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Extracellular cues drive integrin mediated signaling to drive cancer cell invasion

by

Matthew Rubashkin

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Bioengineering

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

AND

UNIVERSITY OF CALIFORNIA, BERKELEY

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By

Matthew Gregory Rubashkin

Dedicated to the memory of Fred Katzmann

Acknowledgements

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Abstract

Extracellular cues drive integrin mediated signaling to drive cancer cell invasion

By:

Matthew Gregory Rubashkin

Doctor of Philosophy in Bioengineering

University of California, Berkeley

&

University of California, San Francisco

Professor Valerie Weaver, Chair

For several decades oncologists have been faced with the obstacle of properly classifying tumors for effective patient treatment. Traditionally, this was accomplished by documenting changes in cancer cells, and through observation of canonical changes to the extracellular matrix (ECM). However, even when I began my graduate studies in 2010, it was still unclear if cancer cells were driven to metastasize by changes to the ECM, or if changes to the ECM were a result of malignant progression. Over the past 5 years I have worked in Dr. Valerie Weaver's laboratory at UCSF to answer how cancer cells interpret extracellular mechanical cues in 3D and how these cues can be translated into oncogenic signals at the cell-ECM interface. In order to study molecular interactions of oncogenic proteins in invasive cancer cells, I participated in the development of a new super-resolution microscopy technique, Scanning Angle Interference Microscopy (SAIM). I then employed this technology to determine how ECM stiffness and composition can drive vinculin mediated PI3-kinase signaling to enhance cell invasion, and studied how paxillin mediated mechano-sensing of the ECM could drive metastasis in difficult to treat triple negative breast cancer patients.

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Chapter 1. Background

1.1. Introduction

1.1.1 Dissertation Overview

As a PhD student in bioengineering, science provides me the opportunity to solve problems in the clinic by asking new questions in the laboratory. For several decades oncologists have been faced with the obstacle of properly classifying tumors for effective patient treatment ¹⁻⁵. Traditionally, this was accomplished by documenting changes in cancer cells, and through observation of canonical changes to the extracellular matrix (ECM) ^{2,4,6}. However, even when I began my graduate studies in 2010, it was still unclear if cancer cells were driven to metastasize by changes to the ECM, or if changes to the ECM were a result of malignant progression ⁷⁻¹¹. Over the past 5 years I have worked in Dr. Valerie Weaver's laboratory at UCSF to answer how cancer cells interpret extracellular mechanical cues in 3D and how these cues can be translated into oncogenic signals at the cell-ECM interface ¹²⁻¹⁴.

1.1.2 Dissertation Approach – Chapter 1

In vivo, cells exist within the context of a multicellular tissue, where their behavior is governed by many interactions ¹⁵⁻¹⁷. These include homo- and heterotypic cell-cell interactions, the material properties of the extracellular matrix, and the distribution and accessibility of soluble and physical factors. However, most of the methods currently used to study and manipulate cellular behavior *in vitro* sacrifice physiological relevance for experimental expediency. In **Chapter 1**, I deconstruct how signaling occurs across multiple dimensions in space and time, and how an enhanced understanding of mechanobiology in 3D, as well as nanoscale molecular interactions, is critical for combatting cancer cell invasion and metastasis. This research was published in *Biochemistry in 2014* ¹⁵.

1.1.3 Dissertation Approach – Chapter 2

In order to study individual proteins in live cells, light microscopy is the tool of choice for cell biologists¹⁸. Several techniques had been developed from 2000-2010 to analyze proteins at the ~20nm scale¹⁹, wherein the diffraction limit of light would be overcome (~300nm). However, there were no methods available in 2010 to analyze how ECM cues could alter the plasma membrane, focal adhesions, and the cytoskeleton at protein level resolution in real time in live cells. To solve this problem, I worked with several post-doctoral researchers to develop Scanning Angle Interference Microscopy (SAIM), which allows for <5nm axial resolution in live cells with a time resolution of 10 seconds, published in *Nature Methods* in 2012¹². This work is featured in **Chapter 2**. My largest contribution to this project was development of software that enabled analysis of changes to molecular distribution of genetically encoded probes, which enabled the first ever measurement of dynamic changes in focal adhesion nanoscale architecture as a function of adhesion maturation.

1.1.4 Dissertation Approach – Chapter 3

Our newly developed super-resolution microscopy technique, SAIM, has facilitated many new studies into the mechanisms of cancer cell invasion and metastasis, both inside and outside of the plasma membrane. In order to study how an altered cancer ECM affected focal adhesion structure, I determined how ECM stiffness and composition can drive vinculin mediated PI3-kinase signaling to enhance cell invasion in 3D (*Cancer Research*, 2014) **Chapter 3**. This work entailed key collaborations that I helped develop, including Michael Davidson at the University of Florida, Dr. Andreas Garcia at Georgia Tech, and Dr. Margaret Gardel at the University of Chicago. This research project could not have been achieved without the polymer science expertise of the Weaver laboratory, which was crucial for the fabrication of novel 3D peptide and collagen matrices. The design of these 3D matrices was informed through the literature review entailed in Chapter 1¹⁵. Moreover, my experiences in light microscopy and cell biology facilitated several experiments that establish a novel oncogenic signaling pathway in breast cancer cells,

wherein ECM cues alter plasma membrane nano-topography, inducing adhesion assembly and Akt signaling.

Partnering with Dr. Matthew Paszek, I helped determine the role of cell tethered extracellular sugars, the glycocalyx, in inducing integrin mediated-growth and survival, published in Nature in 2014¹⁴. For this study I engineered a new glycocalyx Muc1 FRET compression sensor and advanced how the cancer biology community understands cell signaling in a heterogeneous 3D. While this manuscript is not featured in my thesis, its findings will be cited throughout several discussions.

1.1.5 Dissertation Approach – Chapter 4

For the last two years of my PhD, I investigated how cellular mechanosensing of the ECM could drive mesenchymal cell invasion, in difficult to treat triple negative breast cancer patients, featured in **Chapter 4**. Cellular mechanosensing is required for focal adhesion assembly and signaling, which in turn drives mesenchymal-like cell invasion, malignant transformation and tumor metastasis^{20,21}. Specifically, we explored the role of the focal adhesion protein paxillin, a previously identified mechanotransducer^{22,23}, in breast cancer migration, transformation and invasion. Using human bioinformatics data, single and multicellular models of tumor invasion with paxillin mutants, super-resolution analysis of focal adhesion architecture via SAIM and 3B microscopy, as well as cellular mechanical measurements via traction and atomic force microscopy we found that epithelial to mesenchymal transition (EMT) induces alternative paxillin splicing of the paxillin-alpha that enhances cell plasticity in tumor-like ECM, driving cancer cell invasion through a Src-RhoA-Erk signaling axis. Moreover, we establish that the mesenchymal paxillin alpha isoform increased force per integrin, traction on tumor-like matrix, and cellular invasion. These findings reveal that EMT could induce tumor progression by promoting the alternative splicing of the mechanotransducer paxillin; and presents a new series of targets for triple negative breast cancer patients.

1.2. Deconstructing Signaling in Three Dimensions - Background

1.2.1. Abstract

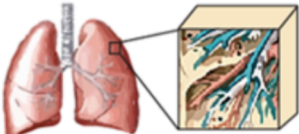
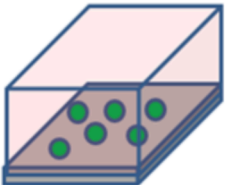
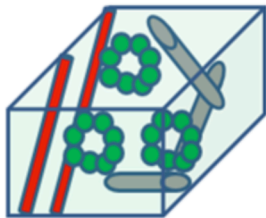
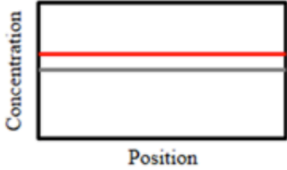
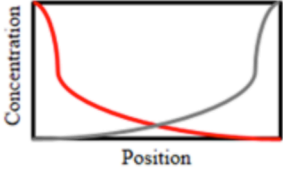
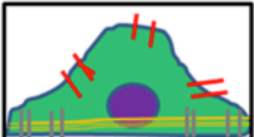
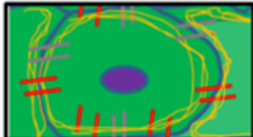
	2D	3D
Model		
Cell Culture System		
Molecular Distribution		
Biophysical Properties		

Fig 1.1. Graphical abstract: Differences in signaling between 2D and 3D. These differences include changes in the cell culture system that alters the molecular distribution of proteins, nutrients and other molecules in the extracellular space. This can lead to variations in the concentration and localization of molecules, which are manifested in different biophysical properties of cell and tissues.

Figure prepared by Matthew Rubashkin

Cells in vivo exist within the context of a multicellular tissue, where their behavior is governed by homo- and heterotypic cell-cell interactions, the material properties of the extracellular matrix, and the distribution and accessibility of various soluble and physical factors (**Fig 1.1**). Yet, most of the methods currently used to study and manipulate cellular behavior in vitro sacrifice physiological relevance for experimental expediency. The fallacy of such approaches to biological investigation has been highlighted

by the recent development and application of three dimensional culture models to cell biology, which have revealed striking phenotypic differences in cell survival, migration, and differentiation in genetically identical cells simply by varying culture conditions. These perplexing findings beg the question: what constitutes a three dimensional culture and why do cells behave so differently in a two versus a three dimensional culture format? In the following review, we dissect the fundamental differences between two and three dimensional culture conditions. We begin by establishing a basic definition of what “three dimensions” means at different biological scales and discuss how dimensionality influences cell signaling across different length scales. We identify which three-dimensional features most potently influence intracellular signaling and distinguish between conserved biological principles that are maintained across culture conditions and cellular behaviors that are sensitive to microenvironmental context. Finally, we highlight state of the art molecular tools amenable to the study of signaling in three dimensions under conditions that facilitate deconstructing cell signaling in a more physiologically relevant manner.

1.2.2. Apoptosis Resistance In Cancer

Programmed cell death by apoptosis is a critical regulator of cell growth and proliferation in both development and cancer. Apoptosis can be onset through various physiologic stresses, including signaling imbalances from elevated oncogene activity, DNA damage associated with hyperproliferation, or changes in extracellular inputs^{9,24,25}. These extracellular inputs can vary from mechanical changes in the extracellular matrix (ECM), such as increased elastic modulus, or altered soluble ligand concentration, such as Ephrin B2. Apoptosis resistance is known to underlie tumorigenesis, metastasis and both classical and effector based drug resistance in breast cancer^{26–29}.

1.2.3. Chemotherapeutic Resistance in Breast Cancer

While chemotherapy has been in use for nearly 60 years in the clinic, it has seen limited success in epithelial-based cancers. These include breast, lung and colon. Targeting of malignant epithelial cells has proven difficult. This is due to a lack of drugs with high specificity, and the development of drug resistance in many patients ^{26,30}. Classical drug resistance arises when the target of interest is no longer accessible or modifiable by a drug. Multiple drug resistance occurs when the secondary effects mediated by the target of interest no longer respond to treatment. This includes cell-cycle checkpoints, mitotic catastrophe, long-term arrest and most notably, apoptosis ²⁶. To this effect, a new class of antibody based drugs has been recently developed that target commonly upregulated trans-membrane growth factor receptors in cancer. These include Avastin®, which targets vascular endothelial growth factor receptor (VEGF), and Herceptin®, which inhibits human epidermal growth factor receptor two (HER2) ^{31,32}.

1.2.4. New Targets For Cancer Therapies

Cancer drugs have traditionally targeted the ‘Hallmarks’ of cancer, including sustained proliferative signaling, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, activating metastasis and resisting cell deaths ²⁴. There is mounting evidence that epithelial cancers contain ‘non-cancerous’ stromal cells that play a role in disease progression through modification of the tumor microenvironment, specifically the extracellular matrix ^{21,33}. Remodeling of the extracellular matrix (ECM) can include alteration of matrix elasticity and growth factor release through protein degradation, increasing the bioavailability of adherence associated ligands, or secretion of factors which remodel the micro or macrostructure of large structural proteins such as laminin ^{8,34}.

1.2.5. Mammary Duct and Luminal Epithelial Cell Biology

Mammary epithelial cells reside within the mammary gland of adult tissue (**Fig 1.2 A**) ³⁵. These cells develop as a dynamic tissue from the epidermis, and require interactions with a stromal cell population to correctly develop in the embryonic and postnatal environment ³⁶. Mammary ducts consist of luminal cells

and myoepithelial cells. Interestingly, it has been shown that myoepithelial and luminal epithelial cells have the intrinsic ability to self-sort³⁷. This self-sorting phenomenon is dysregulated in cancer, and controlled by actomyosin contractility and differential signaling between E-cadherin and P-cadherin³⁷.

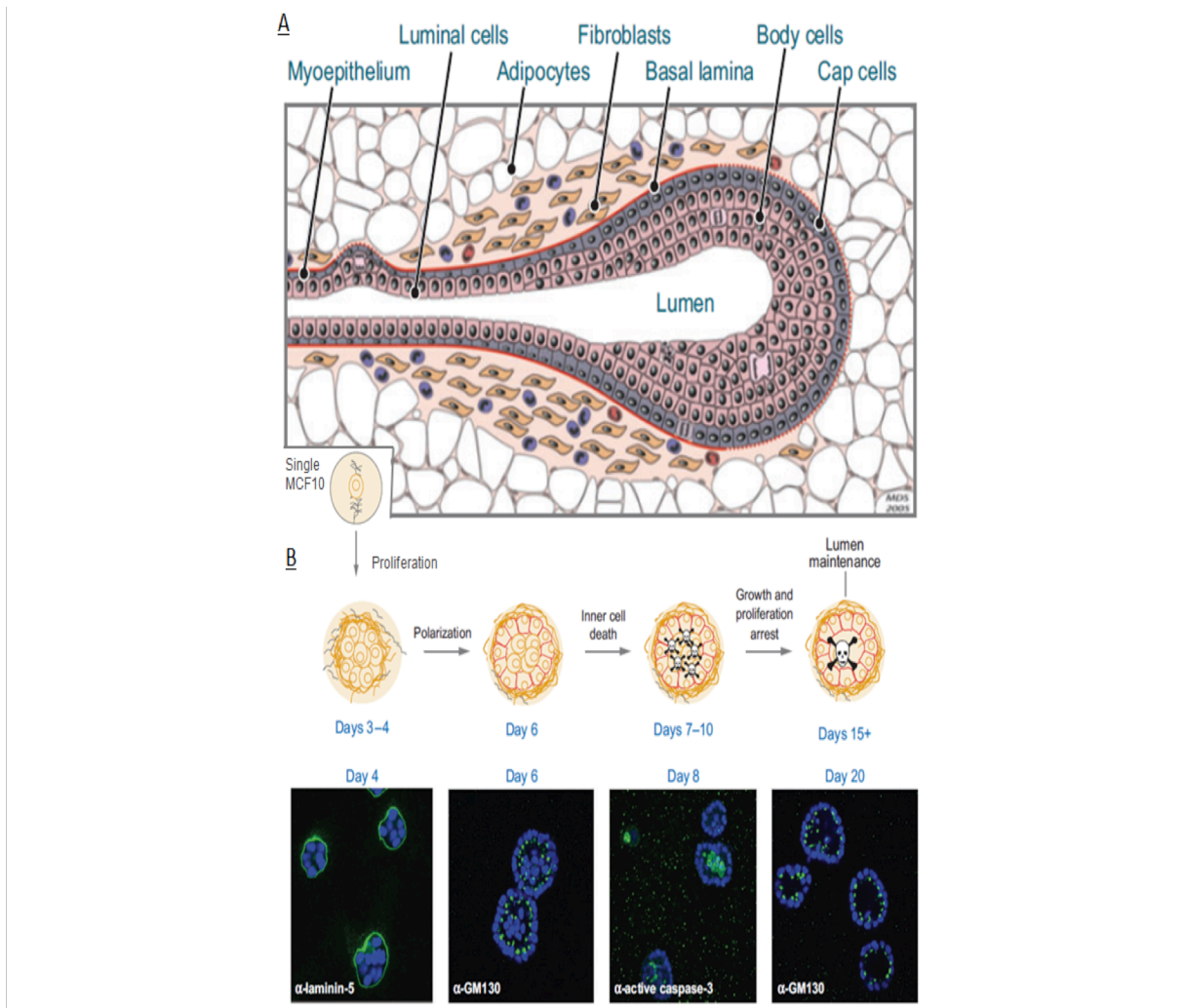


Fig 1.2: In Vivo and In Vitro Mammary Duct/Acini Biology

A. Diagram of the terminal end bud a mammary epithelial duct. Cells of the epithelium such as myoepithelium and luminal epithelial cells, as well as fibroblasts of the stroma are included. *Reproduced from Hebner et al., 2008*³⁵

B. Acini formation *in vitro* using the immortalized cell mammary epithelial cell line MCF-10α. These cells undergo an ordered series of transformative events in 3D culture; including proliferation, polarization, inner cell death and growth arrest. This occurs over the course of three weeks. *Reproduced from Hebner et al., 2008*³⁵

Figure prepared by Matthew Rubashkin

Luminal epithelial cells and myoepithelial cell populations within a duct or terminal end bud are surrounded by a thin basement membrane that consists primarily of laminin [Kass]. Laminin is a heterotrimeric protein consisting of an α , β , and γ subunit. They can combine with either three full short arm (branched portion from main protein body) segments, such as laminin-1, or molecules containing both short and long segments such as laminin-5³⁸. Laminins are able to self-assemble through calcium dependent thermal gelation of N-terminal domains of the short arm domains, producing a meshwork polymer in the basement membrane³⁸. Two integrin populations are well known to interact with laminin in the mammary duct, $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$ ^{39,40}. It has been shown that the ECM components of the basement membrane can modulate mammary epithelial cell growth, differentiation, branching morphogenesis and carcinogenesis³⁵. Stromal cells consisting of fibroblasts, leukocytes and other mesenchymal cells are physically separated from the inner epithelial and myoepithelial cells of the ducts.

