traction stresses exerted by the cells on cadherin coated gels. Cells were cultured in the absence of on soft (0.6 kPa) and semi- rigid (34 kPa) coated with either N-cad-Fc or E-cad-Fc. The filled bars indicate traction stresses in the absence of nocodazole, and the white bars show the increase in traction forces after treating the cells with nocodazole.

3.4.6 Characterization of elastic moduli of polyacrylamide gels

The gel elastic moduli (34 kPa and 1.78 kPa) were quantified with a compression tester. The compressive elastic moduli (E) of the gels were measured, by compressing the hydrogels with a mechanical testing system (MTS) at a rate of 0.1 mm/min and measuring the resulting stress. E was determined from the slope of the linear curve between stress and strain at the first 10% of strain.

3.4.7 Immunofluorescence

MDA-MB-435 cells were initially harvested with 3.5 mM EDTA with 1 w/v% BSA, and then seeded onto the hydrogels coated with either cadherin ectodomains or collagen. After 6 hours at $37^{\circ C}$ in 5% CO_2, the cells were fixed with 4 w/v% paraformaldehyde (Sigma) for 15 min and then washed with PBS. Cells were treated with 0.1 w/v% Triton X-100 in PBS for 10 min, and washed in Tris-Buffered-Saline (TBS) at room temperature. After cell permeabilization, the fixed cells on the hydrogels were blocked with 1 w/v% BSA for 30 min at room temperature, and then washed with TBS.

Cells were then incubated for 1 h at room temperature with mouse monoclonal anti-paxillin antibody (BD Bioscience, San Jose, CA) in TBS with 1 w/v% BSA. Before incubation with secondary antibody, the cells were again washed with TBS. Secondary antibody, antimouse IgG FITC (Sigma-Aldrich (St. Louis, MO), was prepared in TBS containing 1 w/v% BSA. Rhodamine Phalloidin (Invitrogen, Carlsbad, CA) was used to label F-actin. Substrata were incubated in secondary antibody for 1 h and washed three times with TBS containing 0.1 v/v% Tween. Cells were mounted with Antifade^{TM}

(Vectashield, Vector Laboratories, Burlingame, CA), and visualized with an Axiovert 200 inverted fluorescence microscope using a 100x oil immersion objective.

3.4.8 Protein surface densities on polyacrylamide gels

To quantify the cadherin-Fc bound to the different hydrogels, anti-human immunoglobulin Fc was radiolabeled using Iodobeads (Pierce Biotech, Rockford, IL) and carrier free Na^{125} [I] (Perkin Elmer, Waltham, Massachusetts). The ^{125}I-labeled protein was desalted with a PD-10 column (GE Healthcare Bioscience) to remove unbound ^{125}I. The determined specific activity of ^{125}I-labeled anti-Fc antibody was 7 cpm/ng.

Polyacrylamide hydrogels (0.6 kPa and 34 kPa) were prepared as described (10 mm diameter, 1 mm thickness). ^{125}I-labeled immunoglobulin Fc was covalently bound to the gels that were chemically activated with Sulfo-SANPAH. Gels of defined size were then rinsed with buffer to remove unbound protein, and placed in scintillation vials containing 5 ml of scintillation cocktail. The radioactivity in each sample was recorded with an LS 6500 scintillation counter (Beckman Instruments) with specified settings for ^{125}I detection. Control measurements used hydrogels incubated with the labeled protein, but without the Sulpho-SANPAH cross-linker.

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Chapter 4

α-Catenin Cytomechanics: Role in Cadherin-Dependent Adhesion and Mechanotransduction

4.1 Introduction

Cadherins are essential intercellular adhesion proteins that maintain the mechanical integrity of all soft tissues, direct morphogenetic movements, and regulate tissue barriers (Gumbiner, 2005; Takeichi, 1991). They are also signaling proteins, but a principal function is to maintain cell-cell cohesion. During tissue morphogenesis and remodeling, as well as in mature tissues, cell-cell cohesive forces are often dynamic and distributed non-uniformly at intercellular junctions (Cavey et al., 2008; Kasza & Zallen, 2011; Lecuit and Le Goff, L., 2007; Rauzi, Verant, Lecuit, & Lenne, 2008). The magnitudes and distributions of intercellular stresses influence a broad range of tissue properties and processes, including cell shape, morphogenetic movements, neural tube closure, wound healing, cell segregation, and the regulation of tissue barriers (Diz-Munoz et al., 2010; Krieg et al., 2008; Lecuit & Lenne, 2007; Lecuit et al., 2011; Paluch & Heisenberg, 2009b; Papusheva & Heisenberg, 2010). Passive, regulated intercellular linkages would, in principle, be sufficient to mechanically support these different processes. However, consideration of mechanical connectivity alone would overlook the instructive cues inherent in the often-dynamic mechanical properties of the tissue microenvironment and the transduction of those cues into biochemical signaling cascades that regulate cell functions. It is increasingly evident that mechanical forces play central roles in development, tissue homeostasis, and disease progression, and mechanotransduction

processes are the vehicles by which cells communicate with their mechanical environment.

Cadherin complexes were not previously identified as adhesion-based mechanosensors (Vogel & Sheetz, 2006). By contrast, the force sensitivity of focal adhesions, which are prototypical mechanoresponsive adhesion complexes (Geiger et al., 2009), was initially identified on the basis of two measurements. First, direct external mechanical manipulation of ligand-coated beads (for example, fibronectin or RGD peptides) that were bound to cell surface integrins triggered integrin bond strengthening and cytoskeletal remodeling (Choquet, Felsenfeld, & Sheetz, 1997; N. Wang et al., 1993). Alternatively, substratum rigidity sensing via focal adhesions tested the capacity of cells to respond to the stiffness of compliant substrata, typically by altering traction forces or cell migration (Beningo et al., 2002; Wang et al., 2001). In cells on compliant, fibronectin-coated substrata, substratum rigidity increased focal adhesion size, stress fiber formation, and focal-adhesion mediated traction forces, as well as influenced stem cell lineage specification and the onset of metastatic phenotypes (Engler et al., 2006; Geiger et al., 2009; Levental et al., 2009). Similar mechanisms as in acute force transduction were thought to regulate endogenous contractile forces between the cytoskeleton and focal adhesions (Beningo & Wang, 2002a; Discher, Janmey, & Wang, 2005; Guo, Frey, Burnham, & Wang, 2006; Pelham Jr. & Wang, 1997; Vogel & Sheetz, 2006).

Recent measurements analogous to the integrin studies demonstrated that cadherin complexes are also mechanosensitive. Specifically, traction forces exerted through cadherin adhesions were shown to increase with substratum rigidity (Ladoux et al., 2010; Tabdili et al., 2012), and bead twisting on cell surface cadherins triggered actin

and E-cadherin-dependent changes in cell mechanics (Ladoux et al., 2010; Tabdili et al., 2012a). The mechanical perturbation of general cell-cell adhesions was achieved indirectly by myosin II (MyII) activation (le Duc et al., 2010; Liu et al., 2010; Miyake et al., 2006; Twiss et al., 2012; Yonemura et al., 2010), which increases tension across cell-cell junctions (Maruthamuthu et al., 2011), and directly by pulling on cell doublets with dual pipettes (Thomas et al., 2013). My II activation stimulated both vinculin recruitment to cell-cell junctions and the thickening of adhesion zones between endothelial cells (le Duc et al., 2010; Liu et al., 2010; Miyake et al., 2006; Twiss et al., 2012; Yonemura et al., 2010). Direct tugging on cell pairs triggered intercellular adhesion strengthening and vinculin recruitment to the cell contacts (Thomas et al., 2013).

The different studies demonstrated that force alters cadherin-mediated junctions, but whether the observations reflect identical or different mechanisms is an open question. In contrast to shearing specific cadherin bonds, force is transmitted throughout intercellular contacts, and could involve other membrane proteins, in addition to cadherins. For example, the principal force transducer at endothelial junctions responsible for fluid shear sensing is PECAM-1, which forms a functional complex with VE-cadherin and vascular endothelial growth factor receptor 2 (VEGFR2) (Schwartz & DeSimone, 2008; Tzima et al., 2005). Pharmacological activation of My II also stimulates kinases and GTPases that could alter junction protein functions (Braga, 1999; Brunton, MacPherson, & Frame, 2004; Dudek & Garcia, 2001; McLachlan & Yap, 2007; Petrova, Spano, & Gumbiner, 2012; Waschke et al., 2004).

