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E-CADHERIN MECHANOTRANSDUCTION BEYOND CELL-CELL JUNCTIONS

BY
ISMAEEL MUHAMED

DISSEbATION

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Doctoral Committee:

Professor Deborah E. Leckband, Chair
Associate Professor Rutilio A. Fratti
Associate Professor William M. Brieher
Professor Robert B. Gennis
Abstract

Cadherins and integrin receptors form crucial extracellular adhesive connections with, respectively, adjacent cells and the extracellular matrix, and transduce mechanical signals across cell membranes. The role of adhesive complexes in diseases, cardiomyopathies, atherosclerosis, cancer, stem cell fate and morphogenesis has shed light on the importance of these adhesive complexes in human physiology.

Results presented in this thesis identified biochemical processes and signal transduction pathways in cadherin-based mechanotransduction. In this thesis, I investigated the role of E-Cadherin in mechanotransduction, using modified Magnetic Twisting Cytometry (MTC) and Traction Force Microscopy (TFM) coupled with fluorescence imaging. A major focus of Chapter 2 is the role of a cadherin-associated protein α-catenin in force transduction. MTC and TFM studies using α-catenin knockdown cells and knockdown cells rescued with α-catenin mutants demonstrated unambiguously that α-catenin enhances cadherin-mediated traction generation and force sensing. Additionally, I identified the minimal extracellular cadherin domain required for cell-cell adhesion and “outside-in” activation of traction generation and cell spreading.

Studies in Chapter 3, which combined MTC and TFM, identified a new E-Cadherin mechanotransduction mechanism, in addition to the mechanism supported by α-catenin. This new mechanism is not confined to cadherin junctions alone, but triggers signals that globally alter cell mechanics and perturb distal focal adhesions. Using specific fluorescent reporters and chemical inhibitors, we defined key events in the specific signaling pathway that links E-Cadherin force transduction to global changes in cell contractility. These findings expand the current E-Cadherin mechanotransduction model, beyond cell-cell junctions, and elucidate an additional mechanism that integrates integrins with E-Cadherin based mechanotransduction. This finding will enhance
the understanding and treatment of mechanotransduction based diseases and the development of

*in vitro* tissue engineering principles.
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Table of Contents

Chapter 1: Introduction .......................................................................................... 1
  1.1 Mechanotransduction ......................................................................................... 1
  1.2 Epithelial cell adhesion receptors that transduce force ........................................ 3
  1.3 Integrins ............................................................................................................ 4
    1.3.1 Integrin – a cell-matrix adhesive molecule .................................................. 4
    1.3.2 Integrin in Mechanotransduction .................................................................. 5
  1.4 Cadherins ......................................................................................................... 7
    1.4.1 Cadherin - a calcium dependent cell-cell adhesion molecule ......................... 7
    1.4.2 Cadherin in Mechanotransduction .................................................................. 9
  1.5 Global Mechanotransduction ............................................................................ 10
  1.6 Methods for investigating mechanotransduction .............................................. 12
    1.6.1 Traction Force Microscopy (TFM) ................................................................. 12
    1.6.2 Magnetic Twisting Cytometry (MTC) ............................................................ 13
    1.6.3 Fluorescence Resonance Energy Transfer (FRET) ........................................ 14
  1.7 Questions Addressed ......................................................................................... 16
  1.8 References ....................................................................................................... 16

Chapter 2: α-catenin is essential for Cadherin-mediated matrix rigidity sensing, .......... 32
  2.1 Introduction .................................................................................................... 32
  2.2 Materials and Methods .................................................................................... 34
    2.2.1 Cell lines and protein production ................................................................. 34
    2.2.2 Traction Force Microscopy ......................................................................... 35
    2.2.3 Magnetic Twisting Cytometry ..................................................................... 36
    2.2.4 Mammalian cell transfection ....................................................................... 37
    2.2.5 Controls and Inhibitors ................................................................................ 38
    2.2.6 Imaging ....................................................................................................... 38
  2.3 Results ............................................................................................................. 39
    2.3.1 α-Catenin is required for cadherin-based traction generation and rigidity sensing 39
    2.3.2 Epithelial cell spreading on cadherin substrates ........................................... 39
    2.3.3 α-catenin and its vinculin-binding site modulate cadherin-mediated traction forces 40
    2.3.4 Cadherin-mediated adhesion and rigidity sensing requires actomyosin ........... 41
    2.3.5 Role of cytosolic α-catenin in E-Cadherin-mediated mechanosensing ............ 41
  2.4 Discussion ....................................................................................................... 42
  2.5 Table and Figures ........................................................................................... 45
  2.6 References ....................................................................................................... 55

Chapter 3: E-Cadherin-Mediated Force Transduction: not just a local affair ............ 59
  3.1 Introduction .................................................................................................... 59
  3.2 Materials and Methods .................................................................................... 61
    3.2.1 Cell lines and protein production ................................................................. 61
    3.2.2 Mimicking cadherin – cadherin adhesion in vitro ......................................... 62
    3.2.3 Cell area analysis ......................................................................................... 62
    3.2.4 Orienting cadherin ectodomain on polyacrylamide hydrogels ....................... 62
    3.2.5 Magnetic Twisting Cytometry ..................................................................... 63
    3.2.6 Traction Force Microscopy (TFM) and combined MTC/TFM ......................... 64
    3.2.7 Imaging ....................................................................................................... 66
  3.3 Results ............................................................................................................ 69
3.3.1 Cell morphology depends on ligand-functionalized substrates and E-Cadherin signaling ...... 69
3.3.2 E-Cadherin specific mechanotransduction affects global cell contractility .................... 72
3.3.3 E-Cadherin mechanotransduction triggers focal adhesion (FA) remodeling ................ 73
3.3.4 E-Cadherin mediated adaptive stiffening is insensitive to cell prestress..................... 74
3.3.5 E-Cadherin mediated cell stiffening requires integrins ........................................... 75
3.3.6 Global signals are required for E-Cadherin-activated cell stiffening .......................... 76
3.4 Discussion .................................................................................................................. 79
3.5 Table and Figures .................................................................................................... 83
3.6 References ................................................................................................................. 92

Chapter 4: Concluding remarks ...................................................................................... 98

Appendix .......................................................................................................................... 101
A.1 Appendix material and methods ................................................................................. 101
A.2 Appendix cell area analysis ....................................................................................... 102
A.3 Appendix Src analysis .............................................................................................. 108
A.4 Appendix FAK analysis ............................................................................................ 113
Chapter 1: Introduction

1.1 Mechanotransduction

Mechanotransduction is a cellular response to specific mechanical stimuli. The phenomenon can be qualitatively interpreted as transforming mechanical energy into biochemical signals. The process involves force reception (recognition) and response. The biochemical role of proteins (receptors, actomyosin and enzymes) in force sensing and transduction is well accepted. The mechanosensitive elements include but are not limited to membrane proteins (stretch activated ion channels, cadherins, gap junctions, growth factor receptors), cell matrix adhesions (integrins, laminin), cytoskeletal components (actin microfilament, microtubule and intermediate filaments), nuclei (gene expression, ion channels, chromatin and lamins), extracellular matrix proteins (fibronectin, collagen, proteoglycans, basement membrane) and intracellular tension elements and myosin motors. These and more elements work in unison to coordinate mechanosensory responses in cells, tissues, and organs to regulate physiology.

Cells sense their chemical environment using specific cell surface receptors, some of which are also mechanosensors that sense the physical environment. The mechanoreceptors induce biochemical signals causing a conformation change or biochemical activation and recruitment of certain molecules. These mechanically induced events modulate cell functions like migration, proliferation, differentiation, apoptosis, gene expression, and they enable cells to adapt to the environment. Defects or compromised mechanotransduction processes are implicated in muscular dystrophies, cardiomyopathies, atherosclerosis, cancer metastasis and disease progression. Mechanotransduction specific signaling has a critical role in the maintenance of
stressed tissues (muscle, bone, cartilage and vessels\textsuperscript{19}), directing stem cell fate\textsuperscript{17,22} and cell morphogenesis\textsuperscript{23–25}.

A common feature among mechanotransductive related diseases is the disruption in the force transmission between ECM, cytoskeleton or signaling molecules\textsuperscript{19}, which delays or affects the signal threshold in producing the necessary conformation change or activation in the mechanosensory complex. An example of mechanotransduction is ear hair cells (stereocilia), that respond to sound waves (mechanical vibratory force induce displacements producing an electrochemical responses in the stereocilia of hair cells\textsuperscript{26}). The strain (deflection in the serially arranged hair cells) produce tension in the connected tip links\textsuperscript{27} between the organized stereocilia, which open up either mechanically gated ion channels or extend the elastic ankyrin protein repeats\textsuperscript{26}. Defects in tip links extension or mutation in force reception (P-Cadherin 23) causes loss in hearing\textsuperscript{26}. Another example is in blood vessels, where laminar fluid stress experienced at the apical layer of endothelial cells exert an atheroprotective effect on cells, compared with inflammatory responses triggered by turbulent flow within the vessel\textsuperscript{8,19,20}. Cells in contact with blood align in the direction of shear stress, while the orthogonally induced stress (blood pressure) produces strain normal to vessel walls (stretching the vessel diameter). Smooth muscle cells under the endothelial lining in blood vessels align circumferentially and resist vessel stretching\textsuperscript{20}. Altering blood shear or flow can affect cardiac development in an embryo\textsuperscript{28} and cause inflammation and plaques in adults with risk factors (diabetes, obesity, lack of exercise and smoking)\textsuperscript{20}. Atherosclerotic plaques develop in these vessel wall lesions, which narrow the arteries and contribute to vascular diseases and heart failure.

Skeletal and cardiac cells also respond to applied load following Wolff’s principle (increasing the magnitude of applied stress (in a bone), enhances bone growth and remodelling\textsuperscript{29}).
In bone matrices the induced pressure gradients (gravity and compressive force caused by muscle contractions) produce deformations within the bone that drive interstitial fluids through the lacunae-canaliculär network. This fluid flow induces bone remodeling and maintenance. Osteoporosis and loss of bone mass is also affected by altered fluid shear, which causes abnormal nano-mechanical stresses at the receptor or cell level.

1.2 Epithelial cell adhesion receptors that transduce force

A distinguishing feature differentiating multicellular eukaryotic cells from unicellular organisms is their ability to form multicellular aggregates mediated by cell-cell adhesion proteins. These networks depend on the origin and function of the cell in the network. Epithelial cells are classified by their shape (squamous, cuboidal, columnar and transitional epithelium) and structural organization (simple, stratified and pseudostratified). Simple epithelial cells form monolayers, forming cell-cell and cell-matrix adhesions. All epithelial cells can sense external chemical and physical environment using specific membrane-associated receptors. These receptors can be defined as bio-molecular antennas, receiving and transducing external chemical (ligand) and physical (mechanical, electrical) stimuli.

Cells use adhesion receptors to sense the substrate and neighboring cells. The dominant cell adhesion receptors include cadherins, integrins, immunoglobulin like cell Adhesion Molecules (ICAM), and selectins. The cadherin protein family comprises essential cell-cell adhesion proteins, and the integrin family forms cell-extracellular matrix adhesions. This thesis specifically deals with cadherin and integrin.
1.3 Integrins

1.3.1 Integrin – a cell-matrix adhesive molecule

Integrins enable epithelial cells to adhere and spread on extracellular matrices. Each integrin receptor is a cis-heterodimer of α and β transmembrane proteins. There are 18 isoforms of α, and 8 isoforms of β receptor, and each specific α-β heterodimer can recognize multiple matrix proteins. In total there are 24 functional integrin α-β heterodimers that recognize specific extracellular matrix (ECM) molecules. The ECM proteins and their specific peptide domains recognized by integrins include Fibronectin (RGD, LDV), Collagen (GFOGER), Laminin, Vitronectin (RGD), Fibrinogen (RGD), Thrombospondin, and other Glycoproteins.

Among integrin ligands, the RGD peptide is the best characterized and most researched integrin ligand. The RGD domain is found in multiple ECM proteins and is recognized by α5β1, αVβ3, αVβ1, αVβ5, αVβ6, αVβ8, and αIIbβ3. Among ECM proteins Laminin is recognized by α1β1, α2β1, α3β1, α6β1, α7β1 and α6β4, and collagen is recognized by α1β1, α2β1, α3β1, α10β1, and α11β1. The integrin receptor is capable of binding to multiple ECM peptides, and it is the level of surface expression, relative ligand availability, and affinity that determine the subtype of integrin adhesion.

New integrin adhesions (nascent focal adhesions) resulting from lamellopodial membrane extension are very dynamic, and contain talin and paxillin in the nascent complex. Talin serves as an actin tethering site in the nascent adhesion complex which, on maturation, forms Focal complexes (FX). The complex (FX) includes a 100 nm diameter cluster of active Vinculin, focal adhesion kinas (FAK), Src family kinases (SFK), α-actinin, actin related protein 2 - actin related protein 3 complex (Arp2/3), and vasodilator-stimulated phosphoprotein (VASP), in addition to...
nascent adhesion complex proteins and Vinculin, which aid in clustering the integrin molecules. Mature Focal Adhesions (FA) contain active Zyxin and Tensin molecules in junction, in addition to focal complex components 46,47. Initial nascent adhesions have low levels of vinculin and focal adhesion kinase with short actin interconnections. Vinculin activation and consequent binding to talin arranges a connection between integrin junctions and actin cytoskeleton 40,48. Once actin fibers are reinforced with actomyosin-based contractility elements, the nascent adhesion matures and grows into focal adhesion 44,49,50. Maturation of focal adhesion is also associated with the accumulation of α-actinin in the complex that crosslinks actin filaments 51, and activation of RHO GEF’s (LARG, GEF H1), which mediate myosin contraction through ROCK and RhoA kinase 6,52–54.

1.3.2 Integrin in Mechanotransduction

The role of cellular tension modulating integrin function (adhesion affinities 55,56), integrin – actin bonds and integrin - ECM adhesion has been investigated only in the last two decades. Tension and maturation of FX recruits talin and vinculin 57, which modulate integrin mechanotransduction process through actin linkages 58. The integrin mechanosensing process begins with cell adhesion and recognition of a substrate. Lamellipodial extensions occur by actin protrusion and generation of membrane tension (force from actin polymerization extends the membrane followed with myosin contraction). Actin is linked to integrins through talin 59 and vinculin 60,61, and the myosin generated actin tensile force is passed onto integrins which can indirectly induce conformation changes in ECM proteins (FN 62).

Myosin dependent traction forces modulate Integrin’s role in sensing the substrate 63. On soft ECM matrices there is reduced traction force, amount of actin stress fiber generation and
tensile force across ECM-Integrin-Cytoskeleton. The link between cortical actin filament and integrin adhesion is perpendicular to one other (peripheral actin fibers is parallel to cell membrane) and the force transfer is recognized as Focal adhesion clutch. The actin moves backwards (retrograde motion) under high intracellular myosin mediated contractility and transfers tensile force to integrins, which then pull on the ECM coated substrate. On stiff substrates the matrix resists the force from the cell and integrins remain immobile, while on softer substrates the matrix is deformed. The FA complexes exhibit dynamic traction forces, and the role of FAK, paxillin and vinculin in the complex is essential for cell migration and rigidity sensing.