1.2.6. *In Vitro* Models of Mammary Duct Biology

Recently, several *in vitro* models of mammary duct development and cancer have been pioneered (**Fig 1.2B**)³⁵. These models offer several advantages over traditional 2D cultures where mammary epithelial cells grown as monolayers do not resemble native *in vivo* physiology. This system can be modified by using basement membrane proteins as a 2D scaffold, enabling these cells to organize into spheres with hollow centers, acini. 3D models may enable more uniform tissue morphogenesis of seeded cells. 3D models involve either seeding cells in unpolymerized matrices, or seeding on 2D polymerized gels followed by the addition of a covering film. In 3D, epithelial cells proliferate till growth arrest in stable acini with polarized cells and a hollow lumen. Structure dissolution is often seen in early stages of breast cancer, where the luminal space and/or the extracellular matrix is invaded by unpolarized and proliferating cells. More complex systems consisting of human adipocytes, fibroblasts and mammary epithelial cells encapsulated in matrigel matrices of varying stiffness have also been recently developed, but not investigated in depth⁴¹. 3D models have proved useful in understanding the homeostasis machinery in glandular lumen, polarity deregulation in pre-metastatic epithelium, how cell-cell and cell-

matrix adhesion pathways can influence the tumorigenic state and the importance of tension in cancer. In latter stages of epithelial cancer, cells often demonstrate an altered phenotype from epithelial to mesenchymal, defined as epithelial-mesenchymal transitions (EMT) ⁴². EMT involves the shedding of epithelial characteristics such as polarity and cell-cell adhesion to acquire motility, invasiveness and apoptosis resistance ⁴².

1.3. Signaling in 3D within the Cell—Intracellular Localization:

1.3.1. Biochemical Reactions, a traditional and modern perspective

Conventionally, signaling events within the cell are modeled as concentration-dependent biochemical reactions. This paradigm assumes that signaling molecules are freely diffusing and uniformly distributed in the cytoplasm and has resulted in a “whole-cell” perspective on signaling, where the cell is considered the reaction vessel in which given concentrations of signaling molecules react with each other. A large body of evidence has arisen to suggest, however, that the localization of a specific signaling molecule within a cell greatly impacts the magnitude and the effect of the resultant signaling event.^{43,44} Signaling molecules can be constrained in space by barriers to free diffusion, scaffolded into multi-protein complexes, or tethered to 2D membranes. Each of these situations significantly alters the diffusivity of signaling molecules, which in turn change the frequency, speed, and duration of biochemical reactions.

1.3.2 Barriers to Diffusion: Scaffolding Proteins

Scaffolding proteins can nucleate large, multi-protein complexes that accelerate reaction kinetics. By bringing multiple components of a signaling cascade together, these proteins alter the reaction kinetics governing a specific signaling cascade. β -arrestin, for example, can bind several components of the MAPK pathway, including RAF, MEK, and ERK.^{45,46} By clustering these proteins, β -arrestin eliminates RAF-MEK binding as a rate-limiting step. Following MEK sequestration by β -arrestin, MEK phosphorylation by RAF occurs at a constant rate.⁴⁵ Physiologically, β -arrestin enhances ERK activation downstream of growth factor receptor signaling by prolonging RAF-MEK-ERK interaction time in the cytoplasm.^{45,47}

Similarly, paxillin serves as a scaffold for proteins at sites of integrin mediated cell-matrix adhesions. These adhesion complexes connect the cytoskeleton to proteins within the extracellular matrix (ECM) and are critical sites for transduction of mechanical inputs to intracellular signaling.⁴⁸ Notably, activated paxillin increases the frequency of interaction between protein kinases such as focal adhesion kinase and Src, which can control downstream Rac activity and increase cell motility.^{49,50} Thus, paxillin mediates mechanotransduction by connecting adhesion proteins with signaling pathways governing motility and proliferation.

1.3.3. Inhibitory Scaffolding Proteins

Scaffolds can also serve to reduce signaling in specific pathways. LKB-1 interacting protein 1 (LIP-1) is a scaffolding protein that sequesters the transcription factor Smad4 from binding to TGF β or BMP promoter sequences, thereby acting as a tumor suppressor.⁵¹ Depending on availability, scaffolding proteins may serve either enhancing or inhibitory functions. An elegant example of this may be found in the MAPK pathway. Kinase suppressor of Ras-1 (KSR1) can scaffold all members of the MAPK cascade.^{52,53} Titrating KSR1 concentration reveals its cooperative role in the pathway until a certain threshold is reached, after which the concentration of scaffolding protein exceeds “signaling-competent complexes” and sequesters individual proteins from interacting with others in the cascade.⁴⁶ In general, however, there is no doubt scaffolding proteins influence signal transduction kinetics by altering the interaction dynamics of signaling components.

1.3.4 Barriers to Diffusion: The Cytoskeleton

When cells are in a 3D environment, the cytoskeleton is usually non-uniform in shape and composition. For example, cells in collagen matrices often exhibit actin cytoskeleton alignment with fibers within the ECM.^{54,55} Changes in cytoskeletal organization, in turn, influence intracellular signaling by acting as barriers to diffusion. The cytoskeleton can be a passive barrier, where increased cross-linking represents more obstacles to free diffusion. Computational molecular models have predicted that components of the MAPK, protein kinase A (PKA) and PI3K-Akt-mTOR pathways have reduced diffusion due to increased molecular crowding.⁴³ Alternatively, cytoskeletal proteins can act as scaffolds to promote or inhibit interaction of signaling molecules. Nanoclustering of epidermal growth factor receptor (EGFR) at the cell periphery can be due to high actin activity at these sites.⁵⁶ Altered clustering and diffusion of receptor tyrosine kinases has been proposed as a molecular mechanism employed by cells in 3D environments to resist drug therapies, such as resistance to HER2 targeting agents in breast cancer via increased HER2 dimerization in 3D.^{57,58} Thus, external stimuli leading to changes in actin density can alter the spatiotemporal behavior of intracellular signaling.

1.3.5 Altered Dimensionality: Membrane Sequestration

In some cases, transition from 3D diffusion in the cytoplasm to 2D diffusion in a membrane can enhance cellular signaling. A good example of this is given by the BCL-2 family of proteins, which mediate caspase driven apoptosis by regulating cytochrome c release from mitochondria.⁵⁹ Bax, a BCL-2 protein typically found freely diffusing in the cytoplasm, activates mitochondrial membrane permeabilization via oligomerization. When cytokine activation of apoptosis occurs,

Bax is recruited to the mitochondrial membrane surface via upstream signaling events.⁶⁰ When Bax is in the cytoplasm, diffusing in a 3D environment, the probability that it will oligomerize with itself is quite low to negligible. However, once it is limited to 2D diffusion in the mitochondrial membrane, the likelihood of interaction with other Bax proteins also anchored in the membrane greatly increases.⁶⁰ This leads to Bax oligomerization, release of cytochrome c, and progression of apoptotic signaling.

1.3.6 Novel Tools for Characterization of Spatial Control of Signaling

As can be seen, the spatial organization of signaling molecules can greatly impact the dynamics of intracellular signaling. Moreover, there are many mechanisms by which the molecular localization can be affected. Traditional biochemical assays like Western blots and ELISAs lack the subcellular resolution to capture this information (Table 1A). Electron microscopy has long been used to characterize the molecular organization of the cytoskeleton, membranes, and organelles in 2D and 3D, including changes in epithelial cell structure after hormone stimulation or altered mechanical properties of the ECM^{61–63} (Table 1B). However, 3D samples must be fixed, dehydrated, and sectioned, prohibiting studies of spatial or temporal dynamics of cells in 3D environment. Light microscopy, on the other hand, is highly conducive to characterizing how protein localization influences cellular signaling. To empirically determine the diffusion coefficients and dissociation constants, fluorescence recovery after photobleaching (FRAP) can be used. FRAP involves tagging a protein of interest with a fluorescent molecule, taking an image of the basal fluorescence level, photobleaching a specific area of interest with high intensity laser illumination, then quantifying the recovery in fluorescence in intensity as unbleached molecules switch places in the area of interest with the bleached molecules (Table 1D). FRAP is a technique that can be used to measure the ensemble change in protein behavior at

a region of interest and has been used to great advantage to determine the binding kinetics of transcription factors and the mobility of receptors in the plasma membrane (Table 1E).^{64,65}

1.3.7. Tools for studying protein-protein interactions

To study protein-protein interactions, techniques like Förster Resonance Energy Transfer (FRET) and Fluorescence-Lifetime Imaging Microscopy (FLIM) can be used. FRET imaging involves using the energy transfer between fluorophores as a proxy for distance between fluorescently tagged molecules; it is sensitive within the 5-10nm range and is often used as an indication of protein-protein binding (Table 1E). FRET has been used to study receptor-ligand binding at the membrane as well as protein conformation.^{66,67} Grashoff *et al.* demonstrated the use of an intramolecular FRET probe that allowed detection of vinculin stretching under cytoskeletal tension.⁶⁸ FLIM, on the other hand, takes advantage of the sensitivity of a fluorescent molecule's lifetime—the exponential rate of decay of its fluorescence—to its environment to characterize protein-protein interactions. Unlike FRET or FRAP, which require a genetically encoded probe or fluorescent antibody, FLIM can use the inherent fluorescence of specific molecules such as NADPH or collagen and is a promising technique for analysis of unmodified and untagged human cancer cells in the future (Table 1F).⁶⁹

	Experiment	Methodology	Sample Needed	Scale Context	Temporal Dynamics	Spatial Dynamics	3D Amenable	Experimental Constraints	Materials / Cost
A	<i>Western Blot</i> 	Biochemical, antibody	~10 ⁶ Cells	Tissue				Difficult to analyze membrane proteins, cross-reactivity of antibodies	Chemi-luminescent imager - \$
B	<i>Electron Microscopy</i> 	Microscopy of endogenous structure or antibody-Au	~100 Cells	Submolecular			✓	Dehydration is standard, but liquid imaging in special environmental chambers is possible	Scanning or Transmission Electron Microscope - \$\$\$
C	<i>Single Molecule Tracking</i> 	Microscopy of GEP (genetically encoded probe), Quantum Dot	10-1000 labeled proteins per cell	Single Protein	✓	✓		Low signal to noise ratio, restricted to molecules at the plasma membrane	Total Internal Reflectance Microscope (TIRF) - \$\$\$
D	<i>FRAP</i> 	Microscopy of antibody/GEP	>10000 labeled proteins per cell	Subcellular	✓	✓	✓	Laser damage to protein of interest and entire cell	Laser Scanning Microscope - \$\$\$
E	<i>FRET</i> 	Microscopy of antibody/GEP	>10000 labeled proteins per cell	Subcellular	✓	✓	✓	1-10nm resolution,	Widefield Microscope - \$\$
F	<i>FLIM</i> 	Microscopy of endogenous structure/ antibody/GEP	>10000 labeled proteins per cell	Subcellular	✓	✓	✓	Special cases: No antibodies or GEP	Pulsed Laser Microscopy - \$\$\$\$

Table 1.1 Quantitative techniques for determining reaction kinetics.

Table prepared by Matthew Rubashkin and Guanqing Ou

1.3.7. Role of protein localization in future studies

The impact of 3D and subcellular distribution of signaling molecules on intracellular signaling is not often discussed despite the fact that localization can profoundly affect cell and tissue behavior and fate^{8,13,70–74}. Nonetheless, as new technologies that allow us to peer into the cell emerge, the need to understand the role of protein localization in signaling cannot be ignored.

1.4. The Cell in 3D—Cell and Matrix Interactions:

1.4.1. Protein localization within cell and matrix interactions

As we zoom out to examine the cell, we must consider it in its natural context. Within the body, cells interact in 3D with the extracellular matrix (ECM) and neighboring cells. Through these interactions, cells gather information about their surroundings, which is integrated to determine cell behavior and fate. In addition, a cell's 3D environment affects the organization of intracellular components and thus the context in which signaling occurs. This section will feature how intracellular signaling is dependent on the context of cell shape, barriers to intracellular diffusion, and protein localization.

1.4.2. Adhesions and Junctions: Gathering Information from the Environment

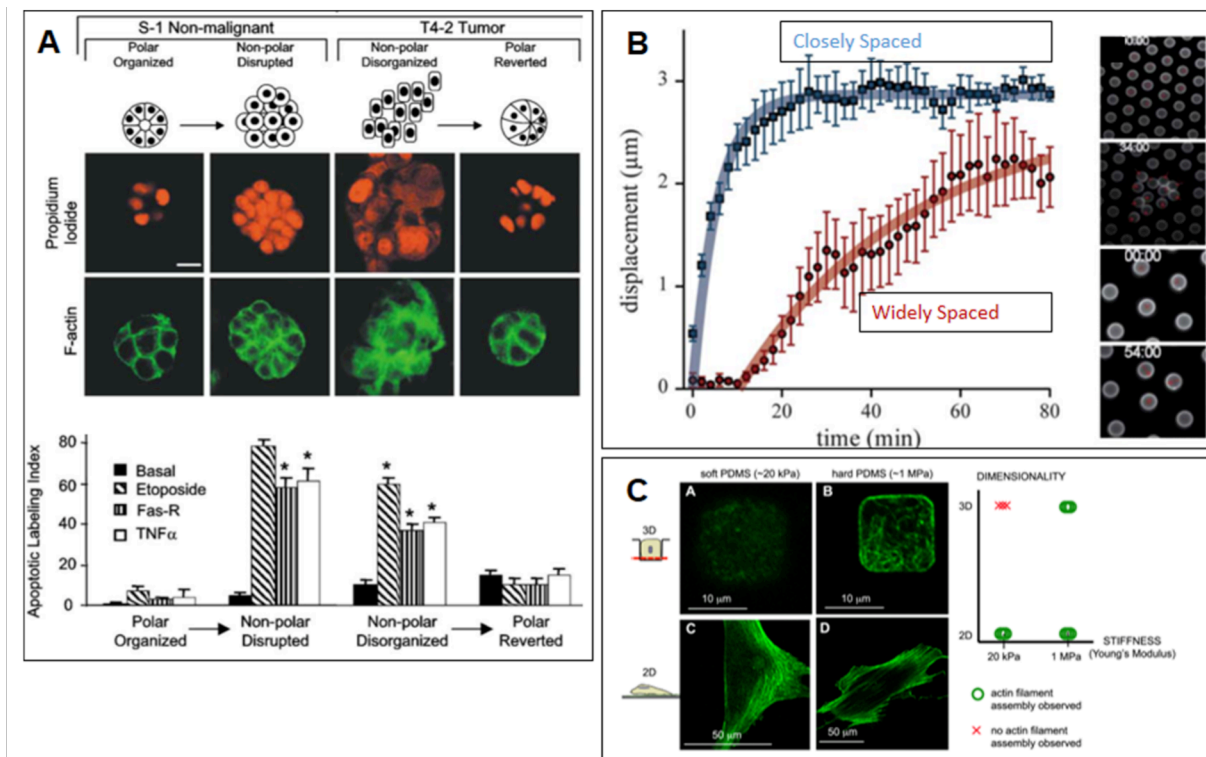


Fig 1.3 A. Changes in cell shape and 3D organization modulate intracellular signaling. (A) Transitions from nonmalignant S1s to malignant T4-2 epithelial cells show a reversal in cell polarity and 3D organization.⁴¹⁷ B. Cells on closely spaced pillars behave as though on a 2D environment, whereas those on widely spaced pillars exhibit 3D-like behaviors⁴¹⁸. C. Actin organization in cells on pillar-based 2D substrates vs. inside 3D microwell scaffolds⁸⁰.