Central questions concern the mechanism(s) underlying different apparent manifestations of cadherin-based mechanotransduction, the identity of the

mechanosensor(s), and the force-dependent protein cascades underlying the experimental outcomes. Biochemical and biophysical evidence strongly suggest that α-catenin is a force-activatable protein in cadherin complexes. α-Catenin is an actin binding protein, which also binds the complex between α -catenin and the cadherin cytoplasmic domain, and is required for mechanical coupling between cadherin and actomyosin (Cavey et al., 2008; Desai et al., 2013; Gumbiner & McCrea, 1993; Imamura, Itoh, Maeno, Tsukita, & Nagafuchi, 1999). Yonemura et al. (Yonemura et al., 2010) first demonstrated that My II activation stimulated both vinculin recruitment to cadherin adhesions and increased -catenin reactivity towards an epitope specific antibody. Based on those observations and α -catenin domain mapping studies, Yonemura et al. (Yonemura et al., 2010) proposed a mechanism in which force triggers the exposure of a cryptic vinculin-binding-site in α-catenin that in turn recruits vinculin and actin to cadherin junctions. This model is supported by adhesion strengthening and vinculin recruitment to stressed intercellular junctions, which both require α-catenin and its vinculin-binding-site (Thomas et al., 2013; Twiss et al., 2012). Studies of α-catenin mutants in *Drosophila* are also consistent with the proposed mechanism (Desai et al., 2013). However, whether the same mechanism(s) accounts for all reported, putative cadherin-based mechanotransduction behavior such as adhesion strengthening, junctional remodeling, and cell stiffening has yet to be established.

Here we used the model of α-catenin force transduction at cadherin complexes as the framework for investigating the role of α-catenin in cadherin-based mechanotransduction, in different mechanical contexts. Bead-twisting measurements in conjunction with imaging, and the use of different cell lines and α-catenin mutants

directly tested whether the mechanical manipulation of cadherin bonds triggers vinculin and actin recruitment, in an actin- and α-catenin dependent manner. Traction force measurements investigated the role of α-catenin in substrate rigidity sensing, and identified differences between acute mechanotransduction and rigidity sensing. Finally, cadherin affinity measurements tested whether α-catenin modulates cadherin affinity (adhesion) through inside-out signaling. Distinct from other reports, these findings directly demonstrate the role of α-catenin in cadherin-specific mechanotransduction, directly verify features of the proposed mechanism, and reveal aspects of cadherin-based force transduction that differ from expected behavior.

4.2 Results

4.2.1 α-catenin is required for cadherin-dependent mechanosensing

Magnetic twisting cytometry was used to investigate the role of α-catenin in cadherin-dependent mechano-sensing (Figure 4.1A). This approach quantifies the viscoelastic properties of the adhesive junction between ferromagnetic beads modified with human immunoglobulin Fc-tagged extracellular domains of E-cadherin (E-cad-Fc) and cadherins expressed on cell surfaces. The oscillating torque applied to the ferromagnetic bead bound to the cell surface displaces the bead and generates shear stress on the cadherin bonds. In the absence of an active response, the bead displacement would not change over time, but active remodeling would alter the cell/junction stiffness and the bead displacement amplitude (Figure 4.1B).

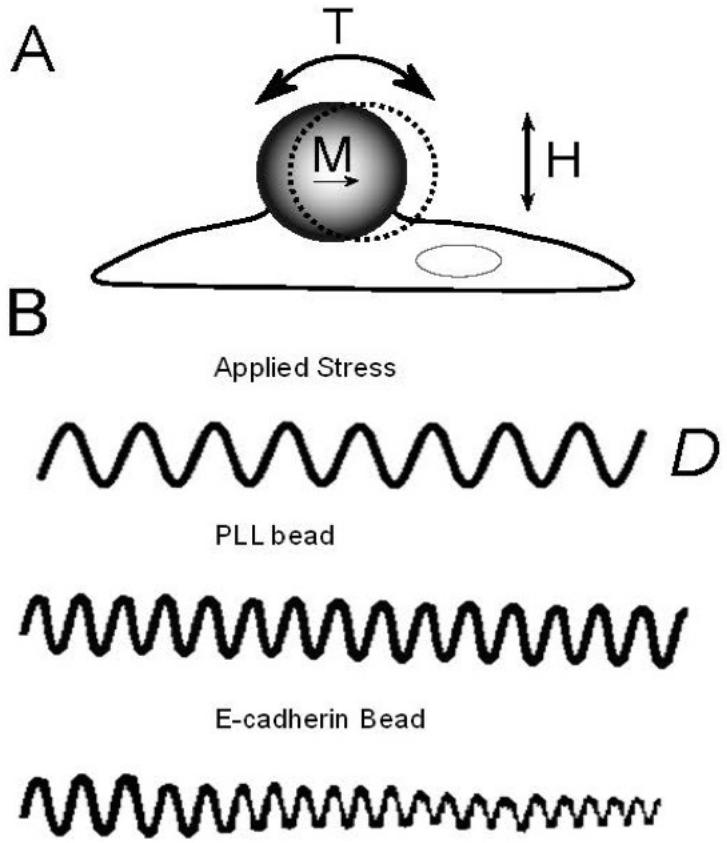

Figure 4.1 Magnetic twisting cytometry. (A) Schematic of magnetic twisting cytometry experiment. An oscillating field (*H)* is applied to the ferromagnetic beads coated with ligand of interest attached to cell membranes. The beads are magnetized with a magnetic moment M parallel to the substrate. The orthogonal applied field generates a torque (*T)* on the bead, causing a bead displacement *D*. (B) Bead displacement in response to modulated shear stress. The upper panel shows the applied torque. The middle panel shows the corresponding displacement of poly(L-lysine)-coated beads. The lower panel shows the displacements of E-cad-Fc coated beads**.**

Figure 4.2A shows the force-induced stiffening response of MDCK KD cells for α-catenin and MDCK KD cells rescued with a GFP-α-catenin construct (MDCK Rescued). MDCK Rescued cells exhibit ~28% increase in junction stiffness, relative to unperturbed cells, in response to bond shear. By contrast, MDCK KD cells probed with identical E-cad-Fc coated beads failed to induce any stiffening response, and the junction

modulus actually decreased slightly (Figure 4.2A). Because the E-cadherin expression levels were the same in both cases, the difference between the MDCK KD and MDCK Rescued cells is attributed to the loss of α-catenin. In control measurements with poly-L-lysine) (PLL) coated beads or with E-cad-Fc coated beads in the presence of EGTA, which removes the Ca^{2+} ions required for cadherin activity (Figure 4.2A), the junction stiffness was unaltered or decreased (Figure 4.2A). The PLL coated beads failed to induce junction remodeling, despite strong adhesion to the cell surface.

α-catenin dimers in the cytosol affect actin dynamics at the leading edge (Benjamin et al., 2010), and could indirectly affect cadherin-based mechano-transduction, by altering the global cell contractility or by altering the local organizational state of actin at cadherin adhesions. To test this, measurements were done with β-cat Act A MDCK cells (Benjamin et al., 2010). In these cells, the mitochondrial protein Act A is fused to the α-catenin binding region of β-catenin. This sequesters cytosolic α-catenin to mitochondria, while retaining cadherin-bound α-catenin at the membrane. Consistent with the postulate that α-catenin affects mechanotransduction locally at the membrane, the stiffening response measured with β-cat Act A MDCK cells was similar to WT and MDCK Rescued cells, when cells were pulled with E-cad-Fc coated beads (Figure 4.2A).

To test whether α-catenin loss similarly alters mechanotransduction in other cells, MTC measurements were conducted with DLD-1 cells and with the α-catenin null R2/7 sub-clone of the DLD-1 cell line (Watabe-Uchida et al., 1998; Yonemura et al., 2010). DLD-1 is a colon carcinoma line that expresses endogenous E-cadherin. Both the DLD-1 and R2/7 lines express E-cadherin at similar levels, as confirmed by FACS. As with the MDCK cells, mechanical perturbation of DLD-1 cells with E-cad-Fc beads induced a

~60% increase in junction stiffness (Figure 4.2B). The larger % change in the junctional stiffness of DLD-1 cells relative to that observed MDCK cells is due to the greater cell contractility. Similar to the MDCK KD cells, the lack of α-catenin in R2/7 cells correlated with the absence of the force-dependent stiffening response (Figure 4.2B). R2/7 cells rescued with GFP-tagged mouse α-catenin (GFP-α-catenin) exhibited a similar stiffening response to DLD-1 cells. R2/7 cells rescued with a GFP-α-catenin construct lacking the vinculin-binding-domain (GFP-α-catenin-ΔVBS) did not trigger a stiffening response (Twiss et al., 2012), consistent with the postulated role of the vinculin-binding-region in mechanotransduction. In control measurements, beads modified with PLL failed to induce junction remodeling in either DLD-1 cells, despite strong adhesion to the cell surfaces (Figure 4.2B).