Integrin junction is associated with many kinases. Focal Adhesion Kinase (FAK) is a membrane-associated tyrosine kinase, and its activity is coupled with focal adhesion activity and maturation. FAK is reported to regulate focal adhesion turnover, cell migration, crosstalk with growth factors, and other integrin complexes and its activation is recognized as a multistep process. FAK is transiently activated (autophosphorylated at Y397) by integrin clustering and recruits SH2 binding Src kinase which phosphorylates Y576-577 and increases FAK activity 20-fold. FAK is also reported to be biophysically activated by ECM ligands, substrate rigidity and competes with PIP2 for Myosin activity, as shown by Seong et al., using FAK FRET reporter. FAK can phosphorylate Src, and Src is stated to be active at integrin junctions. Src is a tyrosine kinase and has been reported to be mechanically activated under RGD peptide shear and growth factor stimulation. Rac is associated with lamellipodial extension and actin protrusion at the membrane, while the Rho GEF’s, namely LARG and GEF-H1, are activated indirectly with Rho kinase. ROCK (Rho Kinase) indirectly activates actomyosin-based contractility by inhibiting the phosphatase activity of MLCK phosphatase. Cdc42, another GTPase, reportedly suppresses Rho GTPase activity and relieves intracellular tension, avoiding
stress-induced detachment of cell-cell adhesions \(^{70,77}\). While Rac and Rho activity are reciprocally correlated to contractility and actin dynamics in lamellipodial extension \(^{78}\), Src and FAK kinase activation are reported to activate each other and induce MAPK, Erk, and Akt pathways \(^{65,66}\). These molecules have direct significance in cancer (regulating gene expression), and their kinase specific roles in adhesion imply a biomechanical role in cancer regulation. Cadherin complex also show activity and recruitment of FAK, Src, PI3K, Rac and Rho – and may share similar downstream pathways \(^{70,71,79}\). Chapter 3 focuses on the biomechanical crosstalk between cadherin and integrins.

1.4 Cadherins

1.4.1 Cadherin - a calcium dependent cell-cell adhesion molecule

Cell-cell adhesion is mediated by many adhesive receptors through lateral non-covalent interactions between neighboring cells \(^{34}\). These contacts are dominated by the Cadherin family of receptors forming Adherens junctions, Claudins and Occludins at Tight junctions, Gap and Desmosomal junction proteins \(^{12,36,80–82}\). Cadherin has been established as a cell-cell adhesion molecule, and the development of biomechanical adhesion tools has opened new opportunities to investigate the role of mechanical perturbation on cadherin function \(^{83–86}\).

Cadherin is a single pass transmembrane protein, and the Cadherin subfamily includes classical type 1, atypical class 2, desmosomal, protocadherins, Flamingo/Celsr, and Dachsous and Fat cadherins \(^{24,36,87,88}\). Understanding the mechanical role of epithelial Cadherin (a classical type 1 Cadherin) in mechanotransduction is the major focus of this thesis and is discussed in this chapter.
E-Cadherin has 5 extracellular domains (EC1-5), a transmembrane domain, and a cytosolic domain. The extracellular EC1-5 domain forms active cis and trans adhesion complex in the presence of calcium. The cytosolic domain contains a direct binding site for β catenin, which binds to α-catenin and allows the cadherin complex to indirectly associate with actin filaments. Microtubules are also reported to be associated with adherens junctions through PLEKHA NEZH1 complex. The juxtamembrane portion of cadherin interacts with p120 catenin, while the C-terminal (~76 amino acids) binds β-catenin. The N-terminal end of α-catenin binds β-catenin, and its C-terminal binds with actin, which allows Cadherin to be indirectly linked with the actin cytoskeleton. The ability of α-catenin modulating cadherin function is discussed in chapter 2.

α-catenin has an autoinhibited M region between residues 376-633, which is exposed upon tension and myosin II activation. The 906 amino acid long α-catenin shares functional and sequence homology with vinculin. The major α-catenin domains are the N terminal β-catenin binding domain ((57-146), which overlaps with a self-binding auto-inhibited region (82-264)); M domain (376-633), and C terminal domain (697-906, binds to actin). There exists a serine threonine responsive casein kinase (CK1, CK2) substrate within a phospho-linker domain (M domain and C terminal of α-catenin) that enables cell migration in wound healing. A mechanical role of the phospho-linker domain is discussed in chapter 2.

In vitro studies has shown that α-catenin may form a homodimer in the cytosol, and the molecule has binding sites for actin and actin-binding molecules such as EPLIN, vinculin, afadin, α-actinin, ZO-1, formin and zyxin, which can link more actin fibers to cadherin junctions. In vitro reconstitution of actin-α-catenin-β-catenin-cadherin complex revealed the biochemical inability of α-catenin to simultaneously interact with actin and β-catenin, and
required in vivo experimental validation. In vivo studies using time resolved fluorescence microscopy (dynamic FRET and immunofluorescence)\textsuperscript{12,93}, organism level experiments with drosophila embryos\textsuperscript{99}, and electron tomography experiments\textsuperscript{95}, show that α-catenin can simultaneously interact with actin and β catenin at cadherin junctions under tension. Chapter 2 discusses in vivo mechanosensory and modulatory role of α-catenin at cadherin junctions.

Vinculin is another molecule that aids cadherin adhesive activity. It is well known that vinculin shares sequence and structural homology with α-catenin\textsuperscript{100}, and binds to α catenin at the modulatory M domain\textsuperscript{13,93,94,106}. Vinculin is directly recruited and activated in cadherin mechanotransduction\textsuperscript{12,13,93}. Vinculin is also studied as an actin linker protein at focal adhesions\textsuperscript{60,107}. The internal auto-inhibited domain is similar to that of α-catenin\textsuperscript{108}, which is activated through force and ligand dependent activation\textsuperscript{109}. Vinculin’s role at focal adhesion and cadherin junctions has raised the possibility that vinculin can regulate the crosstalk between adhesion receptors and global mechanotransduction\textsuperscript{13,48,103}.

\subsection*{1.4.2 Cadherin in Mechanotransduction}

Cell-cell adhesions resist intracellular contractile and extracellular stresses on the cell. Like integrins, cadherins are also associated with actin (through α and β catenins); and tension across adherens junctions is modulated by actomyosin mediated contractility\textsuperscript{8,110}. In this thesis, I refer to biochemical changes induced in response to Cadherin specific mechanical stimuli as ‘Cadherin mechanotransduction’. N (neural), E (epithelial) and VE (vascular endothelial) Cadherins exhibited local actin dependent junction remodeling and increased junction stiffness following cadherin-receptor perturbation\textsuperscript{93,111}. Cadherin mechanotransduction signals affecting cell stiffening are reported in chapter 3 in this thesis.
The response of cadherin complexes to intracellular and external stress demonstrates that they are mechanosensors \(^{112,113}\). C2C12 myogenic cells and MDCK epithelial cells on N and E Cadherin functionalized substrates or beads respectively, can sense and respond to forces through cadherins \(^{13,111}\). The latter studies demonstrated that cadherin complexes are ligand specific mechanosensors. \(\alpha\)-catenin, vinculin and actin gets specifically activated and or recruited in response to cadherin specific stiffening response \(^{8,12,85}\).

The downstream molecular cascades regulating Cadherin mechanotransduction are yet to be completely elucidated. In the model explained by Yonemura et al \(^{94}\), \(\alpha\)-catenin unfurls in response to increased tension on cadherin complexes, allowing actin engagement through vinculin recruitment \(^{12,94}\). Investigations of in vitro perturbation of E-Cadherin in F9 cells, and fluorescence images of an \(\alpha\)-catenin FRET sensor expressed in DLD1-R2/7 cells showed that \(\alpha\)-catenin, vinculin and tension are required for cadherin-mediated stiffening response \(^{12,13,95,114}\). Calcium, Cadherin ligand-specific adhesion, clustering, and shear activate downstream signaling through Rho GTPase (Rac, Rho, Cdc42) Src, PI3K, FAK family of kinases \(^{6,71,77,115–117}\), and respective protein tyrosine phosphatases \(^{118–120}\). The activity of these kinases depends on the balance of respective activating and inactivating pathways (kinases and phosphatases) in combination with spatial activity \(^{53,77,116,120–122}\). An example of cadherin mechanotransductive disease is VE-Cadherin in endothelial cells, which is involved in cardiomyopathies and lung injury \(^{8,52,113,123}\). VE-Cadherin dominates cell-cell contact and regulate vascular permeability \(^{124,125}\).

### 1.5 Global Mechanotransduction

Typical experimental tools and approaches used for mechanotransduction studies relied on analyzing biochemical changes at the vicinity of mechanical perturbation. Although the readout is
measured at the site of force, the specificity of response and the ensuing biochemical changes define mechanotransduction. The term global mechanotransduction includes larger phenotypic cell-wide changes that occur away from the site of shear application. Examples include change in cell polarity in response to fluid shear in endothelial cells \(^{126}\), shear induced opening of calcium ion channels far from the site of shear \(^{127}\), change in apical PECAM-1 stiffening response by altering basal integrin adhesions \(^{6}\) and global RhoA activation under PECAM-1 shear \(^{128}\). The change in mechanical force balance between tensile (actin and intermediary filaments) and compressive (microtubule) elements in the cell (that resist and or respond to mechanical stresses \(^2,^{129}\)) is liable to induce global mechanical changes in the cell \(^{130}\). Collins et al., had discussed that PECAM-1 induced mechanotransduction required new integrin adhesions suggesting a biochemical phenomenon \(^6\). PECAM-1 was stimulated at the apical membrane, and the observed stiffening response required and induced focal adhesions in the basal membrane. These spatially and functionally detached adhesive receptors (PECAM-1 and integrins) were mechanically interacting to produce PECAM dependent stiffening response. They identified PI3K to be activated downstream of PECAM-1 activation and Rho GEF’s (LARG and GEFH-1) downstream of integrin stimulation. An other example of global mechanotransduction is flow induced stresses in murine kidney cells that activate calcium influx through opening of ion channels \(^{127}\). Polycystin 1 is localized at the base of the primary cilium, which undergoes conformational changes under shear and opens up the calcium ion channel (Polycystin 2) \(^{127}\). The response is amplified by further release of intracellular calcium stores. The medium of force transduction between these spatially isolated receptors was described in a stress-focusing model by Hu et al., using fluorescently labeled fiduciary markers in the cytosol of Human airway smooth muscle cells \(^{130}\). Hu et al., identified small intracellular cytosolic displacements (mitochondrial movements) and focal adhesions
movements in response to apical shear dependent on preexisting cytoskeletal cellular elements. In chapter 3, we combined MTC and TFM with fluorescence microscopy and report a biochemically regulated increase in traction force and focal adhesions in the cell, and at least with results from epithelial cells don’t completely approve the stress focusing model, but rather suggest a global increase in traction with new integrin adhesions.

1.6 Methods for investigating mechanotransduction

To study the biophysical parameters of cell mechanics, we use custom-built magnetic twisting cytometry (MTC) and traction force microscopy (TFM) with respective analysis programs in MATLAB. The aim of these tools is to apply controlled forces (dynamic or static) on precise cellular receptors and acquire the biochemical response in live cells.

1.6.1 Traction Force Microscopy (TFM)

Adherent cells exert forces (traction) on their substrate by holding onto specific ECM proteins during spreading, migration, contraction, and invasion. The biochemical mechanism of traction generation involves a complex interplay among adhesion molecules, cytoskeletal elements, and motor proteins. The traction is measured by averaging the magnitude of forces that produce substrate displacement in a unit area. The measured traction is the local force per unit area (shear in Pa), and is calculated using Fourier Transform Traction Cytometry (FTTC). The displacement in the z direction is ignored, while displacement in the x and y plane is considered after assuming a semi-infinite medium substrate — lateral cell dimensions and displacements are smaller compared to gel thickness.
In this thesis, the tractions developed from single epithelial cells are quantified and compared. The cells are, on average, 30 μm in diameter when grown on collagen functionalized flexible polyacrylamide gels with a thickness of ~200 μm. The gels are embedded with fluorescent beads that serve as displacement markers in the gel. The bead positions (ux, uy) when bound to the cell are known by pixelating the 1032*1024 image into an array of 32 by 32 blocks. The new bead positions (ux2, uy2) after removing the attached cell is acquired, and the displacement in each bead position is measured (Ux, Uy). The traction stresses (σ) are related to displacements (u) by a tensorial version of Hooke’s law \(^{133}\) using the known elastic moduli (E) and Poisson’s ratio (υ) of the gel. For experimental purposes, the chosen Poisson ratio is 0.48 for polyacrylamide gels \(^{134,135}\). For proper data analysis, an image-based correction factor is included to overcome any in plane error (stage vibration in x-y plane) in MATLAB.

1.6.2 Magnetic Twisting Cytometry (MTC)

Dynamic shear force is applied on epithelial cells using ferromagnetic beads under a magnetic field. The beads are magnetized under the MTC setup. To enable shear through specific receptors, the carboxylated ferromagnetic bead is covalently coupled to cell receptor ligands (free amines) using EDC NHS chemistry \(^{12,93}\) and is allowed to adhere onto cells. The optimized coupling chemistry is explained in the material and methods section of each chapter.

The MTC system consists of a Helmholtz coil set around the mechanical stage. The current flowing through the coil is controlled using an electronic controller, which modulates the magnetic field. The controller and the accompanied software setup enable us to regulate the amplitude and frequency of applied shear onto cells. To magnetize the beads, a strong but brief magnetic pulse of 1000 G is applied horizontally (horizontal Helmholtz coil) in the plane of the microscope.
mechanical stage, which aligns the bead’s magnetic dipoles. A second oscillating magnetic field (0-70G) is applied in the orthogonal direction (vertical Helmholtz coil), which twists the cell-bound beads. The bead twist applies forces on cells through the attached cell receptors. These forces apply focused shear at the bead-cell junction (the area of contact between the cell and the bead). The produced bead displacement (> 30 nm) is tracked using a bead tracking software from which the shear stress is calculated. Knowing the beads’ magnetic moment and neglecting viscous fraction ($G = G' + iG''$, where $G'$ is elastic fraction and $G''$ is viscous fraction), we estimate the rigidity modulus and % change of stiffness at the bead-cell junction. The relative change in elasticity indicates the dynamic cell response to external shear.

1.6.3 Fluorescence Resonance Energy Transfer (FRET)

In physical terms, FRET is the phenomenon occurring when 2 different molecular moieties have overlapping emission and absorptions bands and are engaged in close proximity (within the Förster’s distance, R) with proper orientation ($\kappa$) that enables a non-radiative energy transfer between the 2 molecules. The efficiency of energy transfer varies greatly as the sixth power of distance (Förster distance).

$$\text{FRET efficiency} = \frac{1}{\left(1 + (R/R_0)^6\right)}$$

where,

$R$ is the Förster’s distance, $R_0$ is the Förster’s distance at 50% FRET efficiency and can be theoretically calculated for every FRET pair.

$$R_0 = 8.79 \times 10^{-5} \times Q_d \times \eta^{-4} \times \kappa \times 2J,$$

where $Q_d$ is quantum efficiency, $\eta$ is refractive index of medium, $\kappa$ is orientation between the fluorophores and J is the spectral overlap.\textsuperscript{136}
The efficiency of FRET energy transfer decreases with the sixth power of the distance between the probes \((1/r^6)\) and is used as a molecular ruler. In biology, FRET is used to investigate structural change in orientation \((\kappa)\) or distance \((R)\), and or biochemical activity changes (change in conformation and function). For an effective FRET readout, the FRET pairs are engineered to have maximal quantum yields of donor \((Q_d)\) and overlapping emission and absorption bands \((J)\) between the donor and acceptor molecule. CFP and YFP proteins are engineered for maximal \(Q_d\), with overlapping bands \((J)\) and are named ECFP (enhanced CFP) and YPet. *In vivo* FRET microscopic techniques (not single molecule conformation change experiments), the readout is reported in binary terms with temporal resolution — e.g. high FRET vs low FRET and or active or inactive conformation — and is applied as an *in vivo* activity biosensor in live cells.