Figure prepared by Matthew Rubashkin

Cell adhesion to the external environment can dictate cell shape, growth, proliferation, and apoptosis signaling.^{75,76} Cells attach to neighboring cells and to ECM molecules, including secreted proteins (i.e. collagens and laminins), proteoglycans (heparin and chondroitin sulfate), and glycosaminoglycans (hyaluronic acid). Cells attach to these extracellular molecules via a plethora of adhesion receptors, including integrins, cadherins, selectins, discoidin receptors, and syndecans.⁷⁷⁻⁷⁹ These adhesion receptors bridge the connection between the extracellular environment and the intracellular cytoskeletal and signaling machinery.

Engagement of the ECM in all three dimensions, compared to only at the basal side of cells as in conventional culture, significantly influences cell behavior. Depending on whether cells are cultured in 3D vs. 2D, a cell's response to a given signal can be significantly altered.⁸⁰⁻⁸² Mammary epithelial cells with intact cell-cell junctions in 3D hydrogels are more resistant to apoptosis inducing factors like tumor necrosis factor alpha (TNF- α) when compared to cells in 2D or cells in 3D lacking cell-cell junctions (**Fig 1.3A**).⁸² This resistance highlights the importance of cell-cell interactions in 3D and implies a connection between adhesion-dependent signaling and intracellular signaling directing cell fate.⁸² Culture in 2D vs. 3D also alters cell spreading and traction force.⁸¹ When fibroblasts are plated onto closely spaced small pillars (5 μ m), cells form adhesions on the top and side of the pillars, simulating a 3D environment.⁸¹ Under these conditions, fibroblasts pull with high levels of force on the external environment (**Fig 1.3B**).⁸¹ Conversely, if the cells are on pillars with increased surface area (15 μ m) that are widely spaced, simulating a 2D environment, cells do not form adhesions on the sides of the pillars and traction on the external environment is decreased (**Fig 1.3B**).⁸¹

1.4.3. Manipulating the ECM Can Lead to Changes in Cell Metabolism

The dimensionality, rigidity, and topology of the external environment have been implicated as a regulating factor in cytoskeleton assembly and metabolism.⁸⁰ Through the use of polymeric hydrogels, researchers can control rigidity by altering the ratio of crosslinker to monomer ratio in the gel solution. When fibroblasts are seeded in 3D silicone microwells of varying stiffness, they exhibited a range of novel behaviors compared to 2D environments. In soft 3D environments, fibroblasts downregulate actin filament assembly and upregulate mitochondrial activity, in contrast to in compliant 2D, stiff 2D, or stiff 3D environments (**Fig 1.3C**).⁸⁰

1.4.4 Altered Extracellular Context Leads to Cytoskeletal Reorganization

Many of the affected cell behaviors associated with 3D culture have been attributed to altered cell shape. Altered cell shape leads to reorganization of the cytoskeleton as well as changes to membrane tension, curvature, and composition, changing the context in which intracellular signaling takes place.

Cell shape directly influences the organization of the cytoskeleton, which can have profound effects on intracellular signaling. In mesenchymal stem cells (MSC), cell spreading leads to RhoA activation of Rho activated kinase (ROCK), myosin light chain kinase phosphorylation, elevated myosin contractility, increased traction forces to the external environment, and differentiation to an osteoblast phenotype.⁸³ Conversely, if MSCs are rounded or express a dominant negative RhoA, these cells differentiate into an adipocyte phenotype.^{83,84}

1.4.5 Altered Extracellular Context Leads to Membrane Reorganization

Cell shape, in combination with ECM stiffness, governs membrane properties like curvature and tension.^{85,86} These characteristics of the plasma membrane, in turn, determine membrane composition, membrane protein distribution, and intracellular trafficking rates.^{86,87} Several families of proteins, including the FERM (4.1 protein, Ezrin, Radixin, and Moesin) and BAR (Bin/amphiphysin/Rvs) domain proteins, have molecular motifs that are sensitive to membrane curvature. Proteins containing these domains, which include the ARF (adenosine-ribosylation factor) family,^{88,89} small GTPases like Rac,^{88,90} and guanine exchange factors,⁹¹ can assemble multi-protein complexes and preferentially target these complexes toward a curved membrane, thereby affecting protein localization and signaling. Similarly, membrane tension is a key regulator of endo- and exo-cytosis.^{86,87} Increased membrane tension decreases the probability of vesicular budding and favors exocytic merging of vesicles with the plasma membrane.⁸⁷ This affects the overall balance of vesicular trafficking within the cell, which can influence such diverse signaling pathways as growth factor receptor signaling, reactive oxygen species production, and phagocytosis.

1.4.6. Differences in Membrane Biology in 2D and 3D

The composition and organization of the plasma membrane is different between 2D and 3D, including cholesterol and sphingomyelin content and organization.⁹² This membrane reorganization can alter the distribution of lipids and lipid rafts in the membrane.^{83,93,94} These differences can then affect lipid-dependent signaling, such as signaling through the PI3K-Akt-mTOR pathway, which regulates cell metabolism, migration, and apoptosis.⁹⁵ The PI3K-Akt-

mROR pathway is initiated by phosphatidylinositol 3-kinase (PI3K) recruitment to the membrane and the generation of lipid rafts with phosphatidylinositol-3,4,5-trisphosphates (PtdIns(3,4,5)P₃) and is attenuated by phosphatase and tensin homolog (PTEN).⁹⁶ PI3K and PTEN are usually sequestered in distinct lipid groups in the membrane which display limited diffusion and mixing between lipid rafts.⁹⁷ This lipid raft-induced segregation of PI3K and PTEN is exacerbated in spread cells, where increased adhesions lead to lipid raft formation in the plasma membrane via microtubules and Arf6 recycling.⁹⁸ When cells are rounded via cytoskeletal disruption or via micropatterned surfaces, lipid rafting is decreased, PI3K and PTEN interactions are increased and PI3K signaling is attenuated.^{99,100} When cells are spread and PtdIns(3,4,5)P₃ lipid rafts are present, downstream Akt activity leads to differences in BCL-2 mediated apoptosis and cell migration.^{100,101}

1.4.7. Differences in Cell Signaling in Rounded versus Spread Cells

When cells transit from a rounded to spread morphology, the Rnd family of proteins is targeted to lipid rafts via the KERPA (Lys-Glu-Arg-Pro-Ala) sequence at their N-terminus.¹⁰² Rnd proteins recruit p190RhoGAP to lipid rafts at the plasma membrane, leading to increases in Rho activity that is mediated by filamin cross-linking of the actin.^{102,103} This behavior can be ablated through inhibition of actin polymerization and filamin dependent actin crosslinking.¹⁰³

The pattern of cell-ECM and cell-cell adhesions can dictate the landscape in which other signaling molecules may interact on the cell membrane. Ephrin signaling, for example, is sensitive to the spatial organization of ephrin receptors on the cell membrane.¹⁰⁴ Salaita *et al.* restricted ephrin receptor diffusion in the membrane and showed obstacles to receptor

oligomerization and dramatically influence intracellular signaling. Specifically, limiting ephrinA1 movement led to changes in cytoskeletal organization and metalloprotease secretion in cancer cells.¹⁰⁵ As many membrane-bound proteins act by forming protein complexes, the organization of adhesions on the cell membrane can greatly influence the dynamics of these signaling events.

1.4.8 Adhesions and Junctions: Mediating Cell Response to Dynamic Input

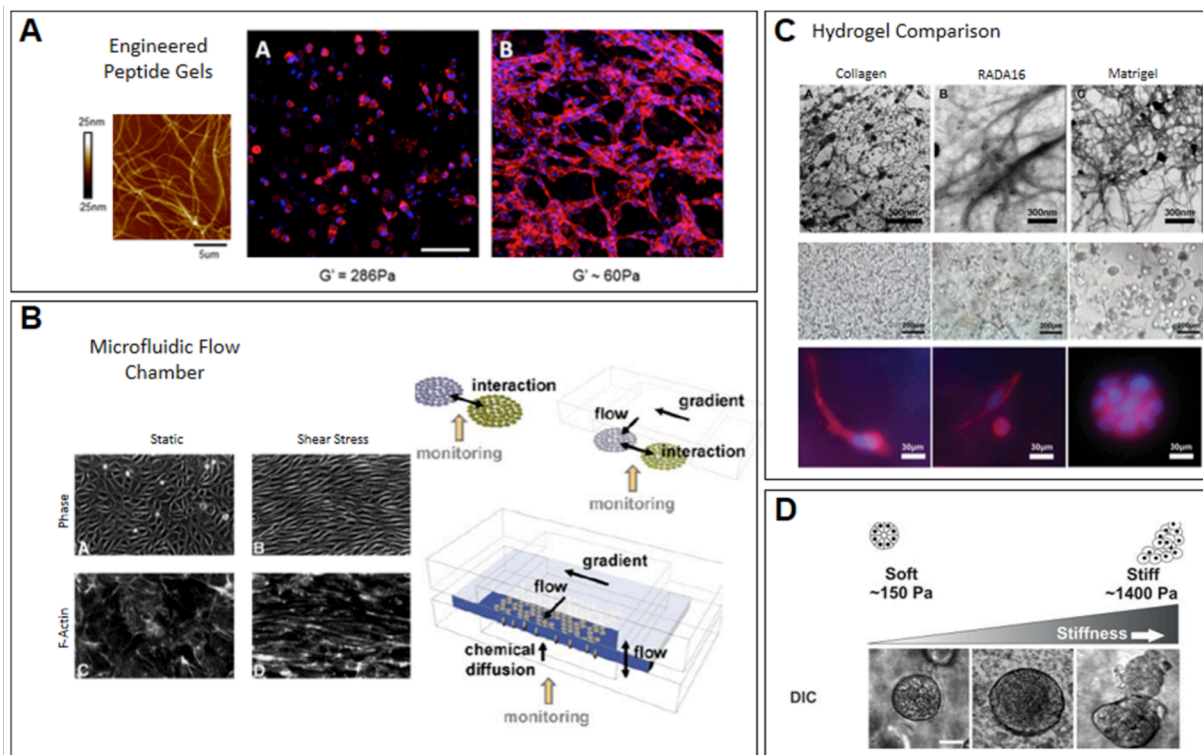


Fig 1.4. Tools for studying cell and matrix mechanical interactions in 3D. **A.** Peptide gels with consistent pore sizes but variable stiffnesses.¹¹⁴ **B.** Microfluidic chambers allow application of flow, chemical gradients, and mechanical forces.^{106,107} **C.** In hydrogels, different materials lead to differential cell behavior.¹¹⁵ **D.** Example of cellular organization on collagen gels with different stiffnesses.⁴¹⁹

Figure prepared by Matthew Rubashkin and Guanqing Ou

In addition to transducing static information about its environment, adhesion molecules can mediate dynamic cellular responses to external input. In 3D environments like the lumen of a blood vessel, signaling at endothelial cell-cell and cell-ECM junctions regulate vessel integrity

and leukocyte extravasation from the vasculature. Upon application of fluid shear stress in vitro, endothelial cells in culture undergo filamentous actin and focal adhesion remodeling in the direction of flow in a VE-cadherin dependent manner (**Fig 1.4B**).^{106,107} Downstream, this results in Rho GTPase activation, which works in conjunction with vascular endothelial growth factor receptor 2 (VEGFR2) to activate p38 MAPK pathway activity.^{108,109} Thus, endothelial cells sense dynamic changes in the environment and feed this information into existing intracellular signaling to influence cell behavior.

The need to process information about the extracellular environment is even more relevant for migrating cells. Leukocyte transit from the vasculature to the subendothelial matrix, for example, relies upon mechanically activated adhesion receptors gathering information about the vascular endothelium upon which leukocytes travel. Endothelial presentation of selectins may be recognized by white blood cells flowing through the blood. Ligation of P-selectin glycoprotein ligand-1 (PSGL-1) with glycoprotein ligands on the surface of a rapidly moving leukocyte can result in strengthening of the P-selectin-PSGL-1 catch bond, leading to leukocyte deceleration.^{110,111} While P-selectin and PSGL-1 bonds are transient on the scale of ~0.5 seconds, they enable stronger, longer lasting integrin-ECM bonds to form.^{110,111} These cell-ECM adhesions then lead to leukocyte polarization, accumulation of the membrane lipids phosphatidylinositol-3,4,5-trisphosphates (PtdIns(3,4,5)P₃), activation of Rac1 and Akt, cytoskeletal remodeling, and transmigration of the endothelium.^{110,112}

1.4.9. Novel tools to study signaling in 3D matrices

To study cell adhesion in 3D environments, many techniques have been developed to encapsulate cells in hydrogels, hydrated polymer networks that behave as viscoelastic solids. Commonly used polymers in hydrogels include naturally derived materials including collagen, proteoglycan such as hyaluronic acid, fibrin gels, cellulose; and synthetic polymers such as polyethylene glycol (PEG), poly-vinyl-alcohol (PVA), poly lactic-glycolic-acid (PLGA), and engineering peptide based biomaterials. Peptide hydrogels are a useful system to study cell signaling in 3D as they allow tuning of individual environment parameters including matrix elasticity, cell adhesion binding sites, and degradation.¹¹³ Examples of different peptides used include elastin and silk like polypeptides, or novel repetitive peptide sequences that form fibrils in ionic environments such as ‘KFE’ gels, which are ((acetyl)-FKFEFKFE-CONH2).^{113,114} By modifying these gels with arginine-glycine-aspartic acid (RGD) sequences for integrin binding and changing the concentration of peptide, KFE gels can be fabricated to allow tuning of cell binding, matrix compliance and pore size (**Fig 1.4A**).¹¹⁴ When these gels are optimized, they enable formation of cell-ECM and cell-cell connections that promote endothelial tube formation, facilitating the study of 3D endothelial cell signaling in an *in vitro* system that far more accurately resembles a capillary bed compared to 2D culture (**Fig 1.4A**).¹¹⁴

Embedding cells in a hydrogel with specific molecular composition and concentration can specifically control the 3D microenvironment *in vitro*. Individual cells can be placed in self-assembling peptide gels, cross-linked collagen matrices or reconstituted basement membrane hydrogels such as Matrigel (**Fig 1.4C**).¹¹⁵ Breast cancer cells such as MDA-MB-231, for example, alter their morphology and adhesion in response to altered ECM properties, while MCF10a cells create stable spheroids in compliant, but not stiff collagen matrices.^{115,116} Furthermore, cell behavior can be influenced by controlling ECM properties like stiffness, which, in the case of collagen gels, can be modulated by increasing collagen concentration or via inhibition of lysyl oxidase mediated collagen crosslinking (**Fig 1.4D**).^{8,117}

1.4.10. Careful selection of cellular environment is critical for the study of cell signaling

The effect of altered cell-matrix and cell-cell interactions on intracellular signaling is undeniable. The cell is continuously gathering information about its surroundings and incorporating this information into its decision making circuits. As such, researchers need to be particularly aware of their phenomena of interest and how cellular context can introduce confounding factors into their studies. Culturing cells in 2D vs. 3D, or one ECM component vs. another, results in such cell-wide changes that choices of experimental conditions must be thoughtfully made.