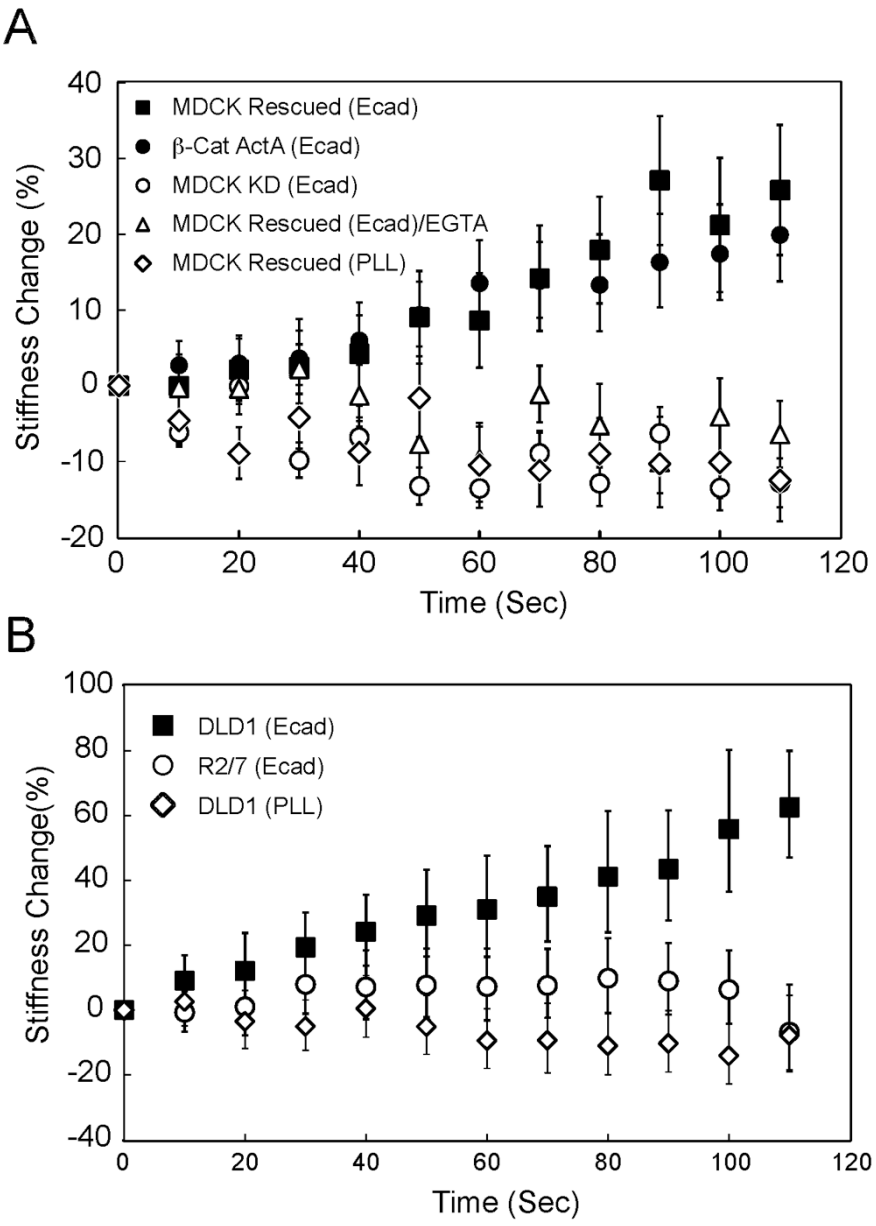

Figure 4.2 α-catenin is required for acute cadherin-dependent mechanotransduction. (A) MTC measurements of the force-induced stiffening response of α-catenin KD MDCK cells (MDCK KD) rescued with mouse α-catenin (MDCK Rescued) were probed with beads coated with E-cad-Fc in the absence (black squares) and presence of 4 mM EGTA (white triangles), and with poly-L-lysine (PLL) coated beads (white diamonds). MDCK KD (white circles) and β-Cat Act A (black circles) MDCK cells were similarly probed with E-cad-Fc-coated. (B) MTC measurements of the force-induced stiffening response of DLD-1 cells probed with beads coated with E-cad-Fc (black squares) or PLL (white diamonds). α-catenin null R2/7 cells were probed with E-cad-Fc-coated beads (white circles). Each time point represents the mean and standard deviation of the mean of measurements with >200 beads.

4.2.2 α-catenin and its vinculin-binding-site modulate traction force generation at cadherin adhesions

Traction force measurements also tested the capacity of cells to sense matrix rigidity via cadherin adhesions subject to endogenous contractile stress. Studies quantified the effect of α-catenin depletion on traction force generation by cells seeded on E-cad-Fc-coated, deformable polyacrylamide (PA) hydrogels. Figure 4.3 compares the root mean square traction force (Pa) exerted by MDCK KD and MDCK Rescued cells, when cultured on PA gels with elastic moduli of 0.6kPa and 34kPa. The softer 0.6kPa stiffness is comparable to that of lung tissue and the 34kPa stiffness is similar to bone (Engler, Rehfeldt, Sen, & Discher, 2007). Similar to rigidity-dependent traction force generation by N-cadherin adhesions (Ladoux et al., 2010; Tabdili et al., 2012), the traction forces generated by MDCK Rescued cells on E-cad-Fc coated substrata depended on the gel stiffness, and decreased from 560 Pa on the stiffer gel to < 20 Pa on the softer gel (Figure 4.3A). Prior measurements with radiolabeled proteins showed that the cadherin surface coverage is independent of the gel stiffness (Tabdili et al., 2012). The loss of α-catenin reduced traction force generation on stiff gels by 45% relative to the MDCK Rescued cells, and by 68% on the softer gels (Figure 4.3A). Interestingly, α-catenin loss did not ablate the rigidity dependence of the traction force generation.

Traction forces exerted by DLD-1 and R2/7 cells were similarly compared. Figure 4.3B shows the traction forces generated by cells on E-cad-Fc substrata. The DLD-1 cells did not detectably deform 34kPa hydrogels, so that these studies used gels with moduli of 1.1kPa and 8.8kPa. The traction force trends of the DLD-1 and R2/7 cells (Figure 4.3B) are similar to the MDCK Rescued and MDCK KD cells. Namely, the DLD-1 cells exert greater traction forces on the semi-rigid hydrogel than on the softer substrate. Similarly,

α-catenin loss reduced the traction force generated by R2/7 cells, relative to DLD-1 cells on both substrata. The loss of α-catenin did not eliminate the dependence of traction forces on substrate rigidity.

The dependence of the traction forces on organized actin and myosin activity differed somewhat from cell responses to acute exogenous force applied to cadherin bonds. In MTC measurements, cytochalasin D treatment abolished the stiffening response, and blebbistatin reduced the stiffening response of E-cadherin on F9 cells by ~50% (le Duc et al., 2010). In traction force measurements of MDCK KD and MDCK Rescued cells, cytochalasin D and blebbistatin reduced the RMS cell tractions on rigid (34kPa) substrata by ~25% and 35%, respectively, relative to untreated cells on identical substrata (Figure 4.3C). We attribute the residual traction forces to other mechanisms, possibly involving microtubules and/or intermediate filaments in the regulation of global cell prestress.