The biosensors discussed in this chapter 3 of this thesis (Src and FAK FRET sensor) are *in vivo* functional reporters of enzyme activity. The respective reporters contain a kinase substrate flanked by a phosphorylated substrate-binding domain (e.g. SH2 for Src kinase) followed by CFP and YFP at the ends of the peptide. Under basal conditions, either reporter is FRET active (CFP is in close proximity to YFP). In the case of Src FRET reporter\(^{11}\), Src phosphorylates the Src kinase substrate (p130 CAS substrate) built within the CFP and YFP domain, causing a conformation change within the reporter and increases the distance between CFP and YFP (the SH2 domain is brought closer to the phosphorylated Src substrate linker region), resulting in a loss of FRET. This change in emission is measured using respective filter cubes (CFP and YFP emission) and indirectly quantifies the level of Src activity. By ratio metric (CFP/YFP) analysis of samples, with internal pre-stimuli normalization controls, and background correction, the heterogeneity in reporter expression from cell to cell and junction to junction is avoided.
1.7 Questions Addressed

This thesis aims at understanding the biochemical and physical parameters that control and limit cadherin mechanotransduction in epithelial cells. Exploiting traction force microscopy on genetically engineered epithelial cells (knock down, rescued and wild type cells), I tested the hypothesis of the immediate role of α-catenin in cadherin mechanosensing (Chapter 2). Using 5 different cell lines and 3 different α-catenin rescued cells; I showed that the activity of α-catenin enhances cadherin mediated rigidity sensing and traction generation. The results aid in addressing the in vivo mechanosensory role of α-catenin at cadherin junctions\(^{137–139}\).

I further identified that the previously stated cadherin mechanosensing and mechanotransduction events is not limited to cadherin junction alone, but is a long-range phenomenon affecting focal adhesions. By adapting traction force microscopy with magnetic twisting cytometry, supplemented with fluorescence imaging, I show that integrins and cadherins form a force-sensitive biochemical network that transduces forces beyond cell-cell adhesions. This result discusses a new global E-Cadherin mechanotransduction pathway beyond cell-cell junction proteins.

1.8 References


12. Kim, T.-J. *et al.* Dynamic Visualization of α-Catenin Reveals Rapid, Reversible Conformation


61. Grashoff, C. et al. Measuring mechanical tension across vinculin reveals regulation of focal


89. Kim, S. A., Tai, C.-Y., Mok, L.-P., Mosser, E. A. & Schuman, E. M. Calcium-dependent


Chapter 2: α-catenin is essential for Cadherin-mediated matrix rigidity sensing

2.1 Introduction

Cadherins are cell adhesion receptors that biochemically connect cells in tissue, and cadherin complexes were recently demonstrated to be force sensors. The absence or disruption of cadherin function contributes to cancer metastasis and developmental dysfunctions. The catenin family of proteins (α, β and p120 catenin) biochemically influence cadherin function, dynamics, signaling and turnover. Experimental evidence showed that cadherins require actin cytoskeleton to effectively transduce force, and that α-catenin was a force transducer in the complex. However, in vivo biochemical evidence that α-catenin could simultaneously associate with actin and cadherin was under debate, until very recently.

This chapter addresses the mechanosensory role of α-catenin in cadherin-mediated traction generation and rigidity sensing. These results show unambiguously, using magnetic twisting cytometry (MTC) and traction force microscopy (TFM), as well as α-catenin KD cells and KD cells rescued with α-catenin-GFP (rescued), α-catenin phosphomutants (5SE and 5SA) or cells transfected with an α-catenin FRET sensor, that α-catenin is required for cadherin-mediated traction generation and rigidity sensing. Using cytoskeletal inhibitors, I further showed that α-catenin-dependent, E-Cadherin traction generation requires an intact actomyosin cytoskeleton.

(First authorship is underlined and * denotes equal contribution)


Bioinformatic (NetPhos) and mass spectroscopic analyses showed that between the α-catenin M domain and the C terminus, that specific serine and threonine residues (within a phospholinker domain, P-linker (629 - 667)) are phosphorylated in vivo. The phosphorylation status of specific amino acids within the P-linker was postulated to significantly affect cell migration and cell-cell adhesion strength. Specifically, four serine and one threonine residues in the phospholinker domain (S641, S652, S654, S655 and T658) were mutated to alanine (5SA) or glutamate (5SE). The 5SA α-catenin mutant served as a constitutive non-phosphorylated phospholinker domain, and conversely 5SE served as a phosphomimetic mutant. The phosphomutants have active β catenin and actin binding sites, along with the modulatory M domain, and are recruited to cadherin junctions. However, these mutants didn’t support any cell stiffening in response to mechanical perturbation of cadherin adhesions. The phosphorylation event may activate the mechanotransduction conformation specific α-catenin structure and this hypothesis is supported by α-catenin conformation FRET sensor that unfurls specifically under E-Cadherin shear.

Vinculin is an actin binding molecule, that is associated with both focal adhesions and adherens junctions. Vinculin shares functional sequence homology with α-catenin in actin binding domain and M domain. The vinculin binding site (VBS) in α-catenin and its effect in α-catenin modulated traction generation were investigated. Studies used R2/7 cells rescued with GFP-tagged α-catenin (Rescued) or with α-catenin in which the VBS was exchanged with the homologous sequence in vinculin (ΔVBS). In the absence of Vinculin recruitment to α-catenin, the traction generated is significantly lower implying a mechanical role for Vinculin in α-catenin mediated E-Cadherin traction generation. These studies show that α-catenin is required for E-Cadherin rigidity sensing and traction generation.
2.2 Materials and Methods

2.2.1 Cell lines and protein production

MCF7 (Michigan Cancer Foundation 7) breast epithelial carcinoma cells were from ATCC; WT MDCK (Madine Darby Canine Kidney) cells were from ATCC; α-catenin KD MDCK cells were from James Nelson (Stanford), and MDCK KD cells rescued with α-catenin mutants were from Cara Gottardi (Northwestern University, Chicago). WT DLD1, DLD1-R2/7 (human intestinal carcinoma), and DLD1-R2/7 cells rescued with α-catenin-GFP or its mutants were from Johan De Rooij (Hubrecht University, Netherlands). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM 4.5 g/L glucose) supplemented with 10% v/v fetal bovine serum (FBS), 1mM Sodium Pyruvate, and 1% v/v penicillin/streptomycin (Corning Cell Grow, Manassas, VA). The respective MDCK α-catenin mutants (KD and rescued cells) were grown in 600 μg/ml of G418 antibiotic with 10% v/v FBS in regular DMEM, and the DLD1-R2/7 α-catenin mutants (α-catenin rescued cells) were grown in regular DMEM growth media supplemented with 10% v/v fetal bovine serum with 6 μg/ml of puromycin. The features of the different cell lines, the respective mutations, and their growth conditions are summarized in Table 2.1.

The soluble E-Cadherin ectodomain tagged with C-terminal Fc domain of human IgG (Ecad-Fc) was stably expressed in engineered human embryonic kidney cells (HEK293T). The protein was purified from cell-harvested media by affinity chromatography with Protein A Affigel (Bio Rad, Hercules, CA). The purified protein was stored in 100 mM HEPES, 100 mM NaCl, 5 mM CaCl₂ buffer and kept at -80°C.
2.2.2 Traction Force Microscopy

Traction force microscopy (TFM) measurements used polyacrylamide hydrogels with Young’s moduli of 1, 9 or 34 kPa, embedded 0.2 μm fluorescent microspheres (Molecular Probes, Eugene, OR) \(^{28,29}\). To immobilize proteins, the hydrogels were chemically activated by bathing the entire gel surface with Sulfo-SANPAH (0.5-1 mg/ml in 100 mM HEPES buffer) (Pierce Biotech, Rockford IL). The gels were irradiated at 365 nm for 8 minutes and washed with excess 100 mM HEPES buffer, by shaking for 5 minutes. The Sulfo-SANPAH and UV treatment was repeated for surface activation. To immobilize Fc-tagged E-Cadherin extracellular domains, 0.2 mg/ml anti-human Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was incubated with the Sulfo-SANPAH activated gels and left overnight at 4°C. The substrates were rinsed to remove unbound antibody and then incubated with E-Cad-Fc (0.2 mg/ml in 100 mM HEPES, 100 mM NaCl, 5 mM CaCl\(_2\), pH 8) for 3-4 hours at 4°C. The orientation and specificity of cell attachment to gels were controlled by orienting the N terminal of E-Cadherin-Fc outward, while the C terminal Fc portion binds to anti-Fc functionalized gel, enabling trans dimerization between E-Cadherin Fc functionalized substrate and endogenously expressed E-Cadherin at the cell surface. By modifying substrate rigidity to match cell intracellular tension (8.8 kPa is muscle cell and 34 kPa is osteoblast), the design mimics static cadherin-mediated cell-cell adhesion (Figure 2.4).

After protein immobilization on gels, the substrates were rinsed twice with 1X phosphate buffered saline (PBS) before incubating with 1% w/v BSA (in 1X PBS) for 20 minutes. In all cases, the modified gels were sterilized by UV illumination (365 nm) for at least 15 minutes before seeding cells on the gels. For E-Cadherin functionalized substrates, the cells were seeded in the presence of integrin blocking antibodies (20 μg/ml for GOH3 and 1:20 dilution of AIIB2). Anti-integrin α6 antibody GOH3 was from Santa Cruz technologies, and AIIB2 antibody was harvested.
from an antibody-secreting hybridoma cell line (a gift from Johan De Rooij, Hubrecht Institute, Netherlands).

Cells were harvested from 25 cm\(^2\) tissue culture flasks with 3.5 mM EDTA in PBS containing 1% w/v BSA\(^1\). Cells were centrifuged and resuspended in DMEM (0.5% v/v FBS) growth medium. About 5000-8000 cells/ml were seeded onto protein-functionalized hydrogels, where they were allowed to adhere and spread for 6 hours at 37 °C in a 5% CO\(_2\) environment. The root mean square (RMS) basal traction force (BTF) was determined from fiduciary bead displacements induced by cells, relative to the traction-free bead positions after cell removal (using cell lysis buffer, 1% SDS, 3.5 mM EDTA, 1% BSA in PBS)\(^2,3,12,28\). The mean and standard error of the mean (s.e.m) are reported. P-values were calculated from two-tailed Student’s t-tests using Microsoft Excel.

2.2.3 Magnetic Twisting Cytometry

Magnetic twisting cytometry was performed as described previously\(^2,12,30\) and in chapter 1. The protein-functionalized ferromagnetic beads bound to the cell surface were magnetized parallel to the cell plane by a 0.1 Tesla magnetic field, and torque on the beads was generated by an applied oscillating orthogonal field of 60 Gauss at a frequency of 0.3 Hz. The bead displacement was tracked using a Leica Microscope 20X 0.6 NA, a charged coupled device (Hamamatsu), and a bead tracking software; and the magnitude of the corresponding bead displacements reflects the complex moduli of bead-cell junctions. The latter is a function of the viscous and elastic storage moduli. In these studies, the magnitude of 60 Gauss corresponds to a shear stress of 7.2 Pascal. The moduli follow a log normal distribution, from which we obtained the mean and standard deviation. Comparisons of junction stiffness measured under different conditions were calculated.
from two-tailed Student t-tests using Microsoft Excel, and \( p \) values < 0.05 defined statistically significant differences, at the 95% confidence level.

About 140 μg of carboxyl terminated ferromagnetic beads (1% w/v, 4.4 - 4.9 μm diameter (Spherotek, Lake forest IL) suspended in 300 μL of MES buffer (50 mM, 2-[morpholino]ethanesulfonic acid, 100 mM NaCl, pH 5), were activated with 60 mg each of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-Hydroxy Succinimide (NHS, Thermo Scientific; Rockford, IL). The bead solution was shaken for 15 minutes at room temperature, centrifuged, and then suspended in 300 μL of coupling buffer (100 mM HEPES, 100 mM NaCl and 5 mM CaCl\(_2\) pH 8). To this we added 12 μg of protein (stock concentration of 1 mg/ml) to give a ratio of ~85 μg of protein (E-Cadherin Fc) per mg of ferromagnetic beads, and incubated the protein/bead suspension for 2 hours on a shaker at 4°C. The coupling reaction was quenched with 60 μL of quenching buffer (100 mM Tris, 20 mM NaCl, 5 mM CaCl\(_2\), pH 8), followed by shaking for 30 minutes at room temperature. The beads were then centrifuged and suspended in 400 μL of coupling buffer.

2.2.4 Mammalian cell transfection

Mammalian cells (MDCK) cells were transfected with specified α-catenin conformation sensor plasmid \(^{13}\) using commercially available liposome-based techniques. The cells were grown on collagen-coated 35mm dishes for a day before transfection was initiated. The transfection protocols were from Fugene HD (Promega, WI) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The chosen ratio of DNA (μg) to Liposome reagent (μl) was between 1:3 and 5:7, and transfections were conducted in Opti-MEM®I with reduced serum (Life Technologies). A 2:3
ratio of DNA (in μg): Lipofectamine (in μL) resulted in 40% transfection efficiency in MDCK cells.

2.2.5 Controls and Inhibitors

Biochemical controls included treatment with 50 μM of blebbistatin for 1 hour to inhibit myosin II, and treatment with 4 μM of Cytochalasin D for 10 minutes abolished actin filaments.

2.2.6 Imaging

The respective α-catenin KD plasmids and KD cells rescued with mouse α-catenin-GFP plasmids were stably transfected and expressed in MDCK cells. Prior to measurements, the cells were checked for GFP expression, and only those cells that were GFP positive were used for traction and MTC analyses. For MDCK KD cells rescued with the α-catenin FRET sensor, CFP expression was checked by monitoring the cyan emission, thereby ensuring transfection with the sensor.

Cells were cultured on E-Cadherin-functionalized hydrogels in DMEM containing 0.5% FBS. The low FBS concentration was used to decrease serum and growth factor induced signaling, and to prevent adhesion to adsorbed serum proteins such as vitronectin. The inclusion of integrin blocking antibodies (GOH3 and AIIB2) prevented integrin interference.

Traction measurements were done within 6 hours of seeding the cells. The transfected cells were imaged with an inverted Leica microscope coupled with a Dual View system (Optical Insights) to acquire CFP and YFP images with a CCD camera (Hamamatsu). The filter sets included CFP (excitation, S430/25, emission S470/30); YFP (excitation, S500/20, emission S535/30). The emission filter set used a 515-nm dichroic mirror to split the two emission images.
The exposure time was set to 300 ms. The RMS (Root Mean Square) traction was calculated as previously explained in chapter 1, and the mean, s.e.m and two-tailed t-test were analyzed in Microsoft Excel.