1.5. 3D Signaling at the Multicellular Level:

1.5.1. Multidimensional interactions at the tissue level

Cells in the body exist in a three-dimensional environment. They interact on all sides with other cells, the extracellular matrix, and interstitial fluid. These interactions provide input that the cell then integrates to determine behavior and fate. At the tissue level, two major factors influence cell behavior--local gradients in signaling molecules and multicellular structures. The following section highlights molecular gradients in development and duct-like multicellular structures.

1.5.2. Molecular Gradients in Multicellular Structures:

Sources and sinks for signaling molecules, combined with interstitial pressure, serve to establish molecular gradients that can differentially influence cells within a tissue based on their location. These gradients are established through a combination of cell secretion, protein diffusion, proteoglycan-mediated stabilization, and endocytic depletion in neighboring cells.^{118–120} Some source, or collection of cells secreting the morphogen, acts as the focal point from which diffusion distributes the morphogen. Proteoglycans in the extracellular matrix, like glycosaminoglycans (GAGs), sequester and maintain local supplies of morphogen.¹²¹ Finally, depletion of the morphogen from interstitial fluid occurs via receptor-binding, endocytosis, and degradation.^{120,122}

1.5.3. Molecular Gradients in Development

Many examples of molecular gradients can be found in development. In the early *Drosophila* embryo, before nuclei are separated by cell membranes, a Bicoid (Bcd) gradient governs the expression of gap genes.^{123,124} These genes dictate the anteroposterior patterning of the embryo,

and if mutated, can lead to a loss of continuous segments in the developed organism.^{123,124} At later stages of development, Decapentaplegic (Dpp) and Wingless (Wg) gradients in the wing imaginal disc have been linked to proper segmentation and wing development.^{120,125} In the imaginal disc, the Dpp gradient arises through a combination of changes to Dpp secretion, diffusion, stabilization and depletion. Dpp at the cell surface is captured at the surface of a cell via low-affinity interactions with heparin sulfate proteoglycans, decreasing the diffusion of Dpp. Improved availability of Dpp leads to increased interactions with its receptor, resulting in amplified intracellular signaling.^{126,127}

Similarly, gradients of the vertebrate Dpp homolog, bone morphogenetic protein (Bmp), is vital for dorsal/ventral patterning.^{128,129} High local BMP levels specify ventral tissues, while low BMP signaling leads to development of dorsal tissues.¹²⁸ Sonic hedgehog (Shh), transforming growth factor- β (TGF β), and fibroblast growth factor, gradients have similar effects on developing cells.^{130,131} In all these cases, progenitors have concentration-dependent responses to morphogens. In chicks, for example, the duration of neural cells' responses to Shh is directly proportional to its local concentration.¹¹⁸ This Shh response controls the expression of important transcription factors, which in turn direct differentiation into specific neuronal subtypes.¹³¹

1.5.4. Molecular Gradients in Lower Organisms

While simple model organisms like *D. melanogaster* and *C. elegans* make investigation of the effects of these morphogen gradients possible, translation and extension of this work into more complex specimens is a major challenge. The deterministic pattern of development in *C. elegans* and the small number of cells in the developed organism, for example, facilitate reliable lineage

tracing, where transfection of specific cells with genetic markers allows identification of their progeny.¹³² This can be combined with secondary markers that indicate the relative expression of genes responsible for driving different cell fates.¹³² The resilience of *D. melanogaster* permits the use of simple knockout systems to identify the role of genes like Dpp and Wg.

While studying molecular gradients in model organisms have led to enormous strides in understanding the processes governing development, the same techniques cannot be applied to understand the establishment and effect of molecular gradient in more complex systems. Many knockouts are embryonic lethal in mammals, precluding analysis of their effects on development. Further, many of these model organisms are transparent and thus particularly amenable to imaging approaches. Most tissues are not transparent and preclude the use of common visualization techniques. The increased genomic complexity of higher order organisms makes systematic screening an often untenable challenge. These factors have hampered researchers' investigation and left an important facet of biological function largely unexplored. While hints of the role of molecular gradients have arisen in studies of human embryonic development, little is known about their importance in normal tissue function. Yet, given that many of the same mechanisms that apply to morphogen gradients apply in normal tissues, it is probable that gradients play an equally important role in signaling in tissues.

1.5.5. Transport phenomena in tissues

A key indication that molecular gradients exist in tissues, and must therefore be processed by cells, is the myriad convective transport phenomena known to be at play in tissues. These transport processes generate interstitial flow that would result in local gradients in signaling

factors. A major driver of convective flow is interstitial pressure. Interstitial pressure results from hydrostatic pressure and osmotic pressure differences among the vasculature, interstitium, and lymphatics.¹³³ Positive pressure from blood vessels, combined with leaky capillaries, pump fluid and soluble factors into the tissue. Negative pressure gradients between the tissue and lymphatic system is maintained by pumping in the draining lymphatic capillaries.¹³³ Tissue stretching and compression due to regular movement can also cause transient interstitial flow. These patterns are altered in tumors, where leaky vasculature and increased interstitial pressure inhibit leakage from capillaries into the interstitium. This altered transport can contribute to oxygen deprivation within the tumor, creating hypoxic conditions that activate HIF-1 (hypoxia-inducible factor 1), a transcription factor implicated in control of metabolism, invasion, and apoptosis.¹³⁴ HIF-1 upregulation, in turn, has been strongly implicated in increased tumor aggression and therapeutic resistance.^{135,136}

1.5.6. Cell and tissue generated fluid flow

In addition to pressure differences, ciliary movement in the lung and intestines can also drive interstitial flow. These small convective flows have been demonstrated to govern normal branching morphogenesis in the embryonic lung by directing points at which splitting should occur.¹³⁷ Interstitial flow has been demonstrated to allow generation of pericellular gradients, where directional flow around a cell secreting some signaling factor leads to asymmetrical distribution of that factor around the cell, thereby facilitating generation of an autologous chemotactic.¹³⁸ On a larger scale, immune cells use molecular gradients established by interstitial flow to home to the lymphatic system. While these chemotactic behaviors are well documented, there is no doubt that non-motile cells are similarly affected by gradients established by

interstitial flow. The presence of extracellular signaling factors and the factors influencing differential exposure to these factors within a multicellular structure are, therefore, relevant aspects to include if we are to gain a full picture of the dynamic nature of signaling *in vivo*.

1.5.7. Cell Organization and Signaling in Multicellular Tissues

Cellular localization is an important aspect of signaling at the multicellular level, affecting its exposure to other cells, the ECM, and soluble signaling molecules within the interstitial fluid. In contrast to monolayer culture, where all cells have essentially the same relationship with one another and the nutrient source, multicellular structures can dramatically alter the environment in which a cell receives signals. In human breast ducts, for example, luminal and myoepithelial cells encounter dramatically different environments. Whereas the inner luminal epithelial cells are exposed to the hollow duct and lined with myoepithelial cells, the myoepithelial cells are sandwiched between the luminal epithelial cells and the basal lamina. Thus, myoepithelial cells are subject to more ECM signals than the luminal epithelial cells. Yet this complexity is not captured in conventional culture of these cells, which could lead to artifactual observations that do not translate to *in vivo* behavior. Culture of transformed mammary epithelial cells in 3D matrix can suppress proliferation and reestablish cell polarity, while oral squamous cell carcinoma cells exhibited higher angiogenic potential under 3D conditions.^{139,140}

1.5.8. Cell paracrine signaling

Similarly, the importance of paracrine signaling from neighboring cell types has been highlighted by the necessity for feeder culture of stem cells. Since the early 1950s, maintenance of the undifferentiated state of embryonic stem cells *in vitro* has been achieved via co-culture

with fibroblasts.^{141,142} Recently, this has been substituted with culture of conditioned media containing fibroblast-secreted factors like BMPs and leukemia inhibitory factor (LIF) or 3D culture of stem cells.^{143–145} Cells are rarely in isolation in the body; there is no doubt that many other such mechanisms of cell control have been lost in the transition to *in vitro* culture.

A functional example of the importance of multicellular structures may be found in the brain, where neuron-glia networks cooperate to transduce signals.^{146–148} Neurotransmitters secreted by neurons can initiate signaling in glial cells, which then secrete neuromodulatory factors that act on neurons within the synapse, including glutamate and thrombospondin.^{146,148–150} Glial cell morphology allows them to contact multiple neuronal cell bodies and extend into thousands of synapses, making them uniquely positioned to act as an integrator and modulator of neuronal activity.¹⁴⁸ As such, the specific positional relationships among neurons and glial cells within a network can be key to how that network responds to and propagates stimuli.

1.5.9. Nutrient accessibility in 3D

In addition, multicellular structures often optimize cellular access to nutrients. In particular, hepatocytes are known for their sensitivity to *in vitro* culture. Compared to the dense populations found *in vivo*, hepatocytes lose viability or transdifferentiate at high densities in monolayer culture.^{151–153} This can be partially addressed by culturing them in a microfluidic device with a geometry that maximizes cell access to a central fluidics chamber that mimics the hepatic artery.¹⁵⁴ This enhanced proximity to nutrients via altered organization allowed high-density hepatocyte culture closer to *in vivo* conditions.¹⁵¹

Cells do not signal in isolation within the body. They are parts of multicellular structures exposed to gradients of signaling molecules and physical forces. Their positions within a highly organized structure dictate their exposure to a specific set of signaling inputs, which may differ from those of neighboring cells. This organization and coordination permits these cells to work as a unit to perform a biological function. Accordingly, a full understanding of cell signaling requires studying the cell as part of a larger unit.

1.5.10. Novel techniques to study multicellular structures in 3D – Hydrogel Systems

Many of the conditions that may govern signaling in 3D have eluded researchers due to inadequacies in culture methods. Recent recognition of the importance of the context in which signaling occurs, including molecular gradients, cell-ECM interactions, and multicellular organization, has led to the development of novel culture techniques that seek to recapitulate 3D conditions seen *in vivo* (**Table 1.2**).

The most basic of these is 3D encapsulation culture. This involves embedding cells in a hydrogel composed of ECM components to better mimic the immersive environment of the body. This technique has spawned the development of many biomimetic or biosynthetic materials, including Matrigel™, hyaluronic acid, collagen, and alginate.¹⁵⁵ However, except in cases of self-organization, this method still fails to recapitulate the complex organization of cells and the ECM *in vivo*.

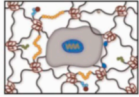
Method	Type	Example(s)	Benefits	Constraints
Hydrogel Systems  Further reading: [118,127,128]	Natural	Collagen, Matrigel, chondroitin sulfate, hyaluronic acid, chitosan, cellulose, alginate, fibrin, elastin ¹²⁹	Can be derived from polymers naturally found in-vivo in a tissue of interest, degradable	Polymer variability with heterogeneous ligand presentation and matrix properties.
	Biomimetic	Poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(hydroxyethyl-methacrylate), (poly(HEMA)), self assembling peptide gels (SAP) ¹³⁰	Permits extensive chemical functionalization and better control of mechanics and architecture	Cell toxicity, biocompatibility issues
	Biosynthetic	Collagen peptide Arg-Gly-Asp (RGD) conjugated biomimetics, and matrix metallo-proteinase (MMP) sensitive biomimetics ¹³⁰	Can combine positive features of natural and biomimetic scaffolds	Often difficult and expensive to synthesize
Tissue-Specific Microfabricated Culture Systems  Further reading: [131]	Geometric mimicry	Liver Subunit ¹¹⁷	Allows dense hepatocyte culture	Requires micro-fabrication facilities and constant perfusion; elastomeric materials not always representative of ECM components and properties; difficult to scale size of culture
		Vascular Beds ¹²²	Controlled geometry and cell seeding; coculture	
	Functional mimicry	“Lung on a chip” ¹²³	Simulated “breathing” via pressure-mediated dilation of PDMS membrane	

Table 1.2: Summary of 3D Culture Systems

Table prepared by Guanqing Ou and Matthew Rubashkin

1.5.11. Novel techniques to study multicellular structures in 3D – Lithography Systems

Soft lithography is proving to be a boon for researchers seeking more precise control of their culture conditions (**Fig 1.5**). This family of methods adapts approaches used in manufacturing microelectronic chips to fabricate or replicate nanometer scale structures and patterns on elastomeric materials like polydimethylsiloxane (PDMS).¹⁵⁶ These methods offer unprecedented control over features in a culture system, including geometry, patterns of ECM protein functionalization, and application of flow.^{156,157} Soft lithography has been used to generate features that mimic the layout of a blood vessel, or facilitate multi-cell type culture (**Fig 1.5C, 1.5D**).^{158,159} The open design aspect of soft lithography also allows researchers to model *in vivo*

multicellular organization, leading to fabrication of devices that recapitulate the shape and layout of major organ subunits within the liver and lung **(Fig 1.5A, 1.5B)**.^{154,160,161} In addition, microfluidic culture permits introduction of flows, in contrast to conventional static culture. This allows recapitulation of both vascular flow and interstitial flow, facilitating the establishment of molecular gradients **(Fig 1.4B and 1.5C)**.^{162,163} 3D printing confers even greater control over culture conditions, allowing layer by layer construction of extracellular matrix and cells. Biodegradable carbohydrate scaffolds can be used to create architecturally complex “organs”. Miller *et al.* used this method to create 3D cylindrical networks of cells within an ECM network **(Fig 1.5C and 1.5D)**.¹⁵⁹

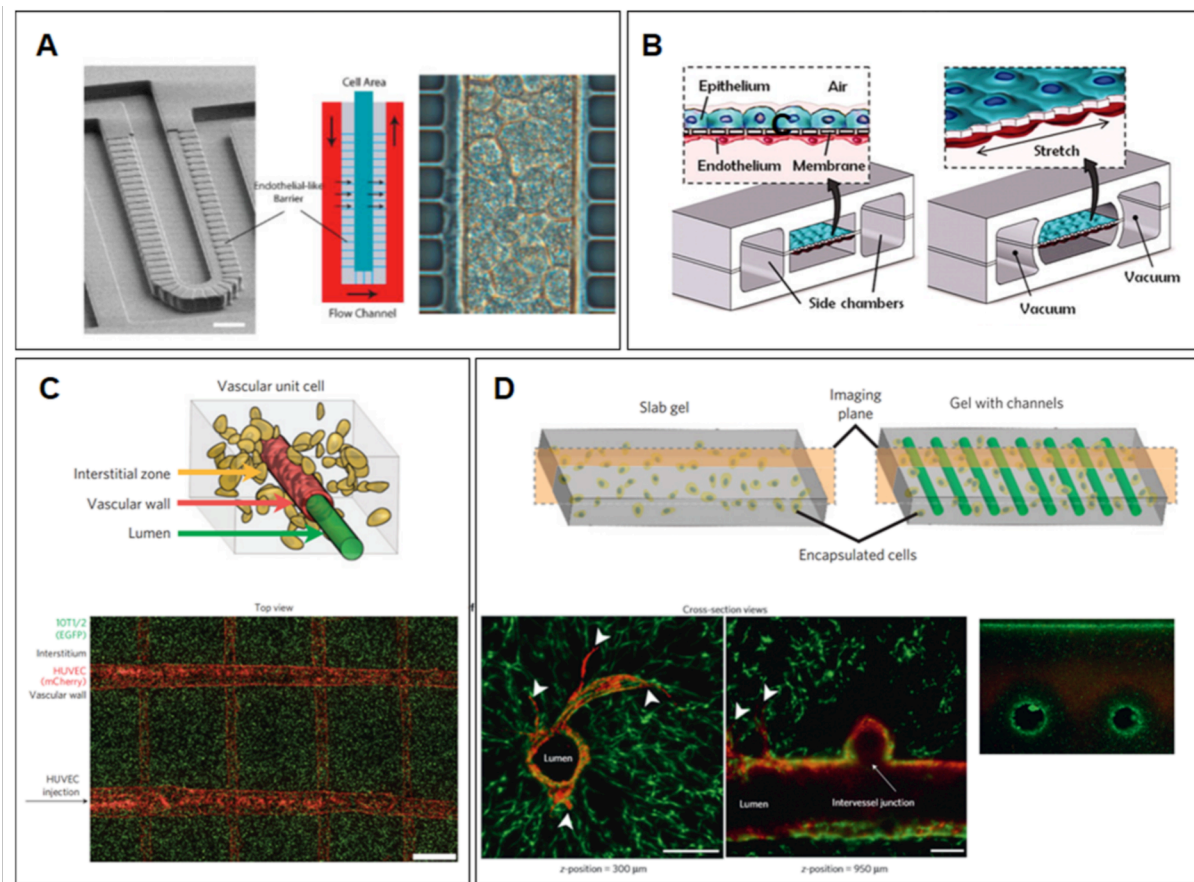


Fig 1.5 Methods for mimicking in vivo conditions of a 3D environment. **A.** Imposing tissue like organization through microfabrication. This example mimics liver ducts in a hepatocyte culture.¹⁵⁴ **B.** Flow application allows recapitulation of regular lung stretching in vitro.¹⁶⁰ **C,D.** 3D printing allows construction of complex matrices and cell seeding. In this case, vascular endothelial cells were encapsulated to form hollow vessels surrounded by an extracellular matrix. **C.** The top panel is a schematic of the relationship of vascular endothelial cells (red) with matrix-encapsulated fibroblasts (yellow) in the interstitium. The bottom panel is a fluorescence image of a printed vascular system, with endothelial cells (mCherry) and fibroblasts (EGFP).¹⁵⁹ **D.** The top panel is a schematic contrasting culture systems. With the novel casting system, randomly encapsulated cells can be combined with regular vascular networks. The bottom panels show cross-sectional views of the vascular network to illustrate both the cleared lumen without cells and the ability to generate intervessel junctions to study the effect of more complicated flow patterns on cell behavior.¹⁵⁹

Figure prepared by Matthew Rubashkin and Guanqing Ou

1.5.12. 3D Systems for Multicellular *In Vitro* Experiments – Future Applications

While these novel microfabrication approaches are both powerful and versatile, in many ways, they are still in their infancy. The elastomeric materials used in soft lithography have a limited range of mechanical properties that may not reflect *in vivo* conditions. Further, while proof of concept cell studies usually accompany technical descriptions of these systems, extensive analysis of intracellular signaling in these culture settings has not been performed. Finally, application of these systems requires a minimum level of commitment and investment that may not be feasible for everyone. Nonetheless, the field is a rapidly growing one with exciting potential.