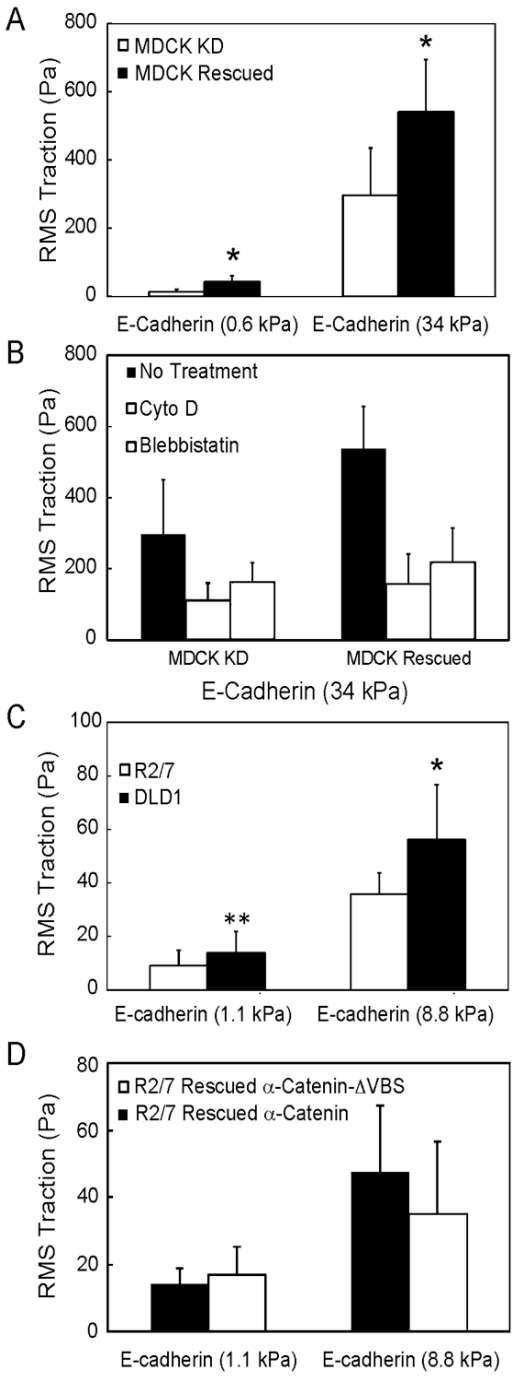

Figure 4.3 α-catenin modulates traction forces at cadherin adhesions. (A) RMS (root-mean-square) traction forces (Pa) exerted by MDCK Rescued and MDCK KD cells on soft (0.6 kPa) and rigid (34 kPa) hydrogels coated with immobilized, oriented E-cad-Fc. (B) RMS traction force exerted by MDCK Rescued and MDCK cells on arigid (34 kPa) hydrogel coated with oriented E-cad-Fc, after treatment with blebbistatin or cytochalasin D (Cyto D). (C) RMS traction forces exerted by DLD-1 and R2/7 cells on soft (1.1 kPa) and semi-rigid (8.8 kPa) gels coated with oriented E-cad-Fc. (D) RMS traction forces exerted by R2/7 Rescued cells and R2/7 Rescued ΔVBS cells on soft (1.1 kPa) and semi-rigid (8.8 kPa) gels coated with oriented E-cad-Fc**.**

4.2.3 Cytosolic α-catenin alters global cell contractility

Interestingly, both global α-catenin knock down and the depletion of cytosolic α-catenin in β-cat-ActA MDCK cells altered traction forces exerted by both cadherin-based adhesions (Figure 4.4A) and focal adhesions to fibronectin-coated gels with 34kPa moduli (Figure 4.4B). On E-cad-Fc coated substrata, traction forces exerted by MDCK Rescued cells were significantly higher than either the α-catenin KD MDCK cells or the β-cat-ActA MDCK cells. However, the RMS traction forces exerted by MDCK KD cells and by the β-cat-ActA cells were statistically similar (p=0.76 > *0.05*). This suggests that cytosolic α-catenin does influence the global contractile state of the cells, in addition to playing a critical role in cadherin-based mechanotransduction at the membrane.

The results obtained with both MDCK KD and β-cat ActA MDCK cells on fibronectin-coated substrata (Figure 4.4B) were qualitatively similar to cells on E-cad-fc substrata, although the generated RMS traction forces were lower. Interestingly, the effect of global and cytosolic α-catenin depletion on focal adhesion-dependent traction forces further supports the conclusion that cytsolic α-catenin contributes to the global regulation of cell prestress.

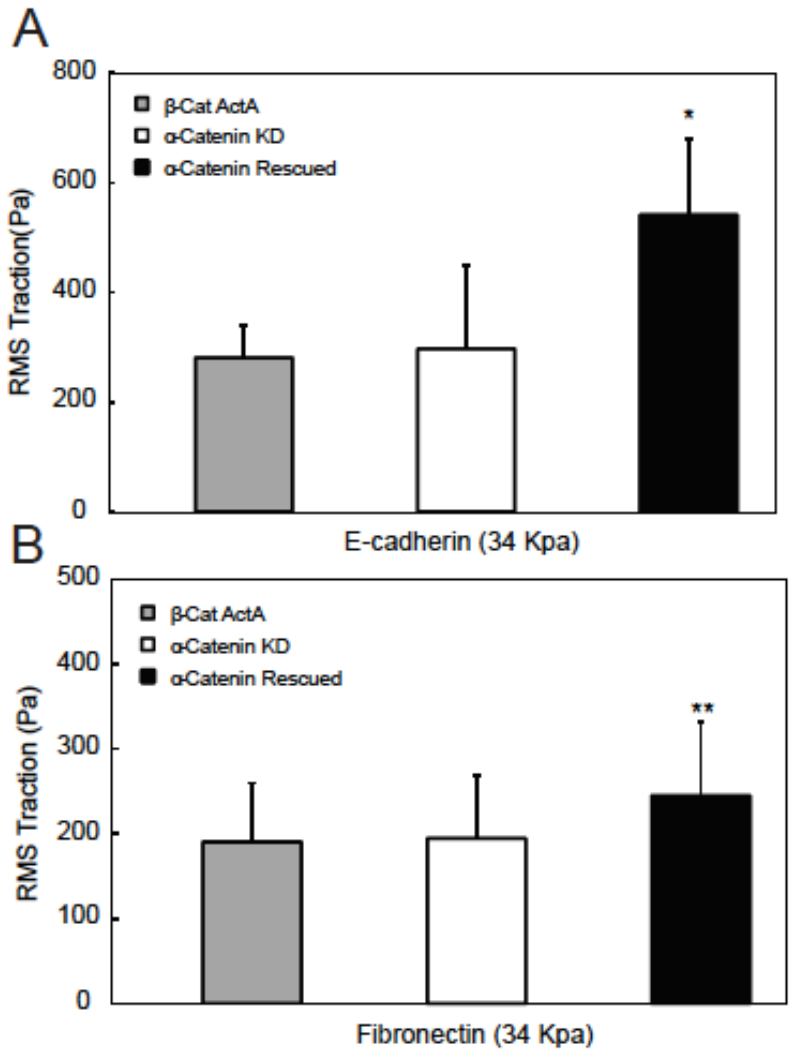

Figure 4.4 Cytosolic depletion of α-catenin affects global cell contractility. (A) RMS traction forces exerted by MDCK Rescued (black), MDCK KD (white), and β-Cat Act A MDCK (gray) cells on rigid (34 kPa) gels coated with E-cad-Fc ligand in the presence of 0.5v/v% bovine serum. (B) RMS traction forces exerted by MDCK Rescued (black), MDCK KD (white), and β-Cat Act A MDCK (gray) cells on rigid (34 kPa) gels coated with fibronectin in the presence of 0.5v/v% bovine serum.

4.2.4 Force-dependent actin recruitment to E-cadherin adhesions requires α-catenin

The postulated force-dependent recruitment of actin to vinculin at stressed intercellular junctions could account for the force-dependent reinforcement of cadherin junctions.

However, besides vinculin recruitment, other force-dependent molecular remodeling at cadherin adhesions could also alter local junction mechanics. Additionally, the molecular basis of the stiffening response detected in MTC measurements was not addressed in earlier studies. Here, laser scanning confocal images acquired at beads on the same time scale as the measured junction stiffening confirmed the spatiotemporal correlation between bond shear, junctional stiffening, α-catenin distributions, and actin recruitment to cadherin adhesions. Figure 4.5A shows the quantitative comparisons of confocal immunofluorescence images of cadherin, actin and α-catenin at beads bound to the apical surface, before and after shearing cadherin adhesions to DLD-1 cells. These data are also compared with force-dependent actin and α-catenin distributions on R2/7 cells. Figure 4.5B shows cadherin, α-catenin, and actin immunostaining at lateral junctions between the cells.

With DLD-1 cells, after bond shear, both cadherin and actin form clearly visible rings around the beads (Figure 4.5A). In unperturbed DLD-1 cells, α-catenin and actin (Figure 4.5A) are both visible at the beads, but the actin intensity and thickness of the actin ring are significantly lower prior to bond shear (Figure 4.5A). There appears to be some α-catenin accumulation after bond shear (Figure 4.5A). We do not attribute the latter to force-dependent α-catenin recruitment, but rather to a small increase in local E-cadherin due to the bead rotation on the cell surface that could pull additional protein into the contact. Controls with PLL coated beads, by contrast, do not exhibit any force-dependent α-catenin or actin accumulation at beads (Figure 4.5A).