2.3 Results

2.3.1 α-Catenin is required for cadherin-based traction generation and rigidity sensing

Cell responses to an oscillating mechanical perturbation of E-Cadherin complexes was measured during application of an oscillating (0.3 Hz) 60 Gauss (7.2 Pa) field to E-Cadherin-coated magnetic beads bound to MDCK cells. We observed a 19 ± 0.1 and 9 ± 0.01 % increase in the stiffness of MDCK WT and MDCK KD α-catenin Rescued (MDCK Rescued) cells. The stiffening response obtained with MDCK α-catenin KD cell (MDCK KD) was -16 ± 0.03 %, and mutant α-catenin 5SA and 5SE rescued cells 16 (-7 ± 0.02 and -3 ± 0.01 % for cells expressing the 5SA and 5SE mutants), indicated that the loss of α-catenin or the phosphorylation mutants ablated E-Cadherin mediated force transduction (Fig 2.1).

2.3.2 Epithelial cell spreading on cadherin substrates

E-Cadherin bond shear mimics cell-cell tugging forces at the region of bead-cell contact. Cadherin mediated adhesion of cells on static E-Cadherin substrates mimic cell-cell lateral adhesions. We designed in vitro lateral E-Cadherin junctions by growing cells on covalently functionalized E-Cadherin hydrogels. Under conditions of reduced serum and integrin blocking antibodies GOH3 and AIIB2 31, MDCK and DLD1 cells displayed a circular morphology and did not show any spreading or lamellipodia-like extension 32 (results discussed further in chapter 3). Cell maps
(traction maps) of respective epithelial lines (Fig. 2.2) show the circular shape of the cells representative of cadherin mediated contact inhibition \textsuperscript{33}.

### 2.3.3 α-catenin and its vinculin-binding site modulate cadherin-mediated traction forces

The α-catenin-dependence of E-Cadherin-mediated traction generation was demonstrated in TFM measurements of MDCK α-catenin KD and MDCK Rescued cells on E-Cadherin modified hydrogels with soft (1.1 kPa) and hard substrate (34 kPa) rigidities. Loss of α-catenin significantly decreased the root mean square (RMS) traction stresses (Pa) exerted by MDCK KD cells on 1 kPa (\( p = 0.02, n = 4 \)), and 34 kPa (\( p = 0.002, n > 10 \)) gels compared with MDCK Rescued (α-catenin rescued) cells (Fig. 2.4). As observed with N-cadherin-modified substrates \textsuperscript{12,34}, the traction generated by MDCK Rescued cells on E-cad-Fc-coated substrata decreased from 162 ± 11 Pa on 34 kPa gels to 43 ± 9 Pa on 1 kPa gels (\( p < 0.001 \)) (Fig. 2.4). Loss of α-catenin significantly reduced the RMS traction force by 39% on 34 kPa gels, from 162 ± 11 Pa for MDCK Rescued cells to 117 ± 8 Pa for MDCK KD cells (\( p = 0.002 \)), and by 70% on 1 kPa gels (43 ± 9 Pa and 13 ± 4 Pa, respectively) (Fig. 2.4). Interestingly, loss of α-catenin in MDCK KD cells reduced, but did not eliminate the rigidity-dependence of the traction forces.

Similarly, traction exerted by DLD-1, R2/7 and R2/7 α-catenin Rescued cells on E-cad-Fc-coated gels were compared on 1, 9 and 34 kPa gels (Fig. 2.5). The DLD-1 and R2/7-Rescued cells exerted greater traction on rigid gels than on softer gels. In addition, the absence of α-catenin (R2/7) reduced the generated traction relative to DLD-1 and R2/7 Rescued cells by 23% on 9 kPa (\( p = 0.03, n > 10 \)) and 34% (\( p < 0.02, n > 10 \)) on 34 kPa gels. The rigidity sensing of R2/7 DVBS and R2/7 cells was similar on 34 kPa (\( p = 0.4, n > 10 \)) and 8.8 kPa (\( p = 0.6, n > 9 \)) gels (Fig. 2.5).
As observed in MDCK KD cells, the lack of α-catenin in R2/7 cells did not eliminate the dependence of traction on substrate rigidity.

Vinculin is associated with both focal adhesions \(^{17-19}\) and adherens junctions \(^{2,20-22}\). The effect of vinculin at cadherin junctions and its effect in α-catenin modulated traction generation were investigated using α-catenin lacking vinculin binding site (R2/7 cells rescued with α-catenin ΔVBS mutant). The traction generated by these cells (121 ± 7), showed significantly lowered traction values compared to DLD1 (174 ± 21) and R2/7 rescued (163 ± 16) cells\((p < 0.05, \text{Fig. 2.5})\).

### 2.3.4 Cadherin-mediated adhesion and rigidity sensing requires actomyosin

We also measured the effect of actomyosin contractility on cadherin traction generation, using chemical inhibitors. Treating MDCK KD and MDCK Rescued cells with 4 μM cytochalasin D and 50 μM blebbistatin, which individually disrupt actin and inhibit myosin II, respectively, reduced the E-Cadherin-mediated traction generation to 94 ± 11 Pa and 95 ± 8 Pa; and 90 ± 11 Pa and 108 ± 6 Pa respectively (Fig. 2.6). The higher traction exerted by MDCK Rescued cells, compared to MDCK KD cells, was abolished by actin disruption of myosin inhibition. This shows that actomyosin plays a significant role in E-Cadherin mediated traction generation.

### 2.3.5 Role of cytosolic α-catenin in E-Cadherin-mediated mechanosensing

To distinguish the role of cytosolic and membrane bound α-catenin in cadherin mechanosensing, we compared traction between MDCK Rescued and MDCK βcat ActA cells. MDCK βcat ActA cells express mitochondrial targeting protein ActA (from \textit{Listeria monocytogenes}) fused with the
minimal α-catenin binding domain of β-catenin and RFP. βcat ActA cells have a portion of cytosolic α-catenin targeted to mitochondria, while the membrane bound α-catenin population remained unaffected. The stiffening response in MDCK β cat ActA cells was similar to MDCK WT cells, but sequestering cytosolic α-catenin in MDCK cells (β cat Act A cells) reduced the generated traction by 31%, from 162 ± 11 Pa in MDCK Rescued cells to 111 ± 10 Pa in MDCK βcat ActA cells (p < 0.01, Fig. 2.7). The traction generated by βcat ActA cells was not significantly different from MDCK KD cells (p = 0.4, Fig. 2.7).

The effect of α-catenin on E-Cadherin-mediated mechanosensing, was further evaluated using a transiently-expressed α-catenin conformation sensor. MDCK KD cells were transfected with the FRET-based α-catenin conformation sensor, where the modulatory M domain is flanked by the ECFP and YFP, FRET pair. The generated traction by MDCK KD cells + α-catenin sensor (206 ± 32 Pa) was compared with MDCK KD (117 ± 8 Pa), MDCK α-catenin Rescue (162 ± 11) and MDCK wild type (201 ± 48 Pa) cells (Fig. 2.8). The results showed that MDCK cells rescued with the α-catenin sensor generated significantly greater traction compared with MDCK KD cells (p < 0.001), and that the magnitude was similar to wild type MDCK WT cells (Fig. 2.8). This result further showed, using a different α-catenin rescue construct, that α-catenin can rescue E-Cadherin traction generation phenomenon utilizing actomyosin related intracellular tension.

2.4 Discussion

TFM measurements demonstrated the modulatory role of α-catenin in cadherin-based mechanosensing. Because α-catenin is crucial for acute mechanotransduction, one might also expect it to mediate traction generation at cadherin adhesions and is shown here (Figs. 2.4 and 2.5). It is somewhat surprising that loss of α-catenin reduced but did not ablate the dependence of
cadherin-based traction forces on substratum stiffness. Using engineered MDCK (non-cancerous canine kidney cell) and DLD1 (human intestinal cell), we observed that \(\alpha\)-catenin rescued cells showed higher traction in comparison to \(\alpha\)-catenin KD cells. On rescuing MDCK KD cells with phosphomimic \(\alpha\)-catenin mutants (5SA and 5SE mutants), the stiffening response was not restored (Fig. 2.3). This result indicates that \(\alpha\)-catenin is not just a physical linker between cadherin and actin, but is dynamically involved in the mechanosensing process as shown using \(\alpha\)-catenin conformation FRET sensor \(^{13}\). The \(\alpha\)-catenin lacking vinculin traction (DVBS) results suggest a mechanosensory role of vinculin at cadherin junction that acts through \(\alpha\)-catenin, which is also supported with \(\alpha\) conformation FRET sensor study \(^{13}\). Comparing \(\alpha\)-catenin rescue results for E-Cadherin stiffening response \(^{3}\), and shear specific unfurling \(^{13}\), the \textit{in vivo} biochemical role of \(\alpha\)-catenin in cadherin mechanotransduction is further elucidated.

To investigate the role of tension in cadherin mechanosensing, the effect of inhibitors (cytochalasin D and blebbistatin) on MDCK traction was compared with untreated cells. Inhibiting intracellular contractility reduced traction generated by MDCK Rescued cells (Fig. 2.8). These results show that \(\alpha\)-catenin mediated substrate sensing requires active cytoskeleton to generate traction, and indicates the generated traction is not an E-Cadherin ligand or membrane tension regulated effect (as the amount of cadherin coated onto the substrate was the same for all conditions and the cell area were similar). The difference in traction can be attributed to an altered ability to sense the substrate. Rigidity sensing would require mechanical connectivity between the substratum and cytoskeleton; besides \(\alpha\)-catenin, possible links between cadherins and the cytoskeleton include the microtubule–Nezha–PLEKHA7 complex \(^{36}\) and the vinculin–\(\beta\)-catenin complex \(^{22}\). Unraveling other mechanisms regulating cell pre-stress is beyond the scope of this
thesis, but α-catenin clearly cooperates to regulate cell contractility in cadherin-controlled traction generation.
### 2.5 Table and Figures

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Species and Lineage</th>
<th>Information</th>
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<tbody>
<tr>
<td><strong>MDCK</strong></td>
<td>Dog kidney epithelial cells, epithelial</td>
<td>Wild type cells (WT) with endogenous α-catenin. Grown in regular DMEM (with 10% FBS, 1mM Sodium Pyruvate, and 1% v/v, penicillin/streptomycin)</td>
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<td><strong>MDCK KD</strong></td>
<td>Dog Kidney cells, epithelial</td>
<td>Stable MDCK cell line with endogenous α-catenin knocked down using shRNA with GFP label. Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate, 1% v/v penicillin/streptomycin and 600 μg/ml of G418)</td>
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<tr>
<td><strong>MDCK Rescue</strong> or <strong>MDCK α-catenin rescue</strong></td>
<td>Dog Kidney cells, epithelial</td>
<td>Stable MDCK KD cell line (with α-catenin knocked down using shRNA) rescued with mouse α-catenin GFP. Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate, 1% v/v penicillin/streptomycin and 600 μg/ml of G418)</td>
</tr>
<tr>
<td><strong>MDCK Mutant 5SA and 5SE</strong></td>
<td>Dog Kidney cells, epithelial</td>
<td>Stable MDCK KD cell line (with α-catenin knocked down using shRNA) rescued with mutant α-catenin (5SE and 5SA mutations) with GFP label. Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate, 1% v/v penicillin/streptomycin and 600 μg/ml of G418)</td>
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Table 2.1 (cont.)
<table>
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<tr>
<th>Cell Line Description</th>
<th>Characteristics</th>
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<tr>
<td><strong>MDCK KD + α-catenin</strong></td>
<td>Dog Kidney cells, epithelial</td>
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<tr>
<td><strong>FRET reporter</strong></td>
<td>Stable MDCK KD cell line (α-catenin knocked down using shRNA) rescued with full-length α-catenin FRET reporter having ECFP and YPet label. Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate, 1% v/v penicillin/streptomycin)</td>
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<td><strong>DLD1</strong></td>
<td>Human intestinal cell, epithelial</td>
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<td></td>
<td>Human intestinal wild type cells (WT). Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate and 1% v/v penicillin/streptomycin)</td>
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<tr>
<td><strong>DLD1- R2/7</strong></td>
<td>Human intestinal cell, epithelial</td>
</tr>
<tr>
<td></td>
<td>A wild type subclone of DLD1 cells lacking α-catenin. Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate and 1% v/v penicillin/streptomycin)</td>
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<tr>
<td><strong>DLD1-R2/7 rescued</strong> and <strong>R2/7 DVBS</strong></td>
<td>Human intestinal cell, epithelial</td>
</tr>
<tr>
<td></td>
<td>Stable DLD1-R2/7 cells lacking human α-catenin rescued with GFP labeled mouse α-catenin (R2/7 rescued) or α-catenin lacking vinculin binding site (R2/7 DVBS). Grown in DMEM (with 10% FBS, 1mM Sodium Pyruvate, 1% v/v penicillin/streptomycin and 6 μg/ml of puromycin)</td>
</tr>
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</table>

Table 2.1 Table explaining the characteristics of the different cell lines and their corresponding mutations and growth conditions
Figure 2.1. Stiffening response of MDCK wild type is compared with α-catenin KD, 5SA, 5SE and rescue cells. The data presented is mean ± s.e.m.
Figure 2.2. Cartoon showing cells grown on functionalized hydrogels. The hydrogels are embedded with fluorescent beads. For E-Cadherin substrates, the hydrogels were modified with Anti Fc antibody that orients the N terminal of Fc tagged E-Cadherin facing outward.
Figure 2.3. Representative traction stress heat maps is shown. The rows represent R2/7, DLD1, R2/7 Rescue and R2/7 DVBS cells, while the columns represent substrate rigidities of 34, 9 and 1 kPa respectively. The heat scales are different to provide resolution to the generated map. It can be noticed that as the substrate rigidity increases the traction generated is stronger.
Figure 2.4. The average traction generated in MDCK KD (α-catenin KD) and MDCK KD + α-catenin Rescue cells grown in 1 and 34kPa gels are compared. The data presented is mean ± s.e.m. The asterisk (*) denotes p-values < 0.05. The table shows traction mean values with s.e.m values in parentheses.
Figure 2.5. Traction developed by Human carcinoma cells DLD1, R2/7, R2/7 Rescued and R2/7 DVBS mutant cells grown in 1, 9 and 34kpa E-Cadherin Fc functionalized PA gels is compared. The absence of α-catenin and DVBS mutation shows significant reduction in traction generation. The data presented is mean ± s.e.m. The asterix (*) denotes \( p-values < 0.05 \). The table shows the traction mean values with s.e.m values in parentheses.
Figure 2.6. Traction generated on MDCK α-catenin Rescued and MDCK KD cells cultured on E-Cadherin-Fc-coated, functionalized polyacrylamide gels (modulus 34 kPa) in the presence of Cytochalasin D (actin depolymerizing agent) or Blebbistatin (myosin II inhibitor) is compared. The data presented is mean ± s.e.m (n). The asterisk (*) denotes p-values < 0.05. The table below shows the average traction values and the s.e.m values in parentheses.
Figure 2.7. Comparison of traction generated by MDCK βcat ActA, MDCK α-catenin KD, and MDCK Rescued cells on E-Cadherin coated gels (34 kPa). The data depicted are the mean and the s.e.m values in parentheses. The asterisk (*) denotes $p$-values < 0.05.
Figure 2.8. Traction generated by MDCK KD transfected with α-catenin sensor (MDCK KD + Sensor) is compared with that generated by wild type (MDCK WT), α-catenin KD (MDCK KD) and that rescued with GFP α-catenin (MDCK KD + α-catenin GFP). All cells were grown on 34kPa E-Cadherin Fc coated gels with integrin-blocking antibodies in media. The asterisk (*) denotes p-values < 0.05. The table denotes the mean traction values from each cell line and the s.e.m values are in parentheses.
2.6 References


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Chapter 3: E-Cadherin-Mediated Force Transduction: not just a local affair

3.1 Introduction

The transduction of mechanical stimuli to alter biochemical signals is a critical process that enables cells to modulate tissue physiology in response to force fluctuations propagated through the cell environment or to changes in endogenous contractile, protrusive, or compressive forces. Mechanotransduction involves an increasing repertoire of identified force sensitive proteins found in both cells and the extracellular matrix, but cell surface adhesion complexes are common candidates. The role of integrins and their extracellular matrix proteins in mechanotransduction and matrix rigidity sensing is well established. However, recent findings have demonstrated that intercellular adhesive complexes not only regulate cell-to-cell cohesion and tissue barrier integrity, but also transduce force at junctions to instruct cell functions, alter cell shape, and regulate tissue organization.