Studying multicellular structures *in vitro* has only recently become a reality. However, single cell level studies cannot impart information about emergent properties of the organized system. As such, in order to achieve a more thorough understanding of functions within the human body and to design drugs in an informed manner, researchers should test their hypotheses in higher order systems. This step is unnecessary for many—there remain myriad unsolved mysteries within the cell that would not benefit from introduction of the multicellular context. For others with their eye on physiological relevance and translational applications, however, it is entirely apropos.

1.6. Focal Adhesions: Connecting the ECM to the Cytoskeleton

1.6.1 Introduction

Focal adhesions are the means through which cells receive and interpret mechanical signals from the extracellular matrix, and serve a critical role in cell motility^{20,164}. Disregulation of cell-ECM interactions including the proteins integrin, talin and vinculin have been shown to drive cancer progression in many tumor types^{7,13,165–168}. Cell-ECM interactions are dynamic, and involve membrane spanning adhesion proteins, integrins, and cytoplasmic focal adhesion proteins that link integrins to the contractile actin cytoskeleton (**Fig 1.6 A,B**)¹⁶⁹. ECM adhesion proteins have been implicated in force transmission from integrins to actin^{21,170}. Proteins involved in connecting the cell cytoskeleton to the ECM include talin and vinculin for force transduction and signaling scaffold molecules including p130cas and focal adhesion kinase (FAK)^{13,165,171}.

An outstanding question in the field is how do cells employ adhesions molecules in normal or disease like ECMs, and are some of these proteins responsible for biphasic changes in cell behavior in soft and stiff environments^{172–175}. In this sub-chapter I describe the actin linking modular function, wherein I analyzed the input of extracellular ligation of integrins to the output of activation of the actin myosin contractility module. I then identified distinct steps in the function's process, detail the proteins and associated molecules involved in each step, described the temporal and spatial locations of these proteins, and established how these proteins communicated with other upstream and downstream extracellular matrix (ECM) and cytoskeleton modules. The engineering principles that govern the actin linking modular function are the conversion of analog input to digital output, and term limits that regulate the 'on' state. A critical finding for my investigations in **Chapters 3 and 4**, is that talin acts as a molecular clutch between ECM-cytoskeleton force transduction¹⁷⁶.

1.6.2 Integrin Biology

The integrin family of adhesion receptors consists of hetero-dimers of single α and β subunits (**Fig 1.6 A**). Different dimer combinations serve to bind ECM ligands including fibronectin, collagen and laminin. While the β -subunit binds directly to the adhesive residues such as arginine-glycine-aspartic acid (RGD) in fibronectin, the α subunit confers specificity to the particular ligand. The C-terminus of the β subunit confers intracellular specificity, and can bind directly to several structural and signaling molecules¹⁷⁷. These include talin, kindling, vinculin and ilk^{178,179}.

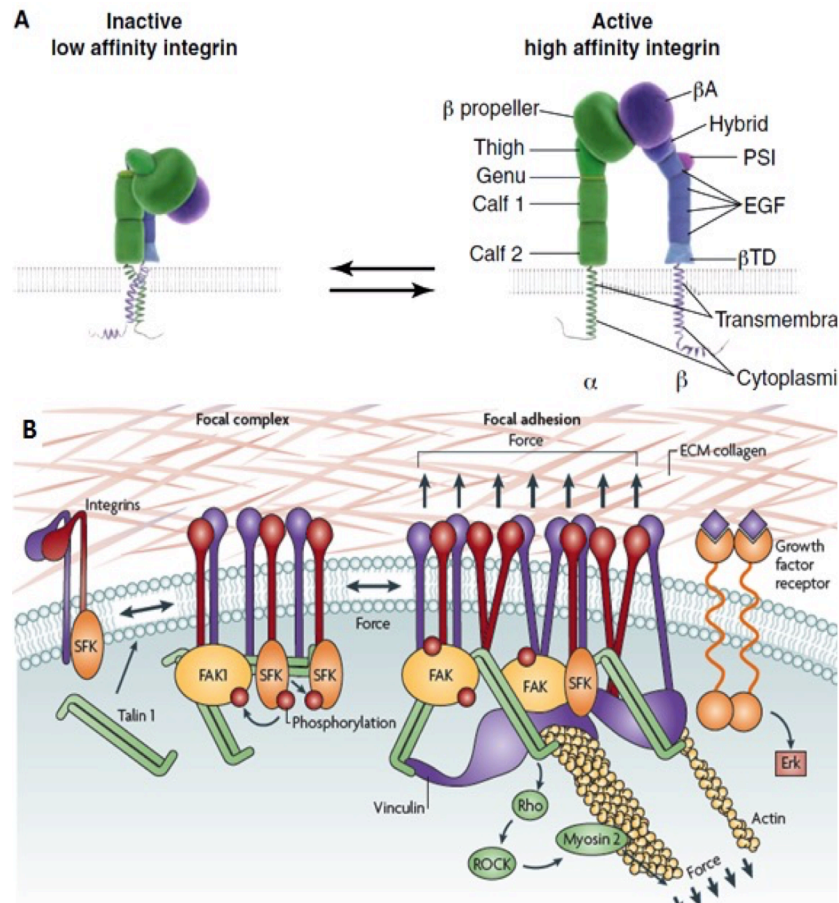


Fig 1.6: Integrin Activation

A. Integrin structure and schematic of activated integrin¹⁸⁰

B. Traditional view of mechanotransduction and focal adhesion maturation. While most integrins exist at the plasma membrane in an inactive state, they can be activated by inside-out or outside-in cues, enabling focal complex and adhesion assembly¹⁶⁹

Figure prepared by Matthew Rubashkin

1.6.3 Connections between the actin cytoskeleton and adhesions

The actin-linking module connects integrins to the actin cytoskeleton. There are two sets of inputs to this module. The first is the availability of β integrin cytoplasmic rods for binding to focal adhesion proteins such as talin¹⁸⁰. The second input is the state of the actin-myosin contractility module. In certain areas of the cell such as lamellapodia and fillopodia, actin is actively assembled and subject to myosin contractility^{164,181–184}. These two inputs are believed to be critical for the outputs of the actin-linking module. These include integrin activation, focal adhesion assembly and downstream signaling^{185,186}. However, recent studies have demonstrated these outputs without tension generated by myosin contractility. An

extracellular input such as shear force can activate focal adhesion assembly even after myosin inhibition¹⁸⁷. With this exception in mind, we will focus on the step processes of the actin linking module with both integrin and actin-myosin inputs.

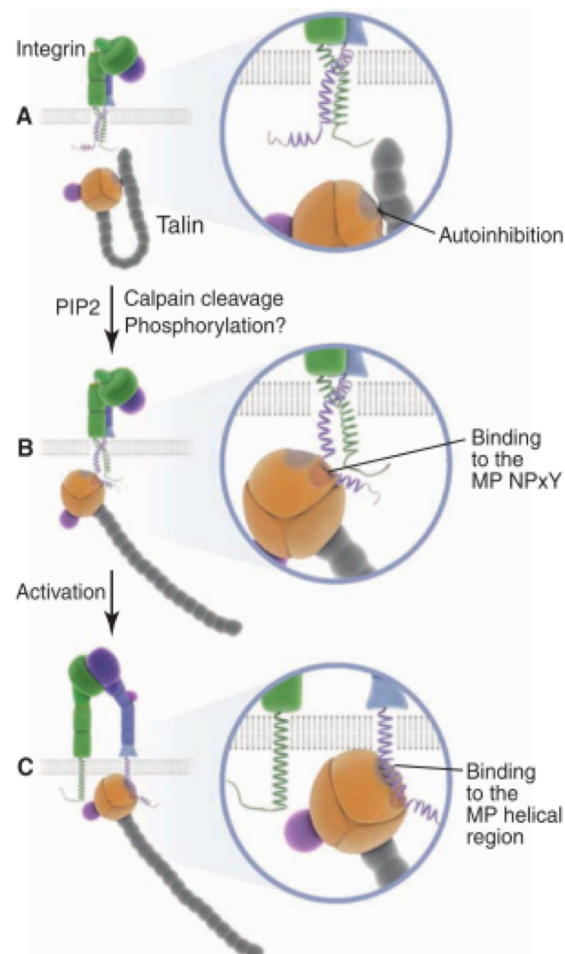


Fig 1.7: Talin-Integrin Interaction

A. Inactivated integrin and autoinhibited talin. **B.** Binding of talin PTB domain to integrin. **C.** Integrin Activation
Figure prepared by Matthew Rubashkin

1.6.4. Adhesion Formation Step 1A – Integrin Activation

Integrin: As seen in **Fig 1.7A**, the inactive integrin heterodimer has two main cytoplasmic binding domain loops, NPxY, and NxxY on the β integrin subunit¹⁸⁸. These domains are well conserved recognition sequence for phosphotyrosine-binding (PTB) domains (**Fig 1.7B**)^{180,182,189}. Two well known focal adhesion plaque proteins with PTB domains are talin and kindlin. It has been shown that impairment of the NPxY domain or talin PTB domains abolish talin binding and decrease integrin affinity; an input for

the actin linking module ^{179,180}. Work by Markus Moser and colleagues has demonstrated that talin also associates to the membrane proximal portion of β -integrin, which is engaged after PTB-NP $\times$ Y activation ^{180,188}. This stabilizes the association between talin and integrin.

1.6.5. Adhesion Formation Step 1B – Talin Activation

Talin: As seen in **Fig 1.7**, Talin has two main components a head that interacts with integrin through a PTB domain, and a rod that binds to actin, vinculin, and other focal adhesion proteins ^{190–193}. Talin is believed to be autoinhibited, unless in the presence of PIP2 lipid, which is at high concentrations in the plasma membrane ^{180,193}. Additionally the rod of talin facilitates cis dimerization to talin and may facilitate the binding of the dimerized talin to a two different integrin heterodimer complexes, leading to integrin clustering ^{193,194}.

1.6.6. Adhesion Formation Step 2– Focal Complex Formation

Actin Ligation: Kindlin interactions with integrins are believed to work in synergy with talin to cluster and prime integrins for activation ^{179,180,194}. Activated integrins are able to engage and ligate extracellular matrix recognition sequences. Work by Michael Sheetz has demonstrated that a cell only expressing the talin head domain can attach to the ECM, but is unable to create stable focal complex around the engaged integrin ¹⁷⁶. In order to create a stable focal complex, talin must ligate its rod domain to actively contracting actin ¹⁷⁶. Further, focal complex formation is dependent on the type of cellular protrusion, and requires active actin formation and treadmilling (**Figure 1.8A**) ^{183,186,195–197}.

1.6.7. Adhesion Formation Step 3 – Focal Adhesion Formation - Tension

Tension: The tension conferred from actin to talin to integrins causes stretching of the talin rod. These conformational changes can have two known effects. First, due to the thermal instability of an unfolded and tensed protein domain, other associated proteins may assemble with talin to energetically stabilize the

focal complex interactions¹⁹⁸. Second, stretching of talin exposes cryptic binding sites for vinculin^{190,199}. Vinculin is then able to transduce tension between actin and talin. For this reason, vinculin can be used as a force sensor to detect focal complex assembly^{13,68,182,200}.

1.6.8. Adhesion Formation Step 3 – Focal Adhesion Formation – Molecular Clutch

Molecular Clutch: Talin serves as an activator of integrins and transducer of tension from the cytoskeleton to the ECM. For this reason, many groups have referred to talin as a ‘molecular clutch’ (**Fig 1.8A**)^{176,186}. Much like a manual transmission car without a clutch cannot engage the motor with the wheels, focal adhesions require talin bidirectional interactions for cell spreading, motility and migration. A key engineering principal to the molecular clutch is analog to digital signal conversion; which in turn is a hallmark of a robust cellular system^{21,164,175}. The actin linking modular function converts stochastic talin-integrin association events into a mechanical transducing and chemically activating focal adhesion. Furthermore, the actin linking module appears to have a digital unit step response to focal adhesion formation. This includes consistent force levels and steady downstream phosphorylation rates^{164,176}.

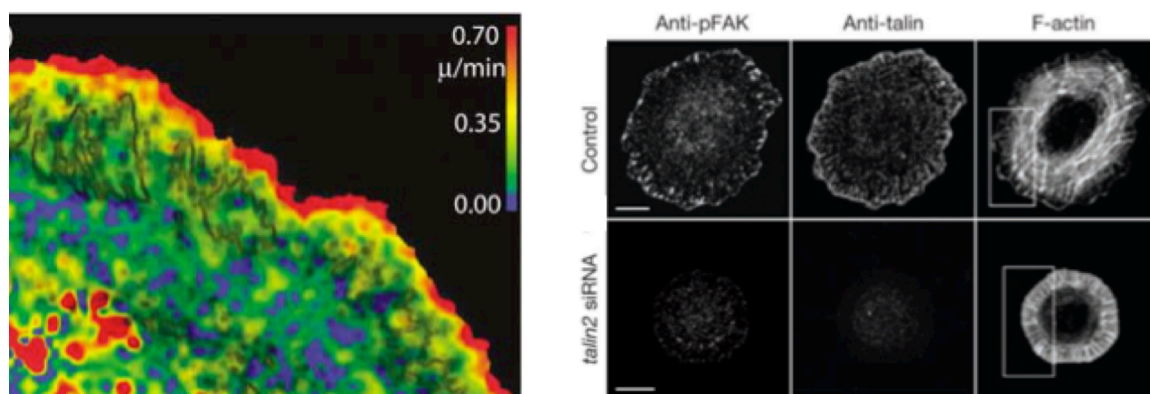


Fig 1.8: Integrin-Talin-Actin Engagement

A. Actin treadmill enables focal complex and focal adhesion assembly. Color map is of actin velocity with focal adhesions outlined in gray¹⁸⁶

B. Cell spreading and focal adhesion maturation requires talin expression. TIRF experiment demonstrating the effects of siRNA knockdown of Talin.¹⁷⁶

Figure prepared by Matthew Rubashkin

1.6.9. Adhesion Formation Step 4 – Downstream Signaling

Focal complex formation enables the scaffolding protein paxillin to bind to either the cytoplasmic tail of β integrin, or other previously recruited proteins in the adhesion complex^{201–203}. In the focal complex, paxillin assembles FAK and other signaling molecules involved in the small G protein signal transduction cascade, such as Rho, Rac and Cdc42, which in turn activate ROCK which upregulates actin-myosin contractility²⁰⁴. Focal Adhesion: Talin stretching directly enables binding of downstream signaling molecules including ILK, p130Cas and p120Cas that positively regulate focal adhesion assembly^{205,206}. For additional information on focal adhesion signaling, see sub-chapters 1.2, 1.3 and 1.4.