In contrast to DLD-1 cells, there is a negligible amount of actin near the beads on R2/7 cells, but the actin intensity did not change after bond shear (Figure 4.5A). There

was no α-catenin surrounding the E-cad-Fc coated beads on R2/7 cells (Figure 4.5A), as expected. The latter results strongly support the postulate that force activates actin recruitment to cadherin adhesions by an α-catenin-dependent mechanism. Moreover, because the imaging and mechanotransduction timescales are identical in these measurements, these images demonstrate direct spatiotemporal correlations between junction reinforcement and force-actuated cytoskeletal remodeling. Conversely, the absence of junctional stiffening coincides with the absence of detectable actin accumulation, following bond shear.

In order to quantify these general observations, we further compared the fluorescence intensities in regions of interest centered on the beads and with radii extending 1 μm from the bead edge, as determined from the DIC images (Figure 4.5A). Figure 4.6C compares the α-catenin and actin intensities surrounding the beads before and after bead twisting and in cells with and without α-catenin. Surrounding the E-cad-Fc coated beads on DLD-1 cells is a statistically significant increase in the intensity of both actin and α-catenin following bead twisting ($P<0.05$). The greatest change in actin densities was before and after bond shear with DLD-1 cells probed with E-cad-Fc coated beads. There is also some increase in α-catenin levels (Figure 4.5C), but the relative change is significantly smaller than the actin accumulation.

The results of line-scan analyses of spatial variations in fluorescence intensity relative to the bead center also quantify changes in actin and α-catenin accumulation (Figures 4.5 D, E). The averages of 25 scans for each condition reveal that the intensity and width of the actin zone at beads on DLD-1 cells increased after 2min of bond shear, relative to unperturbed cells. This behavior contrasts with the lack of any significant,

force-dependent changes when DLD-1 cells were probed with PLL-beads or when R2/7 cells were probed with E-cad-Fc coated beads (Figure 4.6D).

There was more α-catenin at E-cad-Fc coated beads on DLD-1 cells before loading, than at PLL-coated beads on DLD-1 cells, before or after loading (Figure 4.5E). The intensities determined with control PLL-coated beads were near the background fluorescence levels measured with R2/7 cells (Figure 4.5E). Bond shear increased the local α-catenin intensity by ~30%, relative to unperturbed cells, but this is substantially less than the ~400% change in actin intensity measured under identical conditions (Figure 4.5D).

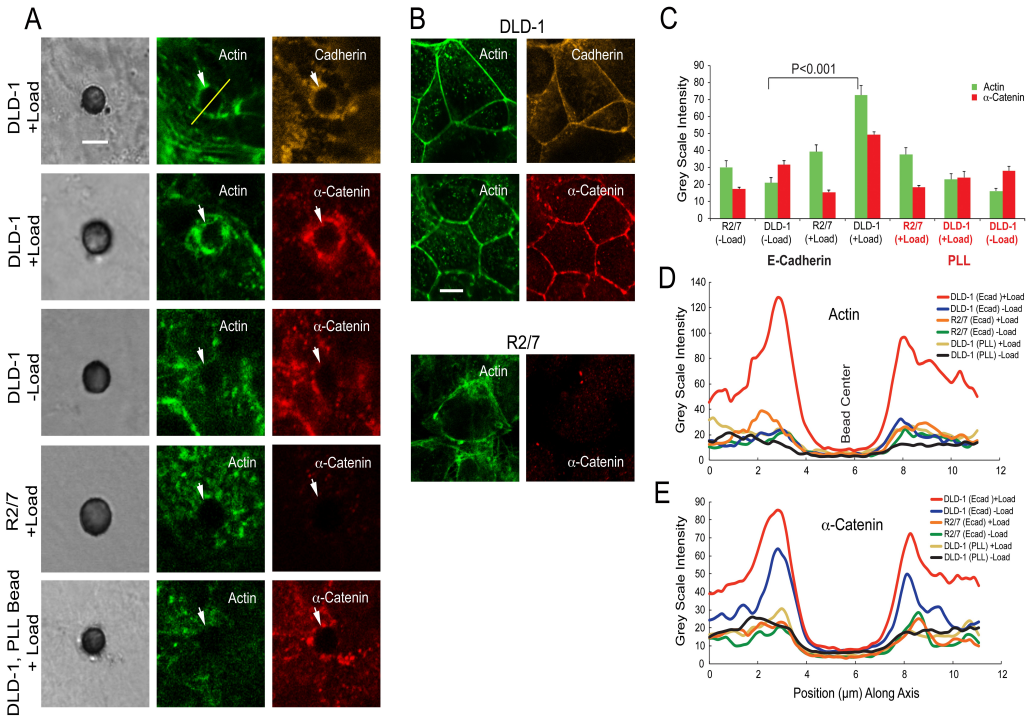

Figure 4.5 Confocal immunofluorescence images of force dependent distributions of cadherin, α-catenin, and actin at E-cadherin coated beads on DLD-1 and R2/7 cells. (A) DIC images of beads on cells, and immunofluorescence images of actin, cadherin, and α-catenin distributions. The scale bar is 5 μm. The fluorescence images were enhanced for presentation using Adobe Photoshop. The top panels show the DIC image of an E-cad-Fc coated bead on a DLD-1 cell after loading, and the corresponding actin and cadherin distributions. The white arrows indicate the bead edge. The panels below show the DIC images, actin, and α-catenin distributions surrounding E-cad-Fc coated bead. The cells and loading conditions are indicated to the left of the images. The bottom panels show the DIC image of a PLL-coated bead on a DLD-1 cell after loading, and the corresponding actin and α-catenin distributions surrounding the bead. (B) Actin, cadherin, and α-catenin distributions at lateral junctions. The scale bar corresponds to 10 μm. The top panels show the protein localization at junctions between DLD-1 cells and the bottom panels show the actin and α-catenin localization at lateral junctions between R2/7 cells. (C) Total fluorescence intensity within rings centered on the beads and extending 1 μm from the edges of beads bound to R2/7 or DLD-1 cells before and after loading. Green bars indicate actin and red bars correspond to α-catenin. The cell type and loading condition are indicated below the bars. The bead coating is indicated by the color of the labeling. Black indicates beads coated with E-cad-Fc and red indicates beads coated with PLL. (D) Line scan analyses of the immunofluorescence intensities for actin over a length of 11 μm, centered on the bead (yellow line, Figure 4.5A). The colored lines correspond to the different experimental conditions indicated in the figure. (E) Line scan analyses of the immunofluorescence intensities for α-catenin over a length of 11 μm, centered on the bead (yellow line, Figure 4.5A). The colored lines correspond to the different experimental conditions indicated in the figure. The color code is the same as in 4.5D.

4.2.5 The vinculin-binding-site of α-catenin is required for actin recruitment

As observed in the MTC measurements, the expression of GFP-α-catenin in R2/7 cells results in qualitatively similar, force-dependent junction remodeling as with DLD-1 cells. Immunofluorescence images (Figure 4.6A) show the levels of actin and α-catenin within a circular region surrounding beads on R2/7 cells restored with GFP-α-catenin or with GFP-α-catenin-ΔVBS after loading. Figure 4.6B shows the actin, cadherin, and α-catenin distributions at lateral junctions between these cells. Figure 4.6A shows that the actin intensity in Rescued R2/7 cells is significantly higher than on cells rescued with GFP-α-catenin-ΔVBS. The results of the immunofluorescence images in Figure 4.6A are confirmed in ring and line-scan analyses in Figures 4.6C-E. These results are consistent with the MTC results and confirm that the vinculin-binding-site of α-catenin is a critical component in the force sensing machinery.