The functional significance of force transduction through intercellular adhesion receptor complexes has been demonstrated in different tissue types and in different mechanical contexts. In endothelia, fluid shear stress results in endothelial cell alignment relative to the direction and magnitude of fluid shear stress. Mechanical stimulation of cleavage stage C-Cadherin was shown to reverse the direction of a collectively migrating cohort of mesendoderm cells isolated from Xenopus embryos. In vivo, tension at the Drosophila wing imaginal disc was shown to increase cell proliferation and organ elongation. Increased intercellular junction tension also triggers cytoskeletal remodeling and the mechanical reinforcement of cell-to-cell contacts in endothelia and in epithelia.

Intercellular force transduction correlates with both global cellular changes and localized
changes at perturbed junctions. Despite its importance, the rudiments of the underlying
mechano-transduction mechanisms have been identified in only a few cases. At interendothelial
junctions, a complex of vascular endothelial growth factor 2 (VEGFR2), platelet endothelial cell
adhesion molecule 1 (PECAM-1), and vascular endothelial cadherin (VE-Cadherin) regulate
endothelial cell alignment in response to fluid shear stress. Studies demonstrated that both
PECAM-1 and VE-Cadherin in those complexes transduce mechanical force. PECAM-1-
mediated force transduction activates global cell stiffening through an integrin-dependent pathway.
VE-Cadherin-specific force transduction similarly increased cell contractility, resulting in the
disruption of peripheral cell-cell junctions, as well as triggered local cytoskeletal remodeling.
However, the physical proximity of VE-Cadherin to PECAM-1 and VEGFR2 complicates
identifying a VE-Cadherin-based mechanism distinct from that of PECAM-1.

In epithelia, E-Cadherin (E-Cad) complexes at intercellular adhesions are force sensitive.
Within these adhesions, an identified force transducer is α-catenin, which is a cytosolic
actin-binding protein that also binds a complex of α-catenin and the E-Cadherin cytoplasmic
domain. α-catenin is thus a crucial mechanical link between homophilic E-Cadherin bonds and
the actin cytoskeleton, and is ideally positioned for mechano-transduction. Substantial experimental
evidence now supports a postulated α-catenin-based mechanism in which the force-dependent
exposure of a cryptic binding site in α-catenin recruits vinculin, and enables localized actin
polymerization by the Mena/VASP complex associated with vinculin. This mechanism
accounts for force-dependent junctional remodeling and changes in the viscoelasticity of force-
loaded E-Cadherin adhesions. However, measured changes in the E-Cadherin junction stiffness
could also reflect a cadherin-based, force-transduction mechanism, beyond α-catenin
conformation switching.
This report presents evidence for an additional E-Cadherin-based mechanotransduction mechanism that triggers global signals that alter cell mechanics and impinge on other adhesion proteins in the cell. A combination of magnetic twisting cytometry, traction force microscopy, and fluorescence imaging exposed E-Cadherin-mediated signals that perturb distant focal adhesions and activate global cell stiffening. This mechanotransduction pathway functions in addition to the α-catenin-dependent, local cytoskeletal remodeling. Our findings reveal a novel mechanism by which cadherins and integrins coordinately regulate global cell mechanics through an integrated, mechano-sensitive network.

3.2 Materials and Methods

3.2.1 Cell lines and protein production

Michigan Cancer Foundation-7 (MCF7) human epithelial breast carcinoma were obtained from ATCC and maintained in Dulbecco’s Modified Eagle Medium (DMEM 4.5 g/L glucose) supplemented with 10 % v/v fetal bovine serum (FBS), 1 mM Sodium Pyruvate, and 1 % v/v penicillin/streptomycin (Corning Cell Grow, Manassas, VA) unless otherwise stated. The soluble, recombinant E-Cadherin ectodomain tagged with C-terminal Fc domain of human IgG (Ecad-Fc) was stably expressed by human embryonic kidney cells (HEK293T), and then purified by affinity chromatography with Protein A Affigel (Bio Rad, Hercules, CA) as described 24.

N-Cadherin 1-2 tagged with biotin and 6XHis was bacterially expressed, lysed and purified by affinity chromatography. The His tag was removed by enzymatic cleavage and biotin tagged NCad12 peptide was further purified and concentrated. The purity of protein was visually quantified by running a SDS PAGE with appropriate ladder. The purified protein was stored in 100mM HEPES, 100mM NaCl, 5mM CaCl$_2$ buffer and preserved at -80°C until use.
3.2.2 Mimicking cadherin – cadherin adhesion in vitro

Intercellular cadherin interactions were mimicked by adhering cells onto functionally oriented E-Cadherin ectodomain on hydrogel substrates\(^{15,25}\) (Fig. A.1). To negate background integrin-mediated substrate adhesions, the growth media contained integrin blocking antibodies (GOH3 against α6, and AIIB2 against β1 integrin). The cells were exposed to cadherin substrates not more than 6 hours to diminish cell secreted ECM proteins which can compromise E-Cadherin mechanosensitivity experiments. The E-Cad bead-cell (Fig. A.3 A) and E-Cadherin gel-cell (Fig. A.1 A) interactions try to mimic in vivo cell-cell interaction. For cadherin ligand controls, the cells were grown on PLL gels and or stimulated with PLL beads.

3.2.3 Cell area analysis

Cell area analysis were performed on single cells and analyzed in ImageJ. Cells were seeded on respective functionalized substrates (8.8 or 34 kPa functionalized with collagen or cadherin) for 6 hours. Only single cells were analyzed to negate the influence of neighboring cell-induced adhesions (tight and desmosomal cadherin junctions) and or contact inhibition that may influence spreading results\(^{26}\). 1344 X 1024 pixel DIC images were acquired from a 40X/1.3 NA objective Leica microscope and were analyzed in ImageJ using a pixel to μm conversion of 0.1632. The mean and standard error of cell area were calculated in Microsoft Excel. P-values were calculated using Student’s two-tailed t-tests, and \(p < 0.05\) is considered statistically significant.

3.2.4 Orienting cadherin ectodomain on polyacrylamide hydrogels
Proper orientation of Ncad1-2 fragment (biotin tagged polypeptide) was achieved using avidin-functionalized hydrogels. The biotin tag was attached to the C terminal and does not sterically interfere the N terminal of EC1-2 domain. Sequence analysis of chicken avidin (uniprot # P02701) shows 28 primary amines (at neutral pH) that can be potentially functionalized on sulfa SUNPAH treated polyacrylamide gels as previously explained \textsuperscript{15}. The coupling conditions were optimized with HEPES (100 mM HEPES, 100 mM NaCl, 5 mM CaCl\textsubscript{2} pH 8) with 0.4 mg/ml of avidin. The coupling efficiency was visually compared using neutravidin conjugated with avidin green under fluorescent microscope.

3.2.5 Magnetic Twisting Cytometry

Magnetic twisting cytometry was performed as documented previously \textsuperscript{4,27,28}. The protein functionalized ferromagnetic beads bound to the cell surface were magnetized parallel to the cell plane by a 0.1 Tesla magnetic field. The torque on the beads were generated by an applied oscillating, orthogonal field of 60 Gauss at a frequency of 0.3 Hz. The magnitude of the corresponding bead displacements reflect the complex moduli of bead-cell junctions. The latter is a function of the viscous and elastic storage moduli of the junctions. In these studies, the magnitude of 60 Gauss, correspond to a shear stress of 7.2 Pascal. The moduli follow a log normal distribution, from which we obtained the mean and standard deviation. Comparisons of junction stiffness measured under different conditions were calculated from two-tailed Student t-tests using Microsoft Excel, and \( p \) values < 0.05 defined statistically significant differences, at the 95% confidence level.

Carboxyl terminated ferromagnetic beads (1\% w/v, 4.4-4.9 \( \mu \)m diameter (Spherotek, Lake forest IL)) in 300 \( \mu \)L of MES buffer (50 mM, 2-[morpholino]ethanesulfonic acid, 100 mM NaCl,
pH 5), were activated with 60 mg each of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-Hydroxy Succinimide (NHS, Thermo scientific; Rockford, IL). The bead solution was shaken for 15 minutes at room temperature, centrifuged, and then suspended in 300 µL of coupling buffer (100 mM HEPES, 100 mM NaCl and 5 mM CaCl₂, pH 8). To this we added 12 µl of protein (stock concentration of 1 mg/ml) to give a ratio of ~85 µg of protein (E-Cadherin Fc) per mg of ferromagnetic beads, and incubated the protein/bead suspension for 2 hours on a shaker at 4°C. The coupling reaction was quenched with 60 µL of quenching buffer (100 mM Tris, 20 mM NaCl, 5 mM CaCl₂, pH 8), followed by shaking for 30 minutes at room temperature. The beads were then centrifuged and suspended in 400 µL of coupling buffer. In controls, beads were coated with Poly-L-lysine (PLL), or with anti-E-Cadherin blocking antibody (DECMA-1) from Sigma-Aldrich (St Louis, MO), or with a neutral (non blocking) anti-E-Cadherin antibody (Clone 76D5, gift from Barry Gumbiner, University of Virginia). The neutral antibody binds the ectodomain, but does not alter E-Cadherin binding activity ²⁹.

3.2.6 Traction Force Microscopy (TFM) and combined MTC/TFM

Traction force microscopy (TFM) measurements used polyacrylamide hydrogels with Young’s moduli of 8.8 kPa or 34 kPa with embedded 0.2 µm fluorescent microspheres (Molecular Probes, Eugene, OR) ³⁰,³¹. To immobilize proteins on the gel surface, the hydrogels were chemically activated by covering the entire gel surface with Sulfo-SANPAH (0.5-1 mg/ml in 100 mM HEPES buffer) (Pierce Biotech, Rockford IL). The gels were irradiated at 365 nm for 8 minutes, and washed with excess 100 mM HEPES buffer by shaking for 5 minutes. The Sulfo-SANPAH and UV treatment was repeated. Gels were incubated with collagen type 1 (Sigma, St Louis, MO) (0.2 mg/ml in 100 mM HEPES, 100 mM NaCl, 5 mM CaCl₂ pH 8) overnight at 4°C. Control gels were
incubated with 0.2 mg/ml Poly-L-lysine (PLL; 100 mM HEPES, 100 mM NaCl, 5 mM CaCl₂, pH 8). The gels were then rinsed with sterile 1X PBS.

In order to immobilize Fc-tagged E-Cadherin extracellular domains, 0.2 mg/ml anti-human Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was incubated with the Sulfo-SANPAH activated gels overnight at 4°C. The substrates were rinsed to remove unbound antibody, and then incubated with E-Cad-Fc (0.2 mg/ml in 100 mM HEPES, 100 mM NaCl, 5 mM CaCl₂, pH 8) for 3-4 hours at 4°C. After protein immobilization on gels, the substrates were rinsed twice with 1X phosphate buffered saline (PBS), before incubating with 1% w/v BSA (in 1X PBS) for 20 minutes. In all cases, the modified gels were sterilized by UV illumination (365 nm), for at least 15 minutes, before seeding cells.

MCF7 cells were harvested from 25 cm² tissue culture flasks with 3.5 mM EDTA in PBS containing 1% w/v BSA. Cells were centrifuged and resuspended in DMEM (10% v/v FBS) growth medium. About 5000-8000 cells/ml were seeded onto protein-functionalized hydrogels, and allowed to adhere and spread for 6 hours.

For combined MTC-TFM measurements, protein-coated ferromagnetic beads were allowed to settle on the cells for 40 minutes, at 37 °C in a 5% CO₂ environment. Unbound beads were washed away with growth medium. These studies measured changes in traction force under three conditions. The root mean square (RMS) basal traction force (BTF) was determined from fiduciary bead displacements induced by cells, relative to the traction-free bead positions after cell removal, as previously described. In combined MTC/TFM measurements (Figs. 3.1 A and B), the positions of the 0.2 μm fiduciary beads in the gel were imaged after incubating cells with ferromagnetic beads for 40 minutes, and then both immediately before and 2 minutes after applying an oscillating shear stress of 8.4 Pa at 0.03 Hz (+Load). In controls, beads were also
imaged after 2 minutes, but without bead twisting (-Load). The constrained traction maps and root
mean square traction stress (Pa; N/m²) were calculated from the resulting bead displacement maps
33. The difference in bead positions were then used to generate bead displacement maps. The time
sequences for these measurements are illustrated within respective figures (Figs. 3.1 B and 3.4 C).
The mean and standard error of the mean are reported. P-values were calculated from two-tailed
Student’s t-tests, using Microsoft Excel.

Integrin-based adhesions were blocked with anti α6 and anti β1 antibodies 15,25,34. The
hybridoma that produces the anti- β1 antibody AIIB2 was a gift from Johan De Rooij (Hubrecht
Institute, Netherlands). The anti-α6 (GOH3) antibody was from Santa Cruz Biotechnology, Inc.
(Santa Cruz, CA) and the anti-α5β1 16G3 antibody was a gift from Kenneth Yamada (NIH
Bethesda, MD) 35. 13G12 is a non-integrin blocking control antibody and is also a gift from
Kenneth Yamada (NIH Bethesda, MD) 35.

3.2.7 Imaging

3.2.7.1 Confocal imaging

Immediately after bead twisting, cells were fixed with 4% w/v paraformaldehyde for 15 minutes
at room temperature. Control cells were not subject to bead twisting, but all conditions were
otherwise identical. Cells were permeabilized with 0.1% Triton X-100 for 5 minutes, blocked in 1
% w/v BSA for 20 minutes, and stained with primary antibodies, followed by rinsing and treatment
with secondary antibodies and phalloidin in 1% w/v BSA for 1 h.