1.6.10. Adhesion Formation Step 4 – Focal Adhesion Disassembly

Regulated focal adhesion disassembly is necessary for many cellular behaviors^{68,207–209}. The actin binding modular function serves a key role in focal adhesion disassembly. Recent work by Mark Ginsberg has demonstrated that talin ubiquitylation and degradation through SMURF1, a ubiquitin ligase, can regulate the rate of adhesion assembly and assembly²¹⁰. This process is dynamic, and can be altered through phosphorylation of talin's ubiquitin ligase pocket via genetic manipulation or CDK5 phosphorylation. Therefore the cell has the ability through SMURF1 upregulation or CDK5 downregulation to promote focal adhesion turnover. Engineering Principle: The actin linking modular function demonstrates strict term limits in its duration through SMURF1 degradation of Talin. Via removal of the only molecular clutch able to connect actin contractility to integrins, the focal adhesion can be switched from active to inactive. Future studies are needed to investigate how SMURF1 interactions regulate the duration of focal adhesion activity in the context of migrating cells.

Chapter 2. SAIM: Developing a new microscopy technique to study cancer cell biology at the nanoscale

2.1. Introduction

2.1.1. Abstract

Emerging questions in cell biology motivate the measurement of fast, nanometer displacements at the cell membrane and cytoskeleton. Here we present a method, Scanning Angle Interference Microscopy (SAIM), able to dynamically image cellular structures, such as the cell membrane, microtubule network, and adhesion complexes with nanometer axial precision. As an example of the utility of SAIM, we observed the temporal evolution and nanoscale mechanical dynamics of focal adhesion complexes, demonstrating the applicability of SAIM for mechanistic investigations of mechanotransduction.

2.1.2 Microscopy for Cancer Cell Biology Applications

Reciprocal communication between cells and their microenvironment is central to development, physiology, and tissue pathology^{211,212}. Cell-microenvironment interactions are enabled by nanoscale reorganization of the plasma membrane, membrane cytoskeleton, and transmembrane receptors and their associated signaling molecules. However, despite recent advances in super-resolution methodologies, a definitive understanding of fundamental processes such as receptor tyrosine kinase signaling and mechanotransduction has been hindered by the inherent difficulty

of dynamically measuring nanometer-scale molecular displacements in living cells. For example, new super-resolution optical techniques (reviewed in¹⁹) have revealed the molecular architecture of force bearing complexes, such as focal adhesions with unprecedented detail, yet the response of these structures to dynamically applied forces remains largely unknown²¹³. Thus, a basic need in cell biology exists for methodologies capable of recording coordinated nano-scale movements of molecules across large spatial regions of the cell^{214,215}.

The leading contemporary approach for measuring the topography of cell membranes and artificial lipid bilayers with z-resolution at the nanometer-scale is fluorescence interference contrast microscopy (FLIC)²¹⁶, an interference-based technique with potential to visualize the rapid movements and spatial organization of proteins associated with the plasma membrane. In FLIC, axially varying structured illumination is used to determine the vertical position of nanometer-sized objects. To eliminate periodic replication artifacts and obtain unambiguous positions, the pattern of structured illumination is manipulated by varying the angle of incidence of excitation light to provide additional spatial information²¹⁷. Current interference contrast methodologies have not been widely adopted in biology, because of the technical challenges needed to implement the methods, which requires the assembly of custom microscopy suites and/or the generation of micro-fabricated cellular substrates. Moreover, FLIC requires strong assumptions about fluorophore orientation and has remained most applicable to imaging structures with fluorophores in known molecular orientations (e.g. dye-labeled membrane).

2.1.3 Overview of New Technique: Scanning Angle Interference Microscopy- SAIM

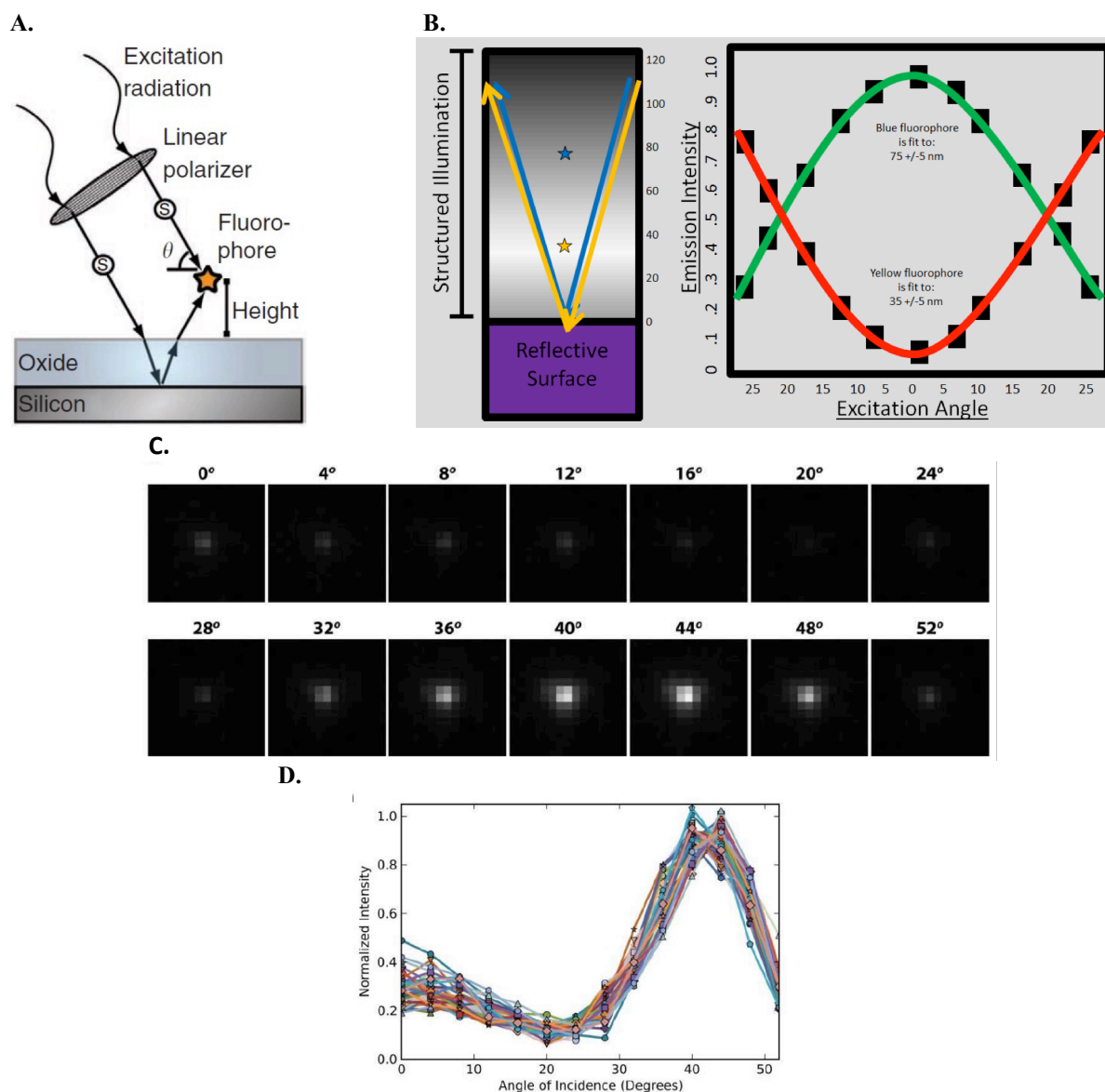


Fig 2.1. In SAIM, axially varying excitation intensity arises from interference between direct and reflected light off a silicon substrate. **A.** The structure of the illumination pattern is controlled by varying the polarization and angle of incidence of the excitation light, and is manipulated to probe the height of a fluorophore above a reflective substrate. A thin layer of oxide (500-nm thick for cell experiments) is used to space the sample above the silicon surface and create additional interference between reflected and reflected light at the oxide/liquid and oxide/silicon interfaces. **B.** For samples stratified axially in space above a reflective surface (here indicated by a yellow and blue fluorophore at 35 and 75 nanometers respectively), the angle of excitation is varied, while the emission intensity per pixel is recorded. Afterwards, *in silico*, pre-calculated curves of angle versus emission intensity are fitted to calculate the axial position of the fluorophore. **C.** Real SAIM data: Representative images of detected emission intensity of a single 20-nm yellow green nano-bead absorbed on the oxide surface of a silicon wafer with 513 nm of SiO₂ with a laser positioned at the indicated angle of incidence. Scale – 0.18 $\mu\text{m}/\text{pixel}$. **D.** Normalized detected emission intensity of 20-nm yellow green nano-beads absorbed on the oxide surface of a silicon wafer with 513 nm of SiO₂. Each curve corresponds to the measurement of an individual bead that was excited with 488 nm laser excitation polarized orthogonal to the plane of incidence.

Here, we describe a novel interference contrast method with nanometer precision, “Scanning Angle Interference Microscopy” (SAIM) that overcomes the inherent limitations of contemporary FLIC (**Fig. 2.1**). SAIM can address questions relating to a multitude of processes that occur proximal to the cell membrane, such as signaling linked to receptor activation and mechanotransduction, as well as endocytosis and remodeling of the extracellular matrix. SAIM can be readily implemented by most users on commercially available total internal reflection (TIRF) microscope systems. The improved method can track axial movements of labeled membrane or protein structures with nanometer precision over ranges approaching 1 micron. By manipulating the polarization state of light, we have eliminated the requirement for prior knowledge of the orientation of the fluorescence dipoles in the labeled structure. The determination of unambiguous positions over a wider axial range is enabled by actively scanning the incidence angle of the excitation light using a commercial TIRF illuminator. SAIM is suitable for live-cell imaging with relatively fast temporal resolutions of several seconds, and is amenable to further improvements with custom hardware designed for fast angle scanning. Importantly, SAIM uses standard fluorophores and laser lines enabling the imaging of multiple color channels simultaneously. These features enable the modified approach to be applied to imaging dynamic cellular processes, where a high-degree of axial localization is required.

2.1.4 Overview of Studies Performed

Focal adhesions are the conduits through which cells receive and interpret mechanical and physical cues from the extracellular matrix and serve a critical role in the transmission of force, wound healing, motility, and morphogenesis^{15,211}. While their role as intricate molecular machines is well appreciated, little is known about their overall organization at the nanoscale²¹⁸.

It has been previously hypothesized that focal adhesion complexes are highly spatially conserved structures. However, our results suggest that there are specific nanoscale changes to focal adhesion architecture that appear to be directly correlated with regions of varying cell-generated contractility and motility phenotypes. To further our understanding of focal adhesion structure and function, the three-dimensional organization and spatial orientation of proteins comprising focal adhesion complexes with precisions of ~ 5 nm along the optical axis and diffraction-limited laterally was revealed with a scanning-angle variant of fluorescence interference contrast microscopy. Utilizing this technique, a library of focal adhesion protein locations including paxillin, focal adhesion kinase, vinculin, N and C-terminal labeled talin, and zyxin was developed. Each protein was found to be stratified to distinct layers and localizations within individual focal adhesion complexes over a vertical range of ~ 60 nm. To observe how these proteins were evolving over time, dynamic studies in 3D of adhesions present in cells with varying motility phenotypes was performed through the development and utilization of adhesion tracking algorithms. We focused our studies on the proteins paxillin and vinculin, which have both been implicated in force-dependent mechanotransduction and focal adhesion maturation. Our results show that paxillin and vinculin are localized into spatially distinct regions relative to one another during processes such as adhesion maturation, where we find that paxillin moves downward relative to vinculin, which stayed in approximately the same vertical position.

2.2. Methods

2.2.1. Optical Theory

The electromagnetic theory describing silicon surface-generated standing waves of excitation radiation has previously been reported¹⁶. The layered silicon oxide/silicon structure is treated as a single virtual layer using transfer matrix methods that account for internal reflections and refractions. Reflection and refraction at known sample interfaces, including the cell membrane and sample buffer or the cell membrane and cell cytoplasm, were determined to have a negligible effect on the structure of the electric field and were therefore excluded from the optical theory (Fig. 2.2A).

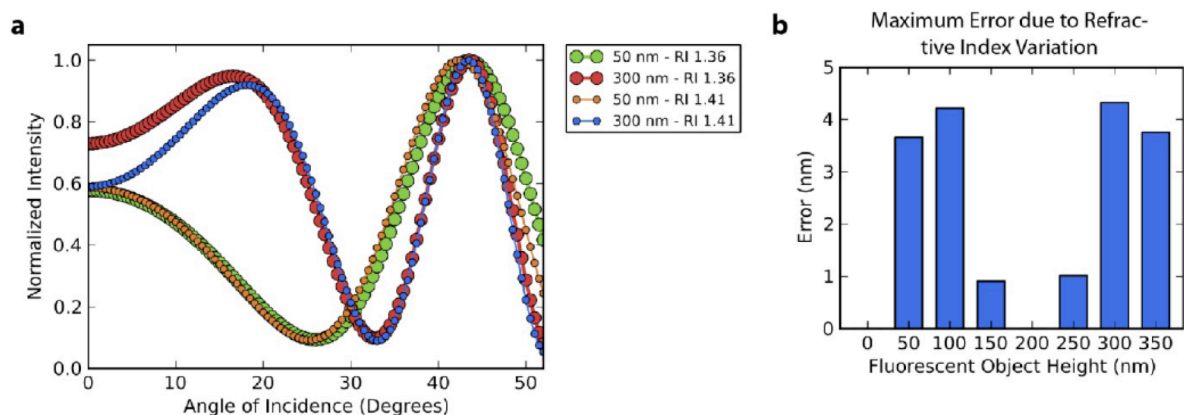


Fig 2.2. Cellular structures have varying refractive index ranging from ~1.36 for normal cell cytoplasm to 1.417 for the cell membrane, nucleus, and cytoplasm of cancer cells. The pattern of structured illumination used to probe the axial height of fluorescent structures in SAIM depends on the refractive index of the sample. Uncertainties in refractive index introduce small errors into the precision of the absolute height localization with SAIM. **(a)** Shows the theoretical variation in intensity as a function of excitation laser angle of incidence for fluorophores residing at 50 nm or 300 nm in a sample with a refractive index (RI) of 1.36 or 1.41. These curves demonstrate the extent of variation in SAIM intensity profiles expected in cellular samples. **(b)** Simulation results showing the error in measurement of fluorescent structures (actual height indicated) that reside in a sample with a refractive index of 1.41 when a refractive index of 1.36 is assumed in SAIM's optical model.

For incident light polarized perpendicular to the plane of incidence, the relative local electric field at a position H in the sample above the silicon oxide is given by:

$$F \sim 1 + r^{TE} e^{i\phi(H)} \quad (1)$$

where r^{TE} is the transverse electric (TE) component, perpendicular to the plane of incidence, of the Fresnel coefficient of reflection between the sample interface and the virtual silicon oxide/silicon layer and ϕ is the phase difference of the direct and reflected light at axial position H given by:

$$\phi(H) = \frac{4\pi}{\lambda} (n_b H \cos \theta_b) \quad (2)$$

The effective Fresnel coefficient is obtained from the characteristic transfer matrix M^{TE} according to:

$$r^{TE} = \frac{(m_{11}^{TE} + m_{12}^{TE} p_o) p_2 - (m_{21}^{TE} + m_{22}^{TE} p_o)}{(m_{11}^{TE} + m_{12}^{TE} p_o) p_2 + (m_{21}^{TE} + m_{22}^{TE} p_o)} \quad (3)$$

$$M^{TE} = \begin{bmatrix} m_{11}^{TE} & m_{12}^{TE} \\ m_{21}^{TE} & m_{22}^{TE} \end{bmatrix} = \begin{bmatrix} \cos(k_{ox} d_{ox} \cos \theta_{ox}) & -\frac{i}{p_1} \sin(k_{ox} d_{ox} \cos \theta_{ox}) \\ -ip_1 \sin(k_{ox} d_{ox} \cos \theta_{ox}) & \cos(k_{ox} d_{ox} \cos \theta_{ox}) \end{bmatrix} \quad (4)$$

$$p_o = n_{Si} \cos \theta_{Si}, \quad p_1 = n_{ox} \cos \theta_{ox}, \quad p_2 = n_b \cos \theta_b \quad (5)$$

$$k_i = \frac{2\pi n_i}{\lambda} \quad (6)$$

where k_i is the wavenumber in material i ; n_{Si} , n_{ox} , n_b are the refractive index of the silicon, silicon oxide, and sample, respectively; θ_{Si} , θ_{ox} , θ_b are the angles of incidence in the silicon,

silicon oxide, and sample, respectively; and d_{ox} is the thickness of the silicon oxide layer. The angles of incidence in silicon oxide and silicon were calculated according to Snell's Law.