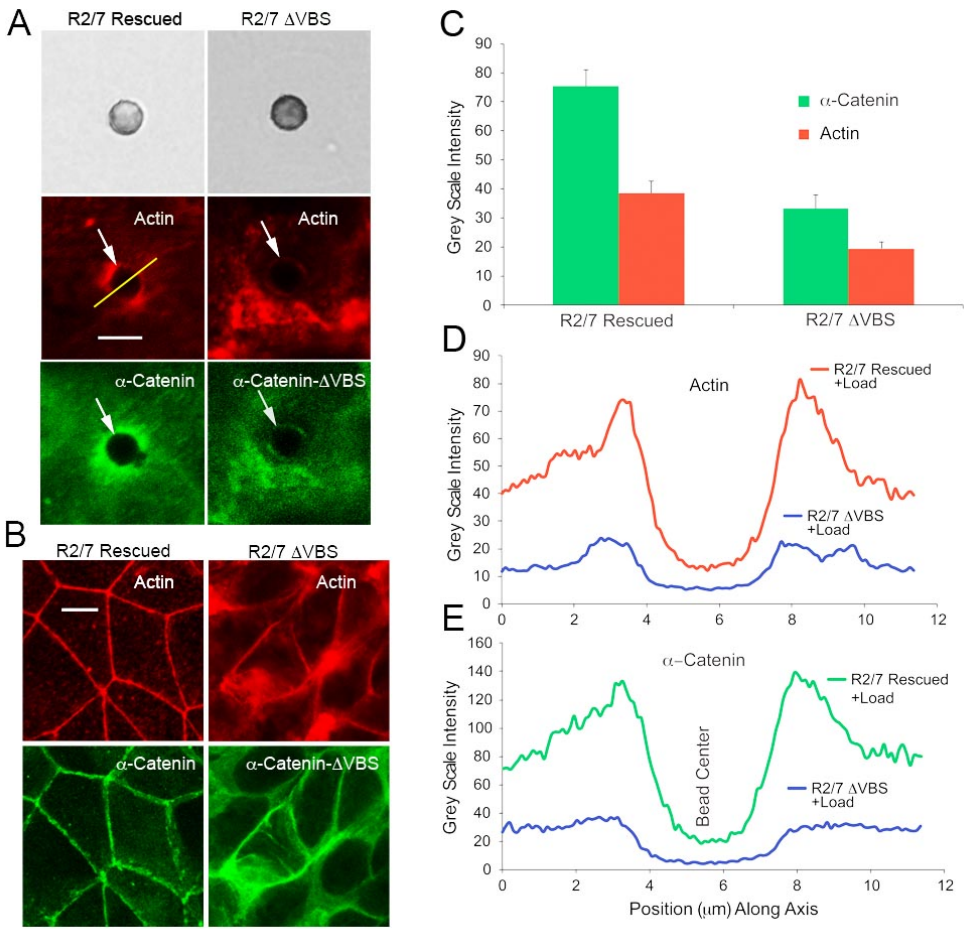

Figure 4.6 Confocal immunofluorescence images of α-catenin and actin at beads on R2/7 cells rescued with GFP-α-catenin and GFP-α-catenin-ΔVBS. (A) DIC images of beads on cells, and immunofluorescence images of actin, cadherin, and α-catenin distributions. Red corresponds to actin and green corresponds to α-catenin. The scale bar is 5 μm. The fluorescence images were enhanced for presentation using Adobe Photoshop. The cells and loading conditions are indicated to the left. The white arrows indicate the bead edge. (B) Actin, cadherin, and α-catenin distributions at lateral junctions. The scale bar corresponds to 10 μm. The top panels show the protein localization in DLD-1 cells and the bottom panels show the actin and α-catenin localization at lateral junctions between R2/7 cells. (C) Total fluorescence intensity within rings extending 1 μm from the edges of beads bound to R2/7 or DLD-1 cells before and after loading. Green bars indicate actin and red bars correspond to α-catenin. The cell type and loading condition are indicated below the bars. The bead coating is indicated by the color of the labeling. Black indicates beads coated with E-cad-Fc and red indicates beads coated with PLL. (D) Line-scan analyses of the immunofluorescence intensities for actin over a distance of 11μm, centered on the bead (yellow line, Figure 4.6A). The colored lines correspond to the different experimental conditions indicated in the figure. (E) Line scan analyses of the immunofluorescence intensities for α-catenin over a length of 11 μm, centered on the bead (yellow line, Figure 4.6A). The colored lines correspond to the different experimental conditions indicated in the figure.

4.3 Discussion

Previously, different experimental approaches were used to investigate cadherin-based mechanotransduction. The results obtained confirmed that mechanical perturbations trigger biochemical changes at intercellular junctions, but there was no general view of how α-catenin influences to cadherin-mediated adhesions, in the different mechanical contexts investigated. The results presented here unify several of those findings and identify mechanistic similarities and differences between observed behaviors.

Different from earlier work, the imaging and bead twisting measurements now demonstrate a causal relationship between direct mechanical stimulation of cadherin adhesions and both vinculin and actin recruitment to stressed cadherin complexes, by an α-catenin- and actin-dependent mechanism. Confocal images of protein distributions at cadherin-modified beads before and after mechanical stimulation directly visualized the molecular cascades triggered by localized, applied force at cadherin-specific bonds. They also directly confirmed that the cadherin-specific remodeling events at the beads involve analogous molecular changes as observed at cell-cell junctions following My II activation or tugging on cell doublets.

The spatial and temporal correlation between applied force, cytoskeletal remodeling at the bead-cell junctions, and the measured cell stiffening also demonstrates that the mechanical reinforcement (stiffening) triggered by cadherin bond shear (le Duc et al., 2010; Tabdili et al., 2012) is due to force-activated actin and vinculin accumulation, rather than to nonspecific effects of, for example, bead twisting. This is similar to the stiffening due to actin recruitment to pulled integrin adhesions (Icard-Arcizet, Cardoso, Richert, & Henon, 2008). In this case, however, the absence of both junctional stiffening

and vinculin or actin accumulation at E-cad-Fc beads on both R2/7 and MDCK KD cells, which lack α-catenin, confirms that α-catenin is essential for acute, cadherin-based mechanotransduction. Furthermore, the data support the postulate that α-catenin is the obligate force sensor in this complex, because loss of its vinculin-binding-site abrogates the tension-dependent responses while otherwise preserving the mechanical connection between cadherin and actin (Twiss et al., 2012).

The complete abrogation of acute force sensing by the α-catenin ΔVBS mutant differs from the 40-50% decrease in the stiffening response of vinculin deficient F9 cells (le Duc et al., 2010). A possible explanation is that the vinculin knockout F9 cells expressed low levels of endogenous vinculin, although none was detected. Alternatively, the vinculin binding segment also harbors sites for other actin binding proteins, including I-afadin, ZO-1, formin, and α-actinin that could also contribute to actin recruitment in the absence of vinculin (Knudsen, Soler, Johnson, & Wheelock, 1995; Kobielak, Pasolli, & Fuchs, 2004; Pokutta, Drees, Takai, Nelson, & Weis, 2002; Provost & Rimm, 1999). So far, however, there is no evidence that the latter proteins accumulate at stressed junctions (Twiss et al., 2012).

The increased width and intensity of the actin zone around the beads parallel the thickening of endothelial cell junctions subject to endogenous tugging forces (Liu et al., 2010), and the co-accumulation of vinculin and radial actin fibers at stressed intercellular junctions (Huveneers et al., 2012; le Duc et al., 2010; Twiss et al., 2012). The molecular mechanism underlying the zonal thickening was not addressed (Liu et al., 2010), but junctional remodeling following bead twisting, Myosin II stimulation, and cell tugging

(Twiss et al., 2012) strongly support the postulate that vinculin and actin recruitment in these cases involves the same α-catenin-dependent pathway.

The increased force to detach cell doublets after initial tugging, with the concurrent junctional accumulation of vinculin is also evidence for cadherin-based mechanotransduction (Thomas et al., 2013). The present results addressed whether α-catenin might contribute to force-activated adhesion strengthening by enhancing cadherin anchorage to the cytoskeleton (Maitre & Heisenberg, 2011), or by altering the cadherin binding affinity (adhesion) by inside-out signaling (Bajpai et al., 2008), analogous to integrins (Geiger et al., 2009).