Focal adhesions were visualized by immunofluorescence imaging of vinculin at the basal
plane. The primary, mouse monoclonal anti-vinculin antibody was from Sigma-Aldrich (St Louis,
MO), and the corresponding secondary antibody was goat anti-mouse Alexa 488 (Sigma-Aldrich,
St Louis, MO). F-actin was stained with Rhodamine-phalloidin (Invitrogen, Carlsbad, CA). Coverslips were mounted using ProLong Gold (Invitrogen). Fluorescence images were acquired with a laser scanning confocal microscope (LSM 700, Zeiss) with a 40x/1.3 NA oil immersion objective and 488 nm and 555 nm lasers. Confocal images of cells stained with anti-vinculin antibody were analyzed with a custom MATLAB code generously shared by Brenton Hoffman (Duke University). Focal adhesion size and number were quantified using the “Analyze Particles” function in ImageJ (NIH) with a minimum size threshold of 0.3 μm².

3.2.7.2 PI3K and Actin accumulation at force-loaded beads

PI3K activity was monitored using the GFP-tagged PH domain of Akt (PH-Akt-GFP) (Ouyang et al. 2008). Cells grown on collagen-coated glass were transfected with PH-Akt GFP plasmid (a gift from Yingxiao Wang, UCSD, San Diego CA) using Lipofectamine 2000 (Invitrogen, Carlsbad CA) following manufacturers protocol. The cells were incubated with functionalized ferromagnetic beads for 70 minutes, to correspond with the time sequence used in measurements with cells treated with PI3K inhibitor (LY294002) (Fig. 3.4 D), and to reduce background due to PI3K signaling induced by E-Cadherin ligation following bead binding 37. Cells with and without 4 minutes of E-Cadherin bond shear were fixed and stained, and bead-cell junctions (n > 150) were imaged by laser scanning confocal microscopy (LSM 700, Zeiss) with a 40x/1.3 NA oil immersion objective. To quantify PH-Akt-GFP accumulation, the background subtracted fluorescence intensity in a region of interest (ROI) defined by the annular region between the bead periphery and a ring 1 μm from the periphery was quantified, as described 15. The same procedure was performed with cells stained for actin (as described in the focal adhesion quantification protocol above), and on control cells transfected with the GFP vector (EGFP-N1 plasmid, Clontech,
Mountain View, CA) (n > 100 bead cell junctions). Data are presented as the normalized mean ± s.e.m, and p-values less than 0.05 were considered statistically significant, using two-tailed Student’s t-test function in Microsoft Excel.

3.2.7.3 Dynamic Fluorescence Imaging

The effect of E-Cadherin bond shear on Src kinase and FAK activity at cadherin adhesions was determined by visualizing Src and FAK kinase activity in real time before and after shear using a FRET-based Src and FAK reporter (a gift from Yingxiao Wang, UCSD, San Diego CA) 38. MCF7 or C2C12 cells were transfected separately with either membrane targeted Src and or FAK reporter (Kras-Src or KRas FAK plasmid generously provided by Yingxiao Wang, UCSD) using Lipofectamine 2000 following modified manufacturer’s instructions. To transflect epithelial cells, the cells were seeded on collagen I coated glass bottom dish (Glass #1.5, Cell E&G, Houston TX) for a day and were incubated in 1.5 ml of pre-warmed Opti-MEM®1 (1X) medium (Life technologies, Grand Island, NY) 30 minutes prior to transfection. We used a ratio of 2 µg DNA to 3 µL Lipofectamine 2000. After transfection the cells were grown in regular growth medium for a minimum of 24 -36 hours before running the MTC-FRET experiment.

Before shearing ferromagnetic beads on cells, the transfected cells were cultured in phenol red free DMEM supplemented with 0.5% FBS, 1 mM Sodium Pyruvate, and 1% v/v penicillin/streptomycin (Corning Cell Grow, Manassas, VA), for 6 hours prior to the experiment 37–39. This treatment reduced serum and growth factor induced kinase activity. Cells were then incubated with E-Cadherin functionalized ferromagnetic beads for 40 minutes. The cells were imaged with an inverted Leica microscope (40X magnification, 0.55 N.A air objective) coupled with a Dual View system (Optical Insights) to acquire CFP and YFP images simultaneously with
a CCD camera (Hamamatsu). The filter sets included CFP: excitation, S430/25, emission S470/30; YFP excitation, S500/20, emission S535/30. The emission filter set uses a 515-nm dichroic mirror to split the two emission images. The exposure time was 300 ms. The cells were imaged for ~10 seconds, prior to applying shear, and then continuously for an additional 10 seconds during bead twisting.

By plotting the CFP/YFP ratio, the average normalized kinase activity prior to shear is tracked in real time. The cells were monitored for 30 seconds, which includes a ~10 second time delay between switching on the oscillating field and acquiring the FRET images. During bead twisting the fluorescent images were monitored continuously. The acquired stacked images were then uniformly contrast enhanced using ImageJ. A region of interest (ROI) was chosen around the beads, and the ratio of the CFP to YFP emission in this ROI was calculated using a custom MATLAB code. Each image was background corrected using a custom MATLAB code, which negates manual bias in choosing an arbitrary region for background correction. The program uniformly subtracts the minimal fluorescent pixel intensity from the image, and this is done for each of the acquired CFP and YFP images. Control data were acquired for the same time period under identical conditions, but without bead loading. In additional controls, cells were treated with inhibitors prior to shear. The CFP/YFP ratios in the ROIs around the beads were normalized to the average CFP/YFP ratio prior to bond shear. The $p$-values were calculated from two-tailed Students’ t-tests, using Microsoft Excel.

3.3 Results

3.3.1 Cell morphology depends on ligand-functionalized substrates and E-Cadherin signaling
Intracellular tension in single epithelial cells is regulated by the rigidity of environment coupled with proteins mediating adhesion and cell contractility. By growing single epithelial cells on functionalized hydrogel substrates (Collagen or E-Cadherin or PLL) of defined rigidity, we can monitor effect of ligands and or substrate rigidity simultaneously. With cells grown on E-Cadherin substrates (under integrin-blocking antibody with reduced serum cell culture media), the basal plane mimics cell-cell lateral adhesion as shown in Figure A.1. Cells spreading and traction generated under these *in vitro* conditions try to mimic minimal cell-cell cadherin mediated intercellular forces.

Integrin based adhesion on collagen substrates show basal focal adhesions with greater traction and higher cell spreading compared with cadherin substrates (Fig. A.1 B), demonstrating that integrin based cell adhesion provide an increased ability to balance intracellular tension. Cells grown on cadherin-coated gels, display reduced spread area (Fig. A.1 B). MCF7 cells spread 152% more on 34 kPa collagen substrates (901 ± 38 μm²) when compared with 34 kPa E-Cadherin substrates (372 ± 34 μm², p < 0.001). It should be noted that as the substrates were not normalized for collagen and cadherin ligand site density, so differences due to density dependent signaling cannot be ruled out.

To investigate the influence of nonspecific adhesion on cell spreading, the average spread area of MCF7 on 34 kPa PLL gels was 257 ± 11 μm², which is also significantly lower than collagen gels (p < 0.001). The influence of cadherin on cell spreading were investigated on other human epithelial cells, R2/7 (328 ± 24 and 345 ± 89 μm²) and the R2/7 rescued with α-catenin (436 ± 62 and 491 ± 26 μm²) on 8.8 kPa and 34 kPa E-Cadherin gels respectively, show reduced spreading compared with collagen gels (data not shown). To compare cadherin isoform specificity and minimal extracellular EC12 domain for cadherin adhesion, C2C12 mouse myoblast were
grown on N-Cadherin coated hydrogels (Fig. A.2). The cells spread and generated traction to a similar extent in either N-Cad FL and N-Cad 1-2 coated gels.

The influence of rigidity on cell spreading was more pronounced with collagen-coated gels (Fig. A.1 C). Comparing MCF7 cell spreading on 8.8 (314 ± 16 μm²) and 34 kPa (901 ± 38 μm²) collagen gels and R2/7 on E-Cadherin coated 8.8 and 34 kPa gels, showed cells are more responsive to rigidity under collagen coated substrates. Positive controls with activated integrin (Mn²⁺ treatment) show increased cell spreading (Fig. A.1 C).

To investigate the influence of cadherin-ligation and associated signaling on integrin mediated focal adhesions and spreading collagen matrix, single cells were grown on collagen substrates (forming basal integrin mediated focal adhesions) and simultaneously bound with E-Cadherin functionalized beads (Fig. A.3). The cellular conditions yielding maximal spreading (collagen coated 34 kPa, Fig. A.1 B) was apically stimulated with cadherin using E-Cadherin coated magnetic beads. Under cadherin bead stimulation, cell spreading on collagen substrates decreased by 44%, from 901 ± 38 μm² to 505 ± 43 μm² (+ E-Cadherin bead, Fig. A.3 C). E-Cadherin bead stimulation cells also spread lower on E-Cadherin (315 ± 21 μm²) and PLL (265 ± 13 μm²) substrates (Fig. A.3 C, p < 0.006, n > 8, in the presence of Anti integrin GOH3 and AIIB2 antibodies). The traction exerted in respective functionalized gels is summarized in Table 3.1, however the spread area of cells on E-Cadherin and PLL gels were not significantly different from one other (p = 0.08, n > 7, Fig. A.3 C). As E-Cadherin mechanotransduction specific negative control, cells stimulated with Neutral E-Cadherin Antibody beads ²⁹ (696 ± 99 μm²), or E-Cadherin blocking antibody DECMA ⁴⁵ (653 ± 63 μm²) did not significantly alter cell spreading on collagen substrates (Fig. A.3 D).
3.3.2 E-Cadherin specific mechanotransduction affects global cell contractility

E-Cadherin-mediated mechanotransduction triggers local vinculin recruitment and actin polymerization at force-loaded E-Cadherin receptors \(^{15,25}\). In prior bead twisting studies, this local cytoskeletal remodeling coincided with an increase in the viscoelasticity of probed bead-cell junctions \(^4\). In order to test whether E-Cadherin mechanotransduction also triggers signals that alter global cell mechanics and possibly other adhesion proteins, we combined magnetic twisting cytometry measurements with traction force microscopy (MTC/TFM) (Fig. 3.1 A). This instrumental set up directly revealed the impact of E-Cadherin receptor loading on cell contractility, and distal focal adhesion remodeling.

Traction forces exerted by MCF7 cells on protein-coated polyacrylamide gels were measured using Traction force microscopy (TFM) and reported in Table 3.1. \(\Delta\)TFM quantifies the change in cell traction forces from the acquired displacement maps of fiduciary beads embedded in the gels, relative to their positions prior to the perturbation (for example, E-Cad bead ligation, or E-Cadherin receptor shear). The basal traction forces (BTF) exerted by adhering cells, prior to the attachment of E-Cadherin beads to the apical cell surface, was lower on 8.8 kPa gels than on 34 kPa gels, as expected (Table 3.1). E-Cadherin-bead ligation to cadherin receptors (-Load) altered integrin-mediated traction forces at the basal surface, supporting previous reports \(^{46}\). The increase in traction force, following the attachment of E-Cadherin-beads (-Load) depended on the substrate rigidity, with the change in the traction being significantly higher on 34 kPa gels compared to softer 8.8 kPa gels (-Load, Fig. 3.1 C). However, force-loading the E-Cadherin beads triggered a statistically significant increase in cell traction forces, relative to the unloaded controls (Fig. 3.1 C). Moreover, E-Cadherin receptor loading triggered a greater increase in cell traction than E-Cadherin ligation alone, on both 34 and 8.8 kPa gels.

Measurements with different magnetic bead coatings demonstrated the E-Cadherin
specificity of the large change in traction following bead loading, as well as the contribution of non-specific membrane tugging (Fig. 3.1 D). Traction heat maps are displayed in Figure 3.2 A. Shearing E-Cadherin beads on MCF7 cells attached to collagen-coated, 34 kPa gels exhibited the greatest increase in traction, relative to control beads coated with Poly-L-lysine (PLL); a neutral, non-blocking anti-E-Cadherin antibody; or the blocking anti-E-Cadherin antibody DECMA-1. PLL bead loading induced significantly lower changes than with E-Cadherin beads (Fig. 3.1 D; \( p = 0.01, n > 11 \)). Shearing beads coated with either neutral antibody or DECMA-1 beads induced significantly lower traction changes than either E-Cadherin-beads (Fig. 3.1 D; \( p = 0.004, n > 8 \)) or PLL-coated beads (\( p < 0.05, n > 8 \)). The distributions of the magnitudes of the local strain vectors beneath cells further illustrate the changes in cell traction following E-Cadherin bond shear (Fig. 3.2 B). E-Cadherin bond shear shifted the mode of the traction vector distribution from 25-50 pN (-Load) to 50-75 pN (+Load). Although nonspecific tugging (PLL) did perturb the substrate strain field, the ligand-specific, E-Cadherin bond shear actuated a much greater increase in cell traction.

Traction changes also depended on the identity of the adhesive coating on the polyacrylamide gels (Fig. 3.1 E). With cells cultured on PLL substrata for 6 hours, in the presence of anti-integrin antibodies to block integrin interference, E-Cadherin bond loading only increased the traction force by 134 ± 19 Pa (Fig. 3.1 E; Table 3.1). Applying shear through PLL beads on cells nonspecifically adhered to PLL-coated gels affected \( \Delta \) RMS traction by 82 ± 12 Pa. The change in traction triggered by E-Cadherin bond shear on cells adhered to E-Cadherin substrata (172 ± 20 Pa) was not statistically different from cells on PLL substrata (\( p = 0.2, n > 7 \)).

### 3.3.3 E-Cadherin mechanotransduction triggers focal adhesion (FA) remodeling

The E-Cadherin-mediated increase in traction forces correlates with FA remodeling. Focal
adhesions were quantified on the basis of immunostained vinculin at the basal plane, and the number and area of FAs were analyzed using the watershed algorithm. FA’s were distributed mainly at the cell perimeter (Fig. 3.1 F). Upon mechanically loading E-Cadherin beads on cells on collagen-coated 8.8 kPa gels, the average FA area and FA number increased by 35% and 20%, respectively (n > 800 analyzed focal adhesions). Figure 3.2 C shows the average normalized values to respective focal adhesions prior to bead twisting. Controls with DECMA-1 coated beads, which trigger neither adaptive stiffening nor changes in traction forces (Fig. 3.1 D), did not generate any statistically significant changes in focal adhesions (Fig. 3.2 C). Thus, the force activated FA remodeling requires E-Cadherin-specific mechanotransduction, and is not due to mere nonspecific tugging on cell surface cadherins.

This combined MTC/TFM approach revealed that, in addition to local, α-catenin-dependent cytoskeletal remodeling, E-Cadherin-mediated force transduction generates global signals that alter integrin-mediated traction forces at the basal plane. In endothelial cells, adaptive cell stiffening activated by the immunoglobulin family protein PECAM-1 required integrins. VE-Cadherin-specific force transduction also mediates endothelial cell stiffening, but PECAM-1, which localizes with vascular endothelial growth factor receptor 2 and VE-Cadherin at interendothelial junctions, could potentially contribute to VE-Cadherin-activated signaling. By contrast, epithelial cells lack PECAM-1, but E-Cadherin-specific force transduction similarly activates global signals that alter cell contractility and remodel focal adhesions.