2.2.2. Image Analysis

For height reconstructions, intensity profiles from acquired image sequences were fit to the optical theory on a pixel-by-pixel basis. To correct for uneven laser illumination across the image, the intensity of each pixel, and for live cell imaging an all-pixel average, in each sample image was adjusted according to the corresponding intensity in the reference slide image sequence:

$$I_{i,n} = \frac{I_{i,n}^S - I^c}{I_{i,n}^R - I^c} \quad (7)$$

$I_{i,n}^S$ is the intensity of pixel i in sample image n , $I_{i,n}^R$ is the intensity of pixel i in reference image n , and I^c is the average background intensity of the camera device obtained by snapping an image on the microscope with the shutters closed and averaging the intensity over all image pixels. Emission by fluorophores on the microscope is proportional to the excitation radiation intensity, which is proportional to the square of Equation 1. The detected pattern of intensity variation at a given pixel in the sample image sequence was thus fit according to:

$$I = A|1 + r^{TE} e^{i\phi(H)}|^2 + B \quad (8)$$

Here the scaling parameter A accounts for variation in detected intensity due to factors including mean excitation laser intensity, fluorophore density, fluorophore photo-physics, self-interference

of emitted photons, and efficiency of emitted photon detection. The offset parameter B accounts for background fluorescence in the sample images. Parameters A, B, and H were fit through constrained nonlinear least-squares optimization using Trust Region algorithms (Math Kernel Library; Intel). Fitted data whose R-squared value was above a maximum defined level or whose scaling parameter was below a defined minimum were excluded from further analysis.

2.2.3. Software

A custom software package was developed for image analysis and height reconstruction of SAIM image sequences. Multi-threaded software was written in C++, compiled for the Linux operating system, and run on an 8-core Dell Precision Workstation with 8 GB of RAM. Additional programs for data analysis and 3D visualization were written in Matlab and Python using libraries distributed by Enthought, Inc.

2.2.4. Preparation of Reflective Substrates for Cell Culture

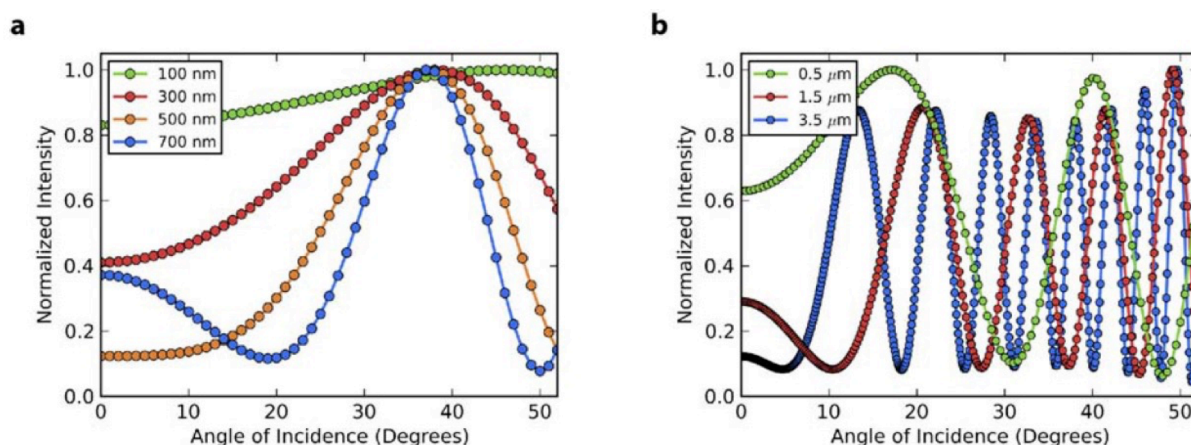


Fig 2.3. On the silicon wafers, the oxide layer functions as both a sample substrate and a spacer between the sample and silicon surface. **(a)** Theoretical variation in intensity as a function of excitation laser angle of incidence for several different thicknesses (100, 300, 500, and 700 nm) of silicon oxide between the silicon surface and sample. All curves were calculated assuming Alexa488 dye and 488-nm laser excitation linearly polarized perpendicular to the plane of incidence. To achieve maximal interference contrast of samples near the substrate, the oxide layer should be ~500 nm thick or greater. For ideal fitting to the optical model, a range of angles over which one complete inversion of sample intensity occurs should be probed. Typically a sampling rate of 8-10 equally spaced angles over this range is sufficient to yield good fits to the optical model. As shown in **(b)**, the interference patterns for samples positioned higher above the substrate undergo more frequent inversions over a given range of angles and should therefore be scanned with a higher angle sampling rate (sample height above the silicon substrate indicated in figure legend).

N-type [100]-orientation silicon wafers with 500 nm or 1.9 μm of silicon oxide were purchased from Addison Engineering. Wafers with 500 nm or greater silicon oxide were selected to maximize interference contrast (**Fig 2.2.3**). Wafers were cut into approximately 1 cm x 1 cm chips by scoring with a diamond-tip pen. They were cleaned by sonicating in acetone for 20 minutes, rinsing with water, sonicating for 20 minutes in 1 M potassium hydroxide, and rinsing with water. The wafers were then chemically activated to enable conjugation of extracellular matrix (ECM) proteins for cell adhesion. The wafers were incubated for one hour in 0.5% (3-aminopropyl)trimethoxysilane (APS) in water, sonicated five times in water for five minutes to remove excess APS, incubated for one hour in 0.5% glutaraldehyde in phosphate buffered saline (PBS; pH = 7.4), sonicated five times in water for five minutes, and dried under nitrogen gas.

The wafers were sterilized under a UV lamp and incubated overnight at 4 °C in 10 µg ml⁻¹ human plasma fibronectin (Millipore FC010).

2.2.5. Preparation of Reflective Substrates with Adsorbed Nanobeads

To validate the setup, silicon wafers were prepared with defined layers of silicon oxide that functioned as spacers between the silicon surface and adsorbed fluorescent nanobeads on the oxide surface. The SiO₂ layers were grown on bare P-type [100]-orientation silicon wafers (Addison Engineering) using silane chemistry (5% SiH₄ / 95 % N₂) of plasma enhanced chemical vapor deposition (PECVD process). The plasma was generated by mixing 170 sccm silane (first precursor) and 710 sccm NO₂ (second precursor) in an Oxford PlasmaLab 100 system. The NO₂ was used as the process oxidant and its flow rate was enough to obtain high quality SiO₂ films with no excess Si and no Si-H bonding. The chamber pressure was maintained at 1000 mT. The radio-frequency (RF) power to generate the plasma of ionized gasses was 20 W. The wafer stage temperature was set at 300 °C and the chiller control was also set at 70 °C to promote the development of nonporous films. Under these conditions, the silicon oxide deposition rate was 78.6 nm min⁻¹. The thickness of each oxide layer was checked using an n & k 1500 system (from n & k Technology, Inc.). 20-nm carboxylate-modified yellow-green fluorescent spheres (FluoSpheres; Invitrogen) were diluted to a final concentration of 5x10⁸ beads ml⁻¹ in PBS + 100 mM NaCl and sonicated for five minutes to disperse aggregates. Silicon wafers were cut and cleaned as above and incubated in the bead solution for five minutes at room temperature to allow beads to adsorb through hydrophobic interaction. The wafers were then washed once with PBS + 100 mM NaCl, dried in air at 37 °C, and stored at room temperature.

2.2.6. Preparation of Fluorescent Reference Slides

Laser illumination in widefield epi-fluorescence is subject to variations in intensity owing to interference fringing. To quantify the variation in intensity, reference slides with a monolayer of fluorescent dye were prepared using silane conjugates of fluorescein and Rhodamine B. The conjugates were synthesized by reacting 1 mg of fluorescein isocyanate or 1.37 mg of Rhodamine B isocyanate with 9.25 mg of APS in 1.25 mL anhydrous ethanol. Reactions were conducted under nitrogen gas at room temperature for two hours with constant stirring. Immediately following the reaction, the conjugates were centrifuged at 20,000g for 10 minutes to pellet unwanted aggregates and then diluted ten-fold in absolute ethanol. Number 1.5 glass coverslips (Corning) were cleaned by sonicating in acetone and potassium hydroxide as described above for the silicon wafers and dried under nitrogen gas. The coverslips were then mounted in imaging chambers (35-mm polystyrene dishes with bottoms drilled out) using Norland Optical Adhesive #68 and incubated with the diluted silane conjugates for 30 minutes at room temperature. The mounted coverslips were rinsed three times with water and stored in water until imaging.

2.2.7. Preparation of Fluorescent Reference Slides

To obtain transcriptionally inducible paxillin-mCherry and paxillin-mEmerald, the fusions were cloned into the 2nd generation lentiviral vector pLV Hygro Tet. pLV Hygro Tet was prepared from pLV tTRKRAB (Addgene #12249; a generous gift of Didier Trono) by inserting in place of the EEF1 α promoter and tTRKRAB transgene, a hygromycin resistance gene expression cassette consisting of an SV40 promoter and bovine growth hormone polyadenylation sequence in

reverse orientation for selection in transduced cells back to back with the regulatable hybrid heptamerized Tet operator minimal CMV promoter followed by a central polypurine tract and the multiple cloning site (MCS) from pcDNA3.1+ (Invitrogen)²¹⁹. The paxillin-mCherry gene was excised from a N1 clonotech-style vector with the restriction enzymes Nhe-I (made blunt) and Not-I and inserted into the PME-I and Not-I sites of the pLV Hygro Tet MCS to make pLV paxillin-mCherry Hygro Tet. Similarly, paxillin-mEmerald was cloned to make pLV paxillin-mEmerald Hygro Tet. A second lentiviral construct, pLV rtTA^s-M2 IRES Neo, was also prepared from pLV tTRKRAB to provide *EEF1 α* promoter mediated constitutive expression of the synthetic tetracycline inducible reverse transcriptional transactivator rtTA^s-M2 (generous gift of Wolfgang Hillen and Hermann Bujard) from a bicistronic mRNA coexpressing neomycin phosphotransferase downstream of an internal ribosome entry site for selection of transduced cells with G418.

A validated shRNAi (TRCN0000299022) in the lentivector pLKO.1 puro and targeting the human Talin1 3'UTR was obtained from Sigma. Murine Talin1 tagged with mCherry at the N-terminus and mEmerald at the C-terminus was prepared from mCherry N-terminally and mEmerald C-terminally tagged murine Talin1 constructs previously described ligating a Sall-NotI fragment from the mEmerald construct containing the C-terminal end of Talin1 fused to mEmerald with a AgeI-Sall fragment from the mCherry construct containing mCherry fused to the N-terminal end of Talin1 with the NotI-AgeI vector fragment from pmCherry-N1 (Clontech)²¹⁸. Murine Talin1 tagged at N and C termini with mCherry was prepared from this latter construct by substituting an AgeI-NotI mCherry fragment from pmCherry-N1 for

mEmerald. The construct for expression of vinculin-mCherry (C-terminal fusion) was described previously¹³.

2.2.8. Cell Culture and Imaging Sample Preparation

MCF10A human mammary epithelial cells were grown at 37 °C and 5% CO₂ in DMEM F12 (Invitrogen) supplemented with 5% donor horse serum (Invitrogen #16050122), 20 ng ml⁻¹ EGF (Peprotech #315-09), 10 µg ml⁻¹ insulin (Sigma #I6634), 0.5 µg ml⁻¹ hydrocortisone (Sigma #H0888), 0.1 µg ml⁻¹ cholera toxin (Sigma #C8052), and 100 units ml⁻¹ penicillin/streptomycin. For stable expression of paxillin-mEmerald or paxillin-mCherry, recombinant lentivirus was prepared by transient transfection into HEK-293T cells and used to transduce MCF10A cells as previously described¹⁴. Stable cell lines were selected in 200 ng ml⁻¹ G418 and 380 ng ml⁻¹ hygromycin and sorted on a FACS Aria II cell sorter (BD Biosciences) for paxillin-mEmerald or paxillin-mCherry expression. Expression of paxillin-mEmerald or paxillin-mCherry was induced with 200 ng ml⁻¹ doxycycline in growth media for one day prior to experimentation. Transient transfection with vinculin-mCherry and talin fusions was with Lonza Nucleofector kit V (program T024). Talin fluorescent protein conjugates were co-transfected with shRNA targeting endogenous MCF10A talin1.

For all experiments, cells were replated on fibronectin-conjugated silicon wafers and cultured overnight before live-cell imaging, fixation, or cell roofing. Except for microtubule imaging, cells were plated on wafers with 500-nm silicon oxide and fixation was performed in 2% paraformaldehyde for 20 minutes. For cell membrane imaging, the ventral cell membrane was removed through cell roofing according to previously described protocols²²⁰. Briefly, cells were

swelled in ice-cold hypotonic buffer (15 mM KCl, 15 mM Hepes, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4) for 4 minutes. Swollen cells were then sonicated with a probe-type sonicator (Microson XL2000; Misonix) using a short pulse (<1 s, Power Setting 8) with the sonicator probe (1/8" diameter) approximately 3 mm above the cells. Following sonication, the remaining ventral plasma membranes were rinsed once in PBS and fixed. Membranes were labeled with fluorescent dye post-fixation by incubating in 1 μ M Vybrant DiO (Invitrogen) in PBS for one hour at room temperature. Rho kinase inhibition was with 10 μ M of the inhibitor Y-27632 (Cayman Chemical #10005583) in growth media. For the microtubule imaging, cells were plated on wafers with 1.9 μ m silicon oxide overnight and fixed by incubating in 0.25% glutaraldehyde for 30 seconds and then transferring them to a solution of 0.25% glutaraldehyde with 0.1% Triton X-100 in PBS. Cells were then rinsed with PBS and covered with a freshly prepared drop of 0.2% sodium borohydride in PBS, which was exchanged three times over 30 minutes. This was followed by two rinses with PBS and a 10 minute incubation with a solution of PBS, 2% BSA, and 0.1% Triton X-100. Microtubules were labeled with rat monoclonal anti-tubulin antibody (AbD serotec cat# MCA77) and Alexa488-conjugated anti-rat secondary antibody (Invitrogen).

2.2.8. Image Acquisition.

SAIM requires the acquisition of images on an epi-fluorescence microscope with multiple defined angles of incidence for excitation illumination. This can be achieved simply on commercial objective-type TIRF microscope systems that have motorized control of the laser position on the back focal plane of the objective. Imaging was performed on a motorized TIRF

inverted microscope system (Ti-E Perfect Focus System; Nikon) equipped with 488 nm and 561 nm lasers, electronic shutters, an electron-multiplying charged-coupled device camera (QuantEM 512; Photometrics) and controlled by NIS-Elements software (Nikon). A linear glass polarizing filter (Edmunds Optics #NT47-316) was placed in the excitation laser path to polarize the light perpendicular to the plane of incidence along which the excitation laser light emanates from the objective. On the Nikon microscope, the polarizer was placed in the filter cube housing the dichroic mirror, where the excitation filter would normally sit. The relationship between motor position of the TIRF illuminator and actual angle of laser light out of the objective was calibrated by varying motor position and measuring the position of the focused laser spot along the ceiling of the room housing the microscope. The corresponding angles in aqueous samples were determined from Snell's Law. Motor units corresponding to desired laser incidence angles for image acquisition were programmed into NIS-Elements to facilitate automated data acquisition. Samples were imaged with a 60x /NA 1.2 water-immersion objective (CFI Plan Apo VC; Nikon) or a 100x /NA 1.45 oil-immersion objective (CFI Plan Apo Lambda; Nikon) with additional magnification using a 1.5x optovar (0.18 $\mu\text{m}/\text{pixel}$ for 60x, 0.11 $\mu\text{m}/\text{pixel}$ for 100x).