Kinetics studies demonstrated that α-catenin loss modestly reduces the intrinsic, two-dimensional cadherin binding affinity by ~30% (Shashikanth Phd thesis, 2014). This is comparable to the 30% reduction in the apparent strength of cadherin-mediated tethers between cells expressing an α-catenin mutant with impaired α--catenin binding (Bajpai et al., 2008). Results showed that, there are differences between the force-independent, two-dimensional affinity measurements and bond rupture measurements (Shashikanth Phd thesis, 2014), which could reflect the force to break homophilic cadherin bonds, cadherin-cytoskeletal bonds, or the force to form membrane tethers (Evans & Calderwood, 2007; Maitre & Heisenberg, 2011). Nevertheless, the limited effect of α-catenin on homophilic cadherin binding would not account for the complete abrogation of acute mechanotransduction in α-catenin deficient cells. Signaling cascades might amplify small binding differences, but the latter processes would likely be slower than the rapid stiffening response observed. The affinity and mechanotransduction measurements were on the same, short timescale (< 2 min). Additionally, inhibitors of Rac and Src,

which are cytoskeletal regulatory proteins at E-cadherin junctions, have similarly minor effects on acute E-cadherin mechanotransduction (Shi & Leckband, 2009). These findings suggest that α-catenin plays a primarily mechanical role in adhesion strengthening, by increasing the extent of cytoskeletal connections by recruiting vinculin and actin, and by mechanically anchoring cadherin to the cytoskeleton, as observed in Zebrafish progenitor cell adhesion measurements (Maitre et al., 2012) and in *Drosophila* (Desai et al., 2013).

Because α-catenin is crucial for acute, cadherin-based mechanotransduction, one might also expect it to control substrate rigidity-sensing at cadherin adhesions (Ladoux et al., 2010), if similar mechanisms control the rigidity-dependence of traction forces. It was therefore somewhat surprising that α-catenin loss reduced cadherin-based traction forces, but that it did not ablate the dependence on substratum stiffness. This result suggests that other mechanisms cooperate with adhesion-based force transducers, to regulate contractility in different mechanical environments, and is consistent with the suggestion that fibroblast traction forces on fibronectin-coated substrata appeared to be modulated by an integrin-independent, presumably cytosolic mechanism (Trichet et al., 2012). Here, cadherins anchor cells to the substrata, and α-catenin regulates the level of tension sustained, but our findings show that α-catenin does not solely regulate cell contractility.

Rigidity sensing would require mechanical connectivity between the substratum and cytoskeleton. However, even without α-catenin, possible links between cadherins and the cytoskeleton include the microtubule/Nezha/PLEKHA7 complex (Meng, Mushika, Ichii, & Takeichi, 2008) and the vinculin/α--catenin complex (Peng, Cuff, Lawton, & DeMali, 2011). Intermediate filaments interact with C-cadherin in *Xenopus* mesendoderm

cells (Weber, Bjerke, & DeSimone, 2012). Determining mechanisms regulating cell prestress is beyond the scope of this study, but α-catenin clearly cooperates with such mechanisms, to regulate cell contractility in different mechanical environments.

In summary, these findings provide direct experimental support for the obligatory role of α-catenin and its vinculin-binding-site in acute force transduction through cadherin adhesions, consequent cytoskeletal remodeling, and force-dependent junction reinforcement. The visualization of molecular cascades triggered by cadherin-specific bead twisting further links different, observed force-dependent changes at intercellular junctions to a common α-catenin-dependent mechanism. Earlier study showed α-catenin has modest effect on cadherin affinity which based on our finding suggests that force-activated adhesion strengthening is due to enhanced cadherin/cytoskeletal interactions rather than to α-catenin-dependent affinity modulation. Thus, in acute, cadherin-based mechanotransduction, force transmission between cadherin and actin triggers local, α-catenin-dependent recruitment of vinculin and actin, which mechanically reinforces cadherin adhesions. Somewhat unexpectedly, these results show that α-catenin affects cadherin-mediated traction forces, but it does not solely regulate cell contractility on compliant, cadherin-coated substrata.

4.4 Materials and Methods

4.4.1 Materials

Blebbistatin, cytochalasin D (Cyto D), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), Poly-L-Lysine (PLL), 3-aminopropyl-triethoxysilane (APTES), and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Ferromagnetic

beads were purchased from Spherotech (Lake Forest, IL). Acrylamide, N,N'-methylene-bis-acrylamide (Bis), TEMED, and ammonium persulfate (APS) were obtained from Bio-Rad (Hercules, CA). N-Hydroxysuccinimide (NHS) and N-Succinimidyl-6-(4'-azido-2'-nitrophenyl-amino) hexanoate (Sulfo-SANPAH) were purchased from Pierce Biotech (Rockford, IL). Anti-human Fc antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA).

4.4.2 Cell lines and protein production

Madin Darby Canine Kidney (MDCK) II and DLD-1 human colon carcinoma cells were obtained from ATCC and maintained in DMEM, which was supplemented with 10 v/v % Fetal Bovine Serum (FBS) and penicillin/streptomycin. The stable knock-down of α-E-catenin was achieved using a hybrid vector kindly provided by Adam Kwiatkowski and James Nelson (Stanford), as described previously Benjamin et al., 2010). It contains an shRNA hairpin that specifically targets canine α-E-catenin (Capaldo & Macara, 2007) pEGFP-C1, and it contains the neomycin resistance gene for selection in G418 (400 μg/ml). The α-E-catenin knock-down line (clone #1) (MDCK KD) was generated by transfecting MDCK cells with Lipofectamine 2000. Subsequent G418 selection of a single MDCK clone showed >90% reduction in α-catenin protein by immunoblot analysis (Figure 4.1). As the EGFP expression in this vector can become "uncoupled" from α-catenin knock-down, this line was periodically re-selected by the serial limiting dilution method. The α-E-catenin-restored MDCK cells (MDCK Rescued) were generated using a vector (also kindly provided by Adam Kwiatkowski and James Nelson) that contains both the dog-specific α-E-catenin shRNA hairpin and a GFP-murine α-E-catenin cDNA that is refractory to the shRNA hairpin. The latter also contains a neomycin selectable marker.

Two stable integrants were selected after transfection with Lipofectamine (clones #10 and 15) and selection in G418. GFP-murine α-catenin expression within 100% of the colony is periodically maintained by fluorescence activated cell sorting (FACS). The R2/7 line is a non-cell-cell adhesive α-catenin null variant of the DLD-1 parental clone (Watabe-Uchida et al., 1998; Yonemura et al., 2010). MDCK β-cat-ActA cells were a gift from James Nelson, (Stanford University, Palo Alto, CA).

Both the DLD-1 subclone R2/7 and the α-catenin knockdown MDCK cells were transfected with lentiviral EGFP-α-catenin and EGFP-α-catenin-ΔVBS constructs described elsewhere (Huveneers et al., 2012), and selected by puromycin. To characterize their cell-cell junction forming capacity, cells were fixed and stained with E-cadherin (clone 36, BD biosciences), α-catenin (rabbit polyclonal, Sigma) and Vinculin (hVin-1, Sigma) antibodies. Cell-cell junctions were restored in these cells by both EGFP-α-catenin and EGFP-α-catenin-ΔVBS expression, although junction maturation seemed somewhat delayed in EGFP-α-catenin-ΔVBS cells. This was not analyzed further. Vinculin was completely absent from junctions in EGFP-α-catenin-ΔVBS cells, whereas it was present in a subset of junctions in EGFP-α-catenin cells.

Recombinant canine E-cadherin ectodomains with C-terminal Fc-tags (E-cad-Fc) were stably expressed in human embryonic kidney cells (293HEK) as described previously (Prakasam et al., 2006). Cells were routinely maintained in DMEM containing 10v/v% FBS. Protein-A Affigel (Bio-Rad, Hercules, CA) was used to affinity-purify the soluble Fc-tagged E-cadherin from the conditioned medium. This was followed by gel-filtration chromatography. SDS-PAGE assessed the protein purity.

4.4.3 Magnetic twisting cytometry (MTC)

Bead twisting measurements were conducted with home-built magnetic twisting cytometer (Wang et al., 1993). The measurements used ferromagnetic beads (Spherotech, Lake Forest, IL) that were covalently modified with E-cad-Fc or poly-L-lysine. To beads were chemically activated by incubating for 15min with EDC (10 mg/ml) / NHS (10 mg/ml) in MES buffer (50 mM MES, 100 mM NaCl, pH 5.0) on a shaker at room temperature. After centrifuging the beads at 12,000 x g for 15 min, the supernatant was removed, and the beads were rinsed with MES buffer. The rinsed, activated beads were then incubated with E-cad-Fc or Poly-L-Lysine (PLL) at 75 μg of protein per mg of beads for 2 hours at 4°C, in coupling buffer (20 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 8.0). Beads were used immediately after protein binding, in order to minimize aggregate formation. In order to disburse small aggregates of beads prior to adding them to cells, the beads were sonicated for 3 seconds. The protein-coated beads were then allowed to settle on a confluent cell monolayer for 20 min, before applying torque.