3.3.4 E-Cadherin mediated adaptive stiffening is insensitive to cell prestress

Previous results showed that blebbistatin significantly diminished the amplitude of the E-Cadherin-dependent stiffening response in F9 cells. To test whether the endogenous contractile
state of cells (cell prestress) modulates cadherin-mediated adaptive stiffening, we altered the substrate elastic moduli to manipulate cell contractility. The BTF (traction in the absence of bead ligation and shear), which indirectly reflect cell contractility are in Table 3.1. The change in cell stiffening triggered by apical E-Cadherin receptor loading were similar, regardless of whether MCF7 cells were seeded on collagen-coated glass (~70 GPa), or on polyacrylamide (PA) gels with elastic moduli of 8.8 kPa or 34 kPa (Fig. 3.3 A). Namely, cell stiffness increased by ~30% during 2 minutes of E-Cadherin receptor loading. The stiffening response was also insensitive to the initial or basal cell stiffness measured by MTC. The latter was slightly lower for cells on hydrogels, being 0.11 ± 0.01 and 0.13 ± 0.01 Pa/nm on 34 and 8.8 kPa gels, respectively, and 0.19 ± 0.01 Pa/nm on glass.

The change in the magnitudes of both traction forces and cell stiffening were insensitive to the range of shear stresses applied to the E-Cadherin bonds. Namely, the overall increase in the cell stiffness did not change when the applied E-Cadherin bond shear doubled from 3.6 Pa to 7.2 Pa (Fig. 3.3 B). Although the ΔRMS traction force increased with the substrate rigidity (Fig. 3.1 C), it did not change significantly when the shear on E-Cadherin beads was increased from 3.6 Pa to 8.4 Pa (Fig. 3.3 C).

3.3.5 E-Cadherin mediated cell stiffening requires integrins

Adaptive cell stiffening response triggered by perturbing PECAM-1 receptors in endothelial cells required the formation of new integrin adhesions 20. To test whether E-Cadherin-activated cell stiffening similarly requires integrins, MTC measurements were conducted with cells adhered to different substrata (Fig. 3.3 D). On fibronectin (FN) and on collagen (Col) coated substrates, E-Cadherin receptor loading triggered stiffness increases of 23 ± 2 % and 32 ± 5%, respectively. On
PLL coated gels, the stiffness decreased to 3 ± 5%. Interestingly, with cells seeded on E-Cadherin-coated gels, the stiffness also decreased to 12 ± 8%. Studies with cells on both PLL and E-Cadherin-Fc coated substrata were done with integrin-blocking antibodies in solution (GOH3 and AIIB2), to reduce integrin interference.

To further investigate the active involvement of integrin requirement, cells on collagen-coated glass were treated with integrin blocking antibodies for 25 minutes, immediately prior to E-Cadherin bead twisting. This brief antibody treatment didn’t affect cell area (Figs. 3.3 E-F), but inhibited free-unbound integrins, and their ability to form new focal adhesions. However, mature focal adhesions were retained, such that the cells remained attached and spread, with clearly visible focal adhesions (Fig. 3.3 E). In cells treated with anti-β1 integrin AIIB2 or with anti- αvβ3 and α5β1 16G3 antibody, the stiffening responses were - 4 ± 5 % and 3 ± 4%, respectively (Fig. 3.4 A). 13G12 is a non-blocking integrin control antibody and incubating cells with 20 μg/ml of 13G12 antibody for 25 minutes didn’t significantly block stiffening response (22 ± 8%, Fig. 3.4 A). Manganese is well known to activate inactive integrins and is used as a positive control for integrin activation in Figure 3.4 A. By activating all background integrins using excess Manganese chloride (0.5 μM) in media, the stiffening response was 12 ± 5 %. Because the surviving focal adhesions did not completely support E-Cadherin-mediated adaptive stiffening, these results indicate that 1) adaptive stiffening reflects changes in global contractility, in addition to α-catenin conformation changes at perturbed E-Cadherin complexes, and 2) the global adaptive stiffening requires the formation of new integrin adhesions.

3.3.6 Global signals are required for E-Cadherin-activated cell stiffening

We further investigated the minimal set of signals required for cadherin-actuated changes in cell
traction and cell stiffness. Nonmuscle myosin II, phosphoinositide 3-kinase, Src kinase, and Rho associated protein kinase one (ROCK1) have been implicated in cross-talk between cadherins and integrins, as well as in PECAM-1 mechanotransduction. Cell treatment with blebbistatin or with the ROCK inhibitor Y27632 reduced the force-dependent changes in traction to 53% and 51% that of untreated cells (Fig. 3.4 D). The latter traction changes were comparable to nonspecific controls with antibody-coated beads (Table 3.1). Treatment with Src or PI3K inhibitors reduced the load-dependent traction force changes to 72% and 69% that of untreated cells (Fig. 3.4 D). The effects of inhibitor treatment on adaptive stiffening were more pronounced than their influence on traction forces. Blebbistatin (MyII) and Y27632 (ROCK1) treatment reduced adaptive stiffening from 32 ± 5 % (untreated cells) to 5 ± 6% and 0 ± 3 %, respectively. PP2 (Src), LY294002 and Wortmanin (PI3K) reduced the response to 6 ± 4 %, -4 ± 6 % and 7 ± 5%, respectively (Fig. 3.4 E). The effect of inhibitors on the basal traction (-beads), relative to untreated cells, is shown in Figure 3.4 F. The effect of inhibitors on cell morphology is compared with the BTF traction generated (Fig. A.4) is plotted for reference.

To determine whether E-Cadherin receptor loading activates PI3K upstream of integrin signaling, the force-dependent accumulation of the PI3K reporter PH-Akt-GFP in regions of interest (ROI) surrounding the E-Cadherin beads (Fig. 3.4 G) was quantified before and after bond shear. Controls included the use of PLL-coated beads and cells expressing GFP. The background-subtracted mean fluorescence intensities (MFI), normalized by the values prior to force-loading (-Load), are summarized in Figure 3.4 G. The use of E-Cadherin beads resulted in a 41 ± 0.1% increase in the normalized MFI, under bead shear. Treatment with anti-integrin antibodies in media abrogated adaptive stiffening (Fig. 3.4 A), but it did not affect PI3K activation at loaded E-Cadherin beads (Fig. 3.4 G). The normalized MFI increased by 54 ± 0.1% and by 49 ± 0.1 % in
cells treated with AIIB2 (Fig. 3.4 G) or 16G3 (not shown), respectively. In controls, GFP did not accumulate at force-loaded E-Cadherin beads, and PH-Akt-GFP did not accumulate at force-loaded PLL beads (Figs. 3.4 G). Thus, E-Cadherin-dependent PI3K activation is upstream of focal adhesion remodeling and myosin contractility.

E-Cadherin ligation activates Src kinase and their activation with calcium is observed using FRET based membrane bound Src sensor (Figs. A.5 and A.6), but mechanical shear (+Load) does not induce Src kinase activity especially at the bead-cell junction (Fig. 3.4 H). Since the location of bead shear (cell rheology) might affect Src kinase activity, comparing the average intracellular Src activity by E-Cadherin bead stimulation above nucleus or cytoplasm or cell periphery does not significantly activate Src. Figure A.7 shows the distribution of Src activity.

Cadherin adhesion induces Src activity, which peaked at ~30 minutes after bead ligation, and then dropped to initial levels at ~45 minutes (Fig. A.6 C). To measure the effect of E-Cadherin shear on Src, we measured the CFP/YFP ratio 40 minutes after bead ligation, to minimize contributions from load-independent signaling. However, ~30 s of E-Cadherin receptor loading did not significantly increase the CFP/YFP ratio, relative to the slow change in the no-load control (Fig. 3.4 H).

Src and FAK are known to function cooperatively in cell adhesion. To investigate the role of Focal Adhesion Kinase (FAK) in cadherin mechanotransduction, we independently checked FAK activity using membrane bound and cytosol localized FAK FRET reporters. We observe shear specific activation of FAK (ratio of ECFP/YPet) under mechanical shear immediately around the ROI with E-Cadherin beads (Fig. A.8 A). To further the role of FAK in cadherin mechanotransduction, we investigated FAK activity in C2C12 cells stimulated by N-Cadherin beads (N-Cadherin FL and N-Cadherin 12) and under FAK inhibition (PF228) (Fig. A.8
We further tested whether the observed phenomenon of actin accumulation at force-loaded E-Cadherin beads attributed to α-catenin unfurling and vinculin recruitment \(^{3,4,14,15,17}\), depends on cell stiffening. Figure 3.4 B shows that impairing the adaptive stiffening by treatment with anti-integrin antibodies did not ablate actin accumulation within ROI’s at force-loaded E-Cadherin beads. Despite absence of adaptive stiffening, there was a statistically significant accumulation of actin at sheared beads. The apparent differences in normalized actin accumulation in cells treated with AIIB2 relative to untreated cells might reflect the inhibition of integrin-mediated actin accumulation at beads, due to nonspecifically bound matrix proteins on beads. However, the use of E-Cadherin beads in the absence of serum resulted in similar actin accumulation (Fig. 3.4 B). Conversely, measurements with PLL beads in the presence of 10% serum did not result in actin accumulation. Thus, actin accumulation at sheared E-Cadherin beads is E-Cadherin specific and does not require adaptive stiffening. The results also suggest that the measured increase in the bead-cell junction stiffness mainly reflects cell contractility rather than local cytoskeletal remodeling.

### 3.4 Discussion

These results expose a new E-Cadherin mechanotransduction pathway beyond the force-dependent α-catenin conformation change and consequent local cytoskeletal remodeling. This new pathway involves global signaling that activates distant integrins and focal adhesion remodeling at the basal plane. Integrin activation can activate ROCK1-dependent global cell contractility. Fluorescence imaging also revealed that PI3K is a downstream effector of E-Cadherin mechanotransduction in epithelial cells, and is required for integrin-dependent adaptive stiffening.
The abrogation of E-Cadherin-mediated stiffening in cells on E-Cadherin substrata and in cells treated with blocking anti-integrin antibodies supports the requirement of integrins in cadherin mechanotransduction. The lack of stiffening in cells on E-Cadherin substrata was unexpected, because cell adhesion through E-Cadherin bonds generates traction forces and also activates GTPase, Src, and PI3K signaling \(^4,28,37,52,57\). Moreover, E-Cadherin receptors are mechanically coupled to actin microfilaments, and could potentially transmit force directly through the cytoskeleton by stress focusing \(^58\). However, the inability of E-Cadherin receptor loading to induce global stiffening in cells on E-Cadherin substrata and growth of focal adhesions in collagen substrates (Figs. 3.1 E and 3.3 D) is evidence that biochemical signals couple cadherin and integrin force transduction signaling to regulate global cell mechanics.

These findings are intriguing in light of reports that force-activated, E-Cadherin-mediated stiffening requires the vinculin-binding site of \(\alpha\)-catenin \(^15,17,25\). Exchanging the vinculin-binding-site for the homologous vinculin domain abolished cell stiffening \(^15\). Yet the absence of cell stiffening did not block local actin accumulation and an \(\alpha\)-catenin mutant lacking this site still undergoes a conformational change at perturbed E-Cadherin adhesions \(^25\). Differential vinculin phosphorylation contributes to its different roles in adherens junctions and focal adhesions \(^59\), but vinculin is not a signaling protein. One possibility is that \(\alpha\)-catenin ligands such as vinculin \(^60\) may act as force-sensitive scaffolds for bonafide signaling proteins.

The abrogation of cell stiffening by anti-integrin antibodies correlated with an apparent reduction in actin accumulation at E-Cadherin beads. This might suggest a positive feedback loop wherein endogenous contractile forces enhance actin polymerization at perturbed E-Cadherin adhesions. In the simplest feedback, increased contractile stress could increase the unfurled \(\alpha\)-catenin population at the beads, with a concomitant increase in local actin polymerization \(^22\). But
there are other possibilities. Zyxin facilitates force-dependent actin polymerization at focal adhesions, and is also enriched at stressed adherens junctions \(^{48,61-63}\). Both tugging on actin fibers \(^{64}\), and mechanically deforming cortical F-actin \(^{65}\) were shown to stimulate actin fiber elongation through formin-dependent mechanisms. Whether any of these mechanisms would account for our observations is an intriguing question, but addressing this issue is outside the scope of the present study.

The dependence of adaptive stiffening on PI3K, Src, and integrins is strikingly similar to PECAM-1 mechanotransduction in endothelia \(^{20}\). E-Cadherin force-loading could activate GTPases at the apical surface through RhoGEFs, analogous to RhoGEF-H1 and LARG at focal adhesions \(^{66}\), but the absence of stiffening by cells on either PLL or E-Cadherin substrata, as well as by cells on extracellular matrix proteins after brief anti-integrin antibody exposure, appears to rule this out. Cadherin loading could also activate integrins, by transmitting force to FAs through microfilaments. Although stress-focusing was invoked to account for global effects of apical integrin loading \(^{58}\), this primarily mechanical mechanism is difficult to reconcile with our finding that apical E-Cadherin receptor loading did not alter basal E-Cadherin adhesions, even though the cadherin adhesions are mechanically linked to actin filaments \(^{4,15,23,67}\). Similarly, use of anti-E-Cadherin antibody DECMA-1 coated beads failed to significantly alter traction forces (Fig. 3.1 D) or trigger stiffening in cells on collagen substrata \(^{15}\). Microtubule disruption also enhanced rather than abrogated adaptive stiffening \(^{28}\). These observations, together with the requirement for specific E-Cadherin ligation, new integrin bond formation, PI3K, and ROCK1 are consistent with the model in Figure 3.5 of global, E-Cadherin force-transduction and mechanical cross-talk between cadherins and integrins in epithelial cells.