Silicon substrates were placed sample-side down in 35-mm #1.5 glass bottom dishes in PBS or phenol red-free growth media with 10 mM Hepes (pH 7.4) to maintain pH for live-cell imaging. A buoyancy adjusted weight of approximately 1.75 g was placed on the wafer to keep the wafer sample surface flat against the glass bottom. Live-cells were maintained at 37 °C using a blower-style heater (ASI 400; Nevtek). For imaging microtubules on silicon substrates with 1.9 μm of silicon oxide, a sequence of 33 epi-fluorescence images of each sample was obtained by scanning through laser incidence angles below the critical angle from -34 degrees to +34 degrees

in two degree increments along the plane of incidence. For all other samples, a sequence of 27 epi-fluorescence images was obtained from -52 to +52 degrees in fourdegree increments. Typical camera exposure times were on the order of 10-100 ms (with an EM gain of 200) resulting in acquisition rates on the order of 3 - 60 s per image sequence. A sequence of images of the reference slides was obtained similarly for each color channel.

2.3. Results

2.3.1. SAIM Control Studies – Technique Development

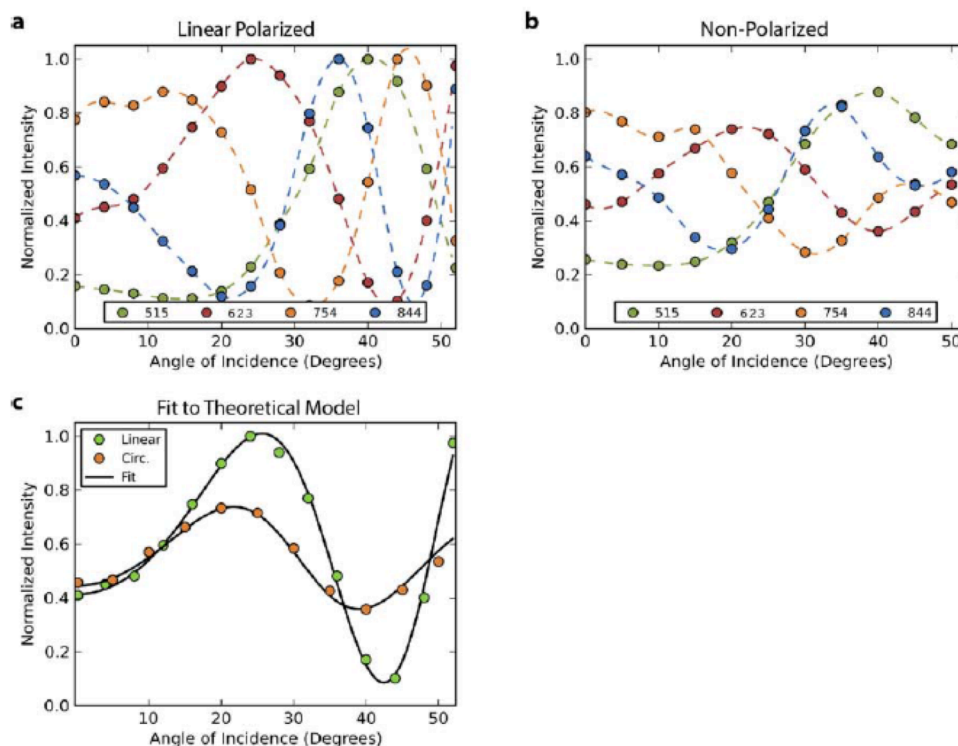


Fig 2.4. Optimal interference intensity contrast is achieved when the excitation laser light is linearly polarized **(a,b)** Experimental results showing the normalized detected emission intensity of 24-nm yellow-green fluorescent beads absorbed onto the SiO₂ of silicon wafers (thickness of SiO₂ in units of nm indicated in the legend) as a function of excitation laser angle of incidence. In **(a)** beads were excited with 488-nm laser light linearly polarized perpendicular to the plane of incidence whereas in **(b)** the excitation light was circularly polarized to mimic the performance of non-polarized light. All intensity points in **(a)** and **(b)** are the average measurement of 45-50 individual beads. **(c)** Best fit lines of the theoretical model to experimental data in **(a,b)** for a silicon substrate having 623 nm of oxide imaged with linear and circularly polarized light.

For SAIM, samples are prepared on commercially available reflective silicon chips with a thin layer of silicon oxide that functions as a sample substrate (**Fig. 2.1**). Axially varying patterns of excitation intensity are generated through the interference of incident and reflected excitation light, and the structure of the pattern is manipulated by controlling the incidence angle of the excitation light (**Fig. 2.1**).

To quantify the performance of SAIM, we adsorbed fluorescent beads with a diameter of 24 nm on silicon oxide substrates at varying heights above the reflective silicon surface. The height of the beads above the silicon was calibrated by precisely varying the thickness of the silicon oxide layer using standard nanofabrication approaches. The adsorbed beads were scanned with excitation light of varying incidence angle using a commercial TIRF illuminator to position a focused laser beam on the back aperture of the microscope objective. The recorded fluorescence intensity profiles across varying laser incidence angles were then fit to an optical model to obtain the axial position of the bead center from the silicon surface with nanometer-scale precision (**Fig. 2.4 A,B,C**). The use of excitation light linearly polarized perpendicular to the plane of incidence (s-polarized) improved interference intensity contrast by as much as five-fold over non-polarized or circularly polarized light (**Fig. 2.5 A,B**), eliminated the complicated dependence of the optical model on fluorophore dipole orientation, and significantly improved the accuracy of object localization.

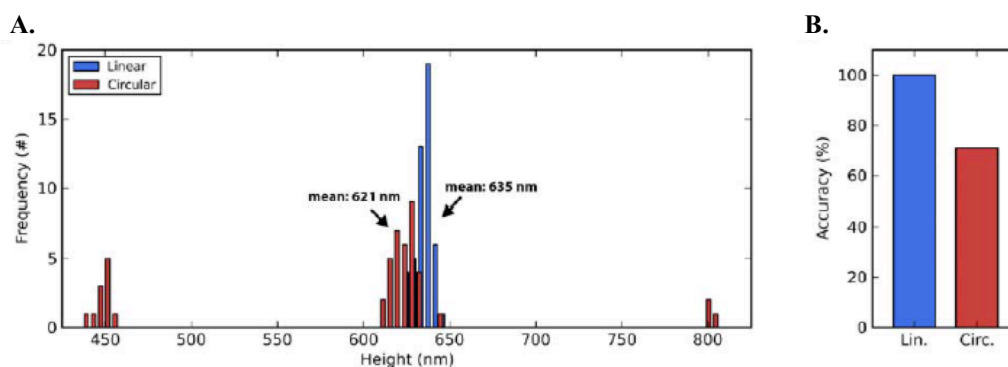


Fig 2.5 Enhanced interference contrast results in higher accuracy of height measurements due to improved fitting of experimental data to the theoretical model. **A.** Histogram of measured bead heights above a silicon substrate with 623 nm of silicon oxide imaged with linear and circularly polarized light. The expected height of the bead center is 635 nm (623 nm oxide + 12 nm bead radius). **B.** Percentage of individual beads accurately measured to within 50-nm of their actual heights using linear and circularly polarized light. Many laser launches already output linearly polarized light of the appropriate orientation, which can be maintained with a polarizing maintaining fiber. Alternatively, a linearly polarizer filter can be introduced into the excitation laser path to achieve s-polarization.

2.3.2. SAIM Control Studies – Technique Verification

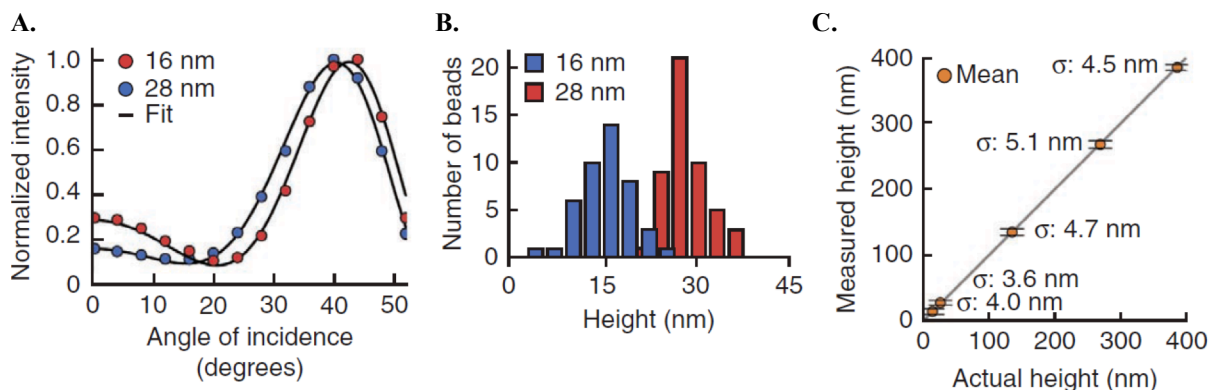


Fig 2.6 In SAIM, axially varying excitation intensity arises from interference between direct and reflected light off a silicon substrate. **A.** Average emission intensity and fits to the optical model for beads differing by 13 nm in axial position, and the corresponding histograms of axial position measured for individual beads excited with 488 nm s-polarized laser light. All illumination angles scanned were below the expected critical angle for total internal reflection. **B.** Axial position of 24 nm fluorescent beads adsorbed on the oxide surface of silicon substrates measured with SAIM and plotted against their expected position based on thin film measurements of the oxide thickness. **C.** The height of beads above the silicon surface was varied by using different oxide thicknesses as spacers. Filled orange dots represent the average of 45-50 individual bead measurements and horizontal bars show the standard deviation, σ , above and below the mean. Height is reported as the axial distance of the bead center above the reference point $z = 500$ nm.

Measured bead heights reflected the high accuracy and precision of the measurement technique and were on average within a few nanometers of the expected heights (**Fig. 2.1 C,D; 2.6 A,B**) with a standard deviation ranging from 3.6 to 5.1 nm (**Fig. 2.6 C**). Beads varying in axial position by as little as 13 nm had distinct interference contrast profiles, which enabled these small height differences to be resolved by fitting the intensity profiles to the optical model (**Fig. 2.6 A**).

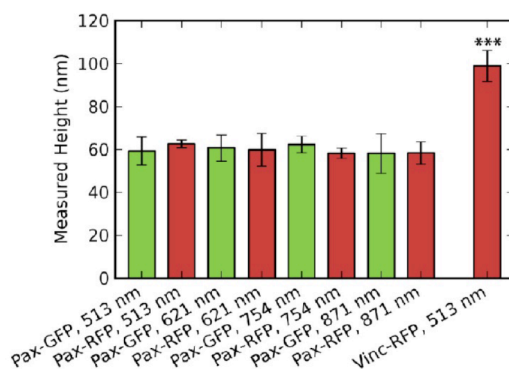


Fig 2.7 Measurement of paxillin-mEmerald (Pax-GFP), paxillin-mCherry (Pax-RFP), and vinculin-mCherry (Vinc-RFP) in fixed epithelial cells with SAIM. Cells were plated on silicon substrates with varying thicknesses of silicon oxide (indicated) to control the absolute spacing of cells above the silicon surface. Measured height is reported as the average vertical position relative to the silicon oxide surface of adhesion-based paxillin or vinculin molecules. Paxillin measurements were comparable across eight independent measurements (two color channels and four silicon oxide thicknesses) with a standard deviation of 1.8 nm.

Similarly, analysis of mCherry and mEmerald conjugates of the focal adhesion protein paxillin in cells, on four different silicon substrates with unique oxide spacer layer thicknesses, revealed a maximum variance of just 1.8 nm across eight independent measurements (**Fig. 2.7 A**). Although the positions of paxillin could be determined from images with surprisingly poor signal to noise ratios, we found that a signal to noise ratio of approximately two or better yielded the most accurate results (**Fig. 2.8 A, B, C**).

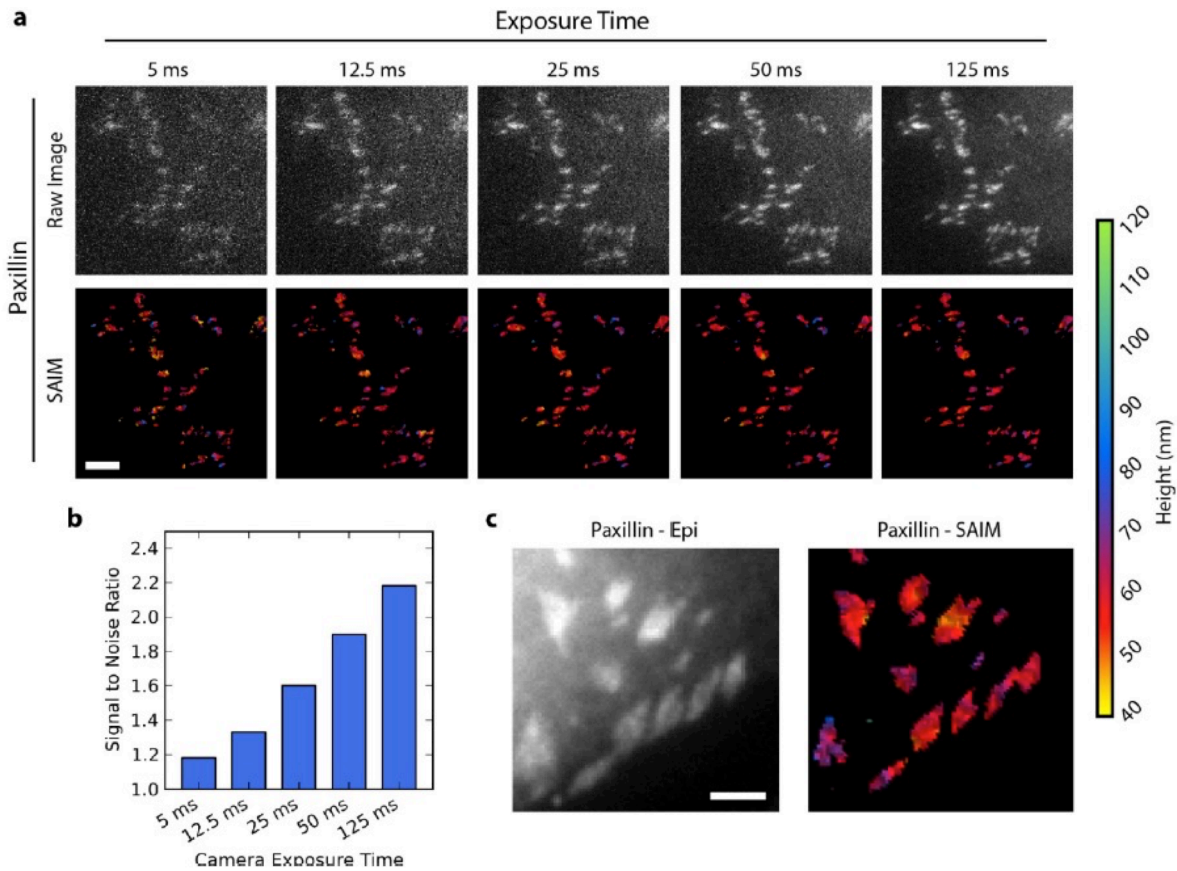


Fig 2.8 (a) Raw epi-fluorescence images of paxillin-mCherry from a SAIM image sequence (upper images) of a fixed epithelial cell and the corresponding SAIM height reconstructions (lower images; Scale bar – 4 μ m). The signal of adhesion-based paxillin-mCherry relative to the background noise was controlled by adjusting the camera detector exposure time (time in ms indicated). **(b)** Quantification of signal to noise ratios for images in **(a)**. **(c)** Epi-fluorescence and SAIM height reconstruction of paxillin-mCherry in a fixed epithelial cell (Scale bar – 2 μ m). Note the similar height measurement of paxillin-mCherry across cellular regions with varying background fluorescence from cytoplasmic molecules.

2.3.3. SAIM – Biological Applicability