Treated beads were allowed to settle on confluent cell monolayers cultured on glass bottomed dishes, which were placed on a microscope stage heated at 37°C. The beads were allowed to sit on the cells for 20 min, prior to twisting the beads. Electric coils around the sample generated a computer-controlled magnetic field. An applied field pulse of 1 Tesla magnetizes the beads with a magnetic moment parallel to the cell substrate. After the initial magnetization, an applied oscillating magnetic field is applied perpendicular to the substrate, to generate a torque T on the beads. The torque causes bead displacements D, which were quantified with an inverted microscope (Leica) with a

20x, 0.6 numerical aperture objective interfaced with a CCD camera (Orca2; Hamamatsu Photonics). The complex modulus of the bead-cell junction was calculated as described (Wang et al., 1993).

Determinations of mechanotransduction in MDCK KD cells restored with GFP-α-catenin, β-cat-ActA MDCK cells, R2/7 cells restored with GFP-α-catenin, or R2/7 cells restored with GFP-α-catenin-ΔVBS only analyzed transfected cells, which were identified by GFP fluorescence or by RFP fluorescence (β-cat-ActA MDCK). Thus, nontransfected cells were excluded from the analyses.

4.4.4 Gel preparation and traction force microscopy

Polyacrylamide gels were prepared as described previously (Beningo & Wang, 2002a; Tse & Engler, 2010). Briefly, red fluorescent microspheres (0.2 μm, Molecular Probes, Eugene, OR) were embedded in the gels. The Young's moduli of the polyacrylamide gels were 0.6 kPa, 1.1 kPa, 8.8 kPa and 34 kPa, as determined with a mechanical testing system (Insight, MTS Systems, Eden Prairie, MN, USA). The mechanotester determined the compressive elastic moduli (E) by compressing the gels at a rate of 0.1 mm/min. The slope of the measured stress versus strain curve within the first 10% of strain determined the elastic modulus. Gels were functionalized as described (Tse and Engler, 2010). Briefly, hydrogels were activated with Sulpho-SANPAH (0.5 mg/ml, 100mM HEPES, pH 7.5). Gels were irradiated twice at 320min for 8min. After each 8min irradiation period, the samples were washed with 100 mM HEPES (pH 7.5). After the second wash, anti-Fc antibody at 0.2 mg/ml in water was added, and incubated with the gels overnight at 4°C. Before incubation with E-cadherin, the substrata were rinsed to remove unbound

antibody. Gels were then incubated with Fc-tagged E-cadherin extracellular domains (E-cad-Fc) at 0.2 mg/ml for 4 hours at 4°C.

Prior to traction force measurements, cells were mechanically detached from tissue culture flasks after treatment with a PBS solution containing 3.5mM EDTA and 1 w/v% BSA, in order to preserve the cell surface cadherin (Takeichi & Nakagawa, 2001). The cells were pelleted by centrifugation and rinsed with buffer lacking EDTA. Then 2000-4000 cells/cm^2 were seeded onto the cadherin-coated hydrogels and allowed to adhere for 6 hours at 37° C, in DMEM supplemented with 0.5 v/v% fetal bovine serum (FBS). After the specified time, fluorescence images of the beads were acquired before and after cell removal by treatment with 1% SDS. The bead positions were compared using an inverted microscope (Leica) with a 40x, 0.6 NA objective. A CCD camera (Orca2; Hamamatsu Photonics) imaged the bead positions. The constrained traction maps were calculated from the bead displacement field, as described (Takeichi & Nakagawa, 2001). The net contractile moment was determined for ~15 cells, and the reported values are the averages and the standard deviations from the mean.

Traction force measurements of MDCK KD cells restored with GFP-α-catenin, β-cat-ActA MDCK cells, R2/7 cells restored with GFP-α-catenin, or R2/7 cells restored with GFP-α-catenin- ΔVBS were only done with transfected cells, which were identified by GFP fluorescence or by RFP fluorescence (β-cat-ActA MDCK). Only data from transfected cells were used for the analyses. In addition to the fluorescence, the nontransfected MDCK KD and R2/7 cells were smaller and more rounded on both rigid and soft gels.

4.4.5 Immunofluorescence and confocal laser-scanning microscopy

Confocal laser-scanning, microscopy was used to image changes in actin and α-catenin distributions at beads on the different cells described above, before and after bond shear with the MTC. In the MTC studies, bead twisting results in displacements along a single axis. To determine whether the bead displacement direction biased the protein distributions, cells were subjected to two, consecutive 2 min loading cycles that displaced the beads along two orthogonal axes. In these cases, bead twisting was done under the identical conditions as in the mechanotransduction measurements: the field was applied 20 min after settling the beads on the cells, and the field oscillated at a frequency of 0.3 Hz for 2 min along each of the twisting axes.

Immediately after bead twisting, the cells were fixed with 4 w/v% paraformaldehyde in PBS for 15 min at room temperature. Cells were then washed with Tris-Buffered-Saline (TBS), treated with 0.25 w/v% Triton X-100 and 50 mM glycine in TBS for 10 min, and washed with TBS at room temperature. After cell permeabilization, the fixed cells were blocked with a solution of 3 w/v% BSA and 10 v/v% goat serum in TBS for 30 min at room temperature, and then washed with TBS. Cells were then incubated with rabbit monoclonal anti-α-catenin antibody (Sigma-Aldrich, St. Louis, MO) in blocking buffer for 1 h at room temperature. After washing, the fixed cells were incubated with anti-rabbit Rhodamine (Millipore) and Alexa Fluor 488 Phalloidin (Invitrogen, Carlsbad, CA). This approach affinity labeled α-catenin and F-actin, respectively.

R2/7 cells that were rescued with either GFP-α-catenin or with GFP-α-catenin-ΔVBS were incubated with Alexa-Fluor 488 anti-GFP, in order to label α-catenin, and

with Rhodamine Phalloidin in order to label actin. Antibody incubations were in 3 w/v% BSA and 10 v/v% goat serum in TBS. After 1 h at room temperature, cells were washed three times with TBS that contained 0.1 w/v% Tween. Cells were mounted with Antifade^{TM} (Vectashield, Vector Laboratories, Burlingame, CA). Images were acquired with a 63x/1.40 NA oil differential contrast microscopy objective lens (Zeiss) and a laser–scanning confocal microscope (LSM710, Zeiss). To image R2/7 cells rescued with either GFP-α-catenin or GFP-α-catenin-ΔVBS the laser power was reduced to prevent pixel saturation, due to the high expression of GFP-α-catenin in these cells.

4.4.6 Confocal image analyses

Quantitative analysis of shear-induced changes in fluorescence intensity associated with α-catenin and actin distributions adjacent to the beads were carried out with data obtained from measurements of beads on 25 cells (one bead/cell). Data were analyzed with two different approaches. Fluorescence intensity changes surrounding the entire bead (ring analyses) were quantified using ImageJ (version 1.46b). Beads were selected at random and the focal plane was adjusted in order to clearly focus on the beads. A region of interest (ROI) was defined by a ring at a distance of 1 μm out from the bead edge. The mask designating the bead edge was defined based on the DIC images. The total fluorescence intensity due to α-catenin and actin at the cell-bead junction on the apical surface of the cells was analyzed with Microsoft Excel. The absolute fluorescence intensities determined for 25 beads were then averaged, and the averages and standard errors of the mean were plotted in histograms that compared the fluorescence intensities measured under the different experimental conditions.

The ring analyses do not take into account non-uniformity in the circumferential actin and α-catenin distributions around the beads, so that line-scan analyses were also used to quantify force dependent variations in fluorescence intensities at cell-bead contacts. These analyses were performed with ImageJ, using the line-scan function. Lines spanning 11 μm (~3 times the bead diameter) and centered on the bead were quantified along a chosen axis, in order to quantify the regions of greatest intensity change. The spatial variation in fluorescence intensity along this axis was quantified, and the intensity profiles across 25 beads were averaged and plotted with Microsoft Excel. The intensities of background regions outside of the 11 μm range were generally non-zero and were not subtracted from the reported fluorescence intensities.

The line-scans obtained with R2/7 cells rescued with GFP-α-catenin or GFP-α-catenin-ΔVBS were plotted separately because the background GFP levels were higher in these cell lines compared to DLD1 and R2/7, due to the over expression of GFP-α-catenin and GFP-α-catenin-ΔVBS. For DLD-1 and R2/7 cells, the green channel actin reports and the red channel reports α-catenin. Conversely, in images of R2/7 cells rescued with GFP-α-catenin or GFP-α-catenin-ΔVBS, the color designation is reversed because GFP-α-catenin is immune-stained with Alexafluor 488.

4.5 References

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