These new findings reveal that E-Cadherin-mediated force transduction actuates a global
signaling pathway, in addition to the local, α-catenin dependent cytoskeletal remodeling. Intriguingly, despite functional and biochemical differences between E-Cadherin and PECAM-1, similar mechanotransduction signaling suggests that these receptors exploit common pathways in different tissues to transduce force throughout mechano-chemically integrated networks in cells.
### 3.5 Table and Figures

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Table 3.1. Root mean square traction forces determined under the different experimental conditions described in the text.
Figure 3.1. E-Cadherin-based mechanotransduction alters cell traction and focal adhesions. (A) Illustration of the experimental setup combining Magnetic Twisting Cytometry (MTC) and traction force microscopy (TFM). An oscillating magnetic field $H$ generates a torque $T$ on the bead. (B) Time sequence of steps in combined MTC/TFM measurements. (C) Bar graph indicating
changes in traction force (± Load) exerted by MCF7 cells on collagen-coated polyacrylamide gels with elastic moduli of 8.8 (–Load, n = 7 cells; +Load, n = 19 cells, *P < 0.01) and 34 kPa (-Load, n = 11 cells, +Load, n = 11 cells, *P < 0.01). (D) Bar graph showing changes in cell traction after force-loading beads modified with E-Cadherin (E-Cad, n = 11 cells), Poly-L Lysine (PLL, n = 18 cells, *P = 0.01), Neutral anti-E-Cadherin antibody (Ntrl Ab, n = 8 cells, *P < 0.01), or blocking anti-E-Cadherin antibody (DECMA-1, n = 8 cells, *P < 0.01). N_exp ≥ 2 (E) Bar graph indicating traction changes (ΔRMS Traction, Pa) after force-loading E-Cad beads on cells adhered to collagen (n = 11 cells), PLL (n = 9 cells, *P < 0.01), or E-Cadherin-coated polyacrylamide gels (34 kPa, n = 7 cells). Results obtained with PLL-coated beads on cells adhered to PLL substrata are also shown (n = 6 cells, *P < 0.01). With cells on either PLL- or E-Cadherin-coated substrata, the medium contained integrin-blocking antibodies. In panels C–E, the black bar denotes the same data used for statistical comparisons. Data presented are the mean ± s.e.m. *P < 0.05 and N_exp ≥ 2. (F) Representative confocal immunofluorescence images of vinculin (green) and actin (gold) at the basal plane of cells on Collagen-functionalized hydrogels. Cells were probed with E-Cad (top) and DECMA-1 (bottom) functionalized beads, with (+Load) and without (-Load) 2 min of force loading. Scale bar, 10 µm.
Figure 3.2. Effect of E-Cadherin bond shear on focal adhesions and basal traction force. (A) Representative heat maps of changes in traction force without load (–Load) and after 2 minutes of loading (+Load) with E-Cadherin beads (top), PLL beads (middle), and DECMA-1 (bottom) coated beads. (B) Distribution of the magnitudes of the traction force vectors without (–Load) and with (+Load) E-Cadherin bond loading. The bars indicate the number of measured traction force vectors within the indicated force ranges. (C) Bar graphs of the focal adhesion area and focal adhesion number (per 100 μm²) on collagen-coated 8.8 kPa substrates, normalized by the initial values prior to E-Cadherin bond loading. Data obtained after bead loading were normalized to the initial, population-averaged values (n > 800). Data depicted are the mean ± s.e.m.
Figure 3.3. Biophysical parameters affecting E-Cadherin-mediated adaptive stiffening (A) Comparison of E-Cadherin mediated cell stiffening (% change) by cells on substrata with different rigidities (glass (▲, n > 50), on 34 kPa gels (■, n > 50), and on 8.8 kPa gels (♦, n > 50)). Results with Poly-Lysine (PLL) beads (○, n > 50) are shown as a negative control. (B) % Stiffness change versus time of E-Cadherin bond loading at applied shear stresses of 3.6 Pa (▲, n = 13) and
7.2 Pa (■, n > 50). (C) Change in the ΔRMS traction force after applying 3.6, 6 or 8.4 Pa of E-Cadherin bond shear. MCF7 cells were seeded on collagen-coated 34 kPa hydrogels. Data depicted are the mean ± s.e.m. (D) % Stiffness change induced by shearing E-Cadherin beads on cells adhered to 34 kPa gels coated with Fibronectin (FN, n = 9), E-Cadherin (E-Cad, n = 16), Poly-L-Lysine (PLL, n= 22), or Collagen (Col, n > 50). Results obtained with PLL beads bound to cells on PLL-coated polyacrylamide are also shown (n = 16). (E) Representative confocal immunofluorescent images of vinculin (green) and actin (gold) in cells treated with anti-integrin antibodies (16G3 and AIIB2) for 25 minutes prior to fixation and imaging. The scale bar is 10 µm. (F) Effect of antibody treatment (16G3 and AIIB2) on cell spread area is compared n > 50 cells.
Figure. 3.4. Biochemical characterization of cadherin mechanotransduction. (A) Effect of compromised integrin adhesion on cadherin % stiffening response is compared using integrin blocking (AIIB2, n > 80 beads, 16G3, n > 80 beads), non-blocking (13G12, n > 50 beads) and excess magnesium (n > 40 beads) conditions. (B) Influence of cadherin stiffening response on...
Actin remodeling is measured by plotting the normalized change in actin intensity around E-Cadherin beads (E-Cad–Ab). Background effect of serum inducing actin accumulation is negated by experimenting in the absence of serum (E-Cad–Serum). The effect of compromised focal adhesions integrin blocking antibodies 16G3 and AIIB2 is shown. Actin intensity under PLL bead shear is shown as control. (C) Time sequence for measurements of the impact of inhibitors on ΔRMS Traction and adaptive stiffening is shown. (D) The change in the root mean square traction forces (ΔRMS Traction, Pa), after force-loading E-Cad beads bound to cells treated with inhibitors of ROCK (Y27632, n = 12 cells) (62), myosin II (Blebbistatin, n = 9 cells) (9), Src kinase (PP2, n = 8 cells) (47), or PI3K (LY294002, n = 7 cells) (10). *P < 0.05; Nexp = 2. (E) % Stiffness change, after force-loading E-Cad beads bound to cells treated with inhibitors of myosin II (Blebbistatin, n > 40 beads), ROCK (Y27632, n > 30 beads), Src kinase (PP2, n > 30 beads), PI3K (LY294002, n > 20 beads; Wortmanin, n > 40 beads) 49, *P < 0.05; Nexp = 2. (F) Bar graphs comparing the traction force (BTF) generated by MCF7 cells (in the absence of beads) on 34 kPa gels under specific inhibitors is plotted, *P < 0.05; Nexp = 2. (G) Bar graph of the mean fluorescence intensity (MFI; PH-Akt-GFP) in ROIs surrounding beads +Load, normalized to values obtained from unperturbed E-Cad beads (−Load). Cells were on collagen-coated glass. Data were obtained with E-Cadherin-coated beads. Results show the normalized change in MFI, measured in the presence of anti-integrin antibody AIIB2 (n > 150 beads, *P < 0.001, Nexp ≥ 2). Controls were with cells expressing GFP or with PLL-coated beads (n > 100 beads, Nexp = 2). (H) CFP/YFP emission ratios in ROIs around E-Cad beads on cells expressing the membrane-bound, FRET-based Src sensor. (●) Bead twisting was activated at t=0 (n = 12 beads, Nexp = 8). (▲) -Load control (n = 18 beads, Nexp = 9). (■) PP2 negative control (n = 9 beads, Nexp = 4). Data shown are the normalized mean ± s.e.m. The break in the time axis indicates the 10s time delay between activating bead twisting (+Load) and fluorescence imaging.
Figure 3.5. Proposed model for E-Cadherin-mediated global mechanotransduction. Mechanically stimulated E-Cadherin receptors activate PI3K through an EGFR-dependent mechanism and trigger α-catenin unfurling. PI3K activation is required for the downstream activation of new integrin adhesions and consequent ROCK-dependent activation of myosin II-dependent, cell contractility and measured adaptive stiffening.
3.6 References


Chapter 4: Concluding remarks

This thesis focuses on dissecting the mechano-sensitivity and the intracellular force transduction ability of epithelial cadherins by investigating cadherin ligand specificity, static and dynamic force sensitivity and immediate early mechanotransduction activity using specific kinase sensors, inhibitors and customized mechanotransduction tools.

In chapter 2, I discussed the modulatory role of α-catenin enabling cadherin specific force sensitivity and transduction. The results shed light on the then prevailing in vitro biochemical conundrum that α-catenin cannot be physically bound to actin and or β-catenin simultaneously. Using different α-catenin rescue constructs, epithelial cell lines and mechanical stimuli inducing tools, I show that α-catenin is required and modulates cadherin’s ability to sense extracellular mechanical stimuli. In addition, I show that in the absence of α-catenin there is reduced traction generation and negation of stiffening response, and using an α-catenin FRET rescue sensor, I show unambiguously that α-catenin can aid cadherin’s ability to exert traction on cadherin substrates. The conformation modules of α-catenin’s phosphorylation state (5SA and 5SE mutants), vinculin bound state using vinculin binding site (VBS) and association with myosin mediated tension during cadherin mechanotransduction, advances an active dynamic role of α-catenin in cadherin mechanotransduction.

In chapter 3, I experimentally demonstrate that the phenomenon of cadherin mechanotransduction is not restricted to cadherin junctions alone, but is a global phenomenon that requires and activates new focal adhesions. This global cadherin mechanotransduction phenomenon was investigated by combining MTC and TFM tool with fluorescently tagged reporters. The discussed pathway is supported by a similar PECAM 1 global mechanotransduction pathway in endothelial cells. This global mechanotransduction network expands the current
cadherin mechanotransduction machinery.

In chapter 2, we do not discuss the role of other possible actin linker elements (ZO-1, ajuba, afadin, α-actinin) that can indirectly connect cadherin to actin cytoskeleton. The minimal cadherin catenin force transduction elements that connect cadherin to actin and or induce mechanotransduction is an open question requiring further experimentation. The allosteric role of extracellular cadherin domains is also not addressed in chapter 2. The mechanotransduction specific cadherin dimer conformation is discussed in chapter 3, where anti E-Cadherin antibody (DECMA), and PLL could not induce stiffening response nor global traction generation or focal adhesion growth. The role of other membrane receptors (EGFR) that may laterally induce or regulate cadherin mechanotransduction alongside cadherin dimerization is not investigated in chapter 3.

The molecular scheme of events in the global mechanotransduction pathway is not completely elucidated and requires further experimentation. The use of inhibitors in the media does not differentiate between activity at cadherin or integrin junctions in the cell requiring techniques that spatially resolves the activity between cadherin and integrin junctions. I did not investigate the role of other cytoskeletal elements (microtubule, intermediary filaments) and their associated linkers in cadherin mechanotransduction. Live cell tracking of global cytoskeletal and or organelle remodeling will further support the global mechanotransduction phenomenon. I have observed cadherin mechanical stiffening events in the order of seconds, which is too fast to be diffusionally or transcriptionally controlled. In such time scales conformationally controlled protein switches, biochemical enzymatic or localization and temporal activity may regulate the process, which requires further experimentation. To investigate the role of substrate rigidity and the crosstalk with other membrane receptors, a high throughput stiffness array and a ligand array
can experimentally decode the mechanosensory elements. I have also not explored the role of extracellular cadherin domains in mechanotransduction. Specific E-Cadherin antibody adhesion and binding has been shown to be blocking, activating or neutral in function. The mechanosensitive role of cadherin transmembrane region and p120 binding juxtamembrane region has also not been explored, and requires the generation of specific truncated cadherin mutants.

The in-depth study of global E-Cadherin mechanotransduction phenomenon with respect to other adhesive junctions and membrane receptors, will help design principles for *in vitro* tissue engineering, controlling or developing models of cancer metastasis and treat mechanotransductive diseases.
Appendix
A.1 Appendix material and methods

The methods followed in the appendix section is discussed in the main text (chapter 3)
A.2 Appendix cell area analysis

Figure A.1. Cell adhesion on different substrates. (A) Cartoon illustrating cell attachment on cadherin or collagen-coated substrates. Cells adhered to Fc tagged cadherin, which was bound to Polyacrylamide Gel. (B) Graph showing cell area on E-Cad gel, PLL gel, and Collagen gel. (C) Graph showing cell area on Untreated, 8.8 kPa, Untreated, 34 kPa, and Mn2+, 34 kPa.
Anti Fc antibody functionalized gels is shown on the left. For collagen-mediated adhesion (right), cells adhered to collagen-functionalized gels. (B) Extent of MCF7 cell spreading on E-Cadherin (E-Cad), Poly L-lysine (PLL) and Collagen coated 34 kPa gel is shown. (C) Spread cell area on collagen-coated gels (8.8 and 34 kPa) or with Mn$^{2+}$ treatment is also shown.
Figure A.2. Effect of N-Cadherin on cell spreading. (A-B) Cartoon showing C2C12 cells adhering onto either N-Cadherin Full length (N-Cad FL) or N-cadherin 1-2 (N-Cad 1-2) functionalized polyacrylamide gels. (A) Biotin tagged N-Cad 1-2 is bound to avidin functionalized gels, and (B) Fc tagged N-Cad FL is bound to Anti Fc antibody functionalized gels. (C-D) C2C12 cells spreading and generating traction on NCad 1-2 and NCad FL functionalized 8.8 kPa gels are shown. Data depicted is the mean ± sem.
Figure A.3. Effect of E-Cadherin ligation on cell spreading. (A) Cartoon depicting a cadherin coated bead bound apically, to a cell grown on collagen functionalized gel (basal integrin adhesion). (B) Effect of substrate rigidity on the spread area of MCF7 cells on collagen-coated gels (34kPa, 8.8kPa), and (C) Effect of ligand influencing cell spreading is checked by growing cells apically bound to E-Cadherin beads, on collagen (Col), E-Cadherin (Ecad) or Poly L-Lysine
(PLL) modified 8.8 kPa gels. (D) Effect of bead interaction (E-Cad-Fc, PLL, Anti E-Cadherin blocking antibody (DECMA) or neutral Anti E-Cadherin antibody (Neutral Ab)) on spread cell area on collagen-coated, 8.8 kPa gels is shown.
Figure A.4. Effect of specific kinase inhibition on MCF7 cell spreading and traction generation on collagen-coated 34 kPa gels. Spread area and corresponding traction by MCF7 cells on collagen-coated gels, after treatment with specific inhibitors against Srk (PP2), PI3k (LY294002), myosin II (Blebbistatin), ROCK kinase (Y27632). Data are also shown for cells treated with excess MnCl$_2$ compared with untreated cells. Data are the mean ± sem.
A.3 Appendix Src analysis

Figure A.5. Control showing Src activity at cell-cell junction following a calcium switch. MCF cells expressed the membrane bound FRET reporter for Src. Representative FRET images of Src

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FRET activity before and at 15 minutes intervals after calcium stimulation. Respective DIC and FRET images of PP2 treated cells before and 30min after calcium addition is shown as reference.
Figure A.6. Calcium-stimulated Src activity at cell junctions. (A) Ratio of CFP/YFP emission, normalized prior to calcium introduction at specific cell-cell junctions. The time points indicate CFP/YFP ratio after calcium addition. Calcium was introduced at ~800 seconds and the following jump in signal (upto 1000 seconds) indicate time lag in acquiring data during calcium media
introduction. (B) Each ROI at cell-cell junctions was background subtracted, and normalized to the average value prior to calcium stimulation. (C) Bar graphs denoting Src activity at E-Cadherin bead-cell junctions in the absence of applied force. Src activity peaked at 30 min, and decayed to the initial value within 45 min. Each data point is the mean ± s.e.m.
Figure A.7. Effect of E-Cadherin mechanotransduction on Src activity. (A) Effect of local cell rheology on Src activity was investigated, by quantifying CFP/YFP ratios in ROI’s surrounding beads at different locations in the cell – above the nucleus, in the cytoplasm or at cell perimeter. (B) Number of cells exhibiting the indicated % changes in the FRET ratio (CFP/YFP). The overall average of CFP/YFP disregarding cell rheology shows no significant difference before and after E-Cadherin shear.
Figure A.8 FAK activity under N and E-Cadherin bead shear. (A) Normalized ratio of membrane bound FAK FRET reporter (ECFP/YPet) tracked under mechanical shear is shown. The chosen ROI is around E-Cadherin bead-cell junctions in MCF7 that dominantly express E-Cadherin. (B) Normalized FRET ratio of membrane bound FAK reporter under N-Cadherin shear (full length N-Cadherin or truncated N-Cadherin 1-2) on C2C12 cells that express N-Cadherin is shown. The
emission intensity at the acquired CFP and YFP channels, were internally normalized prior to shear and collectively averaged. Each data point corresponds to normalized mean ± s.e.m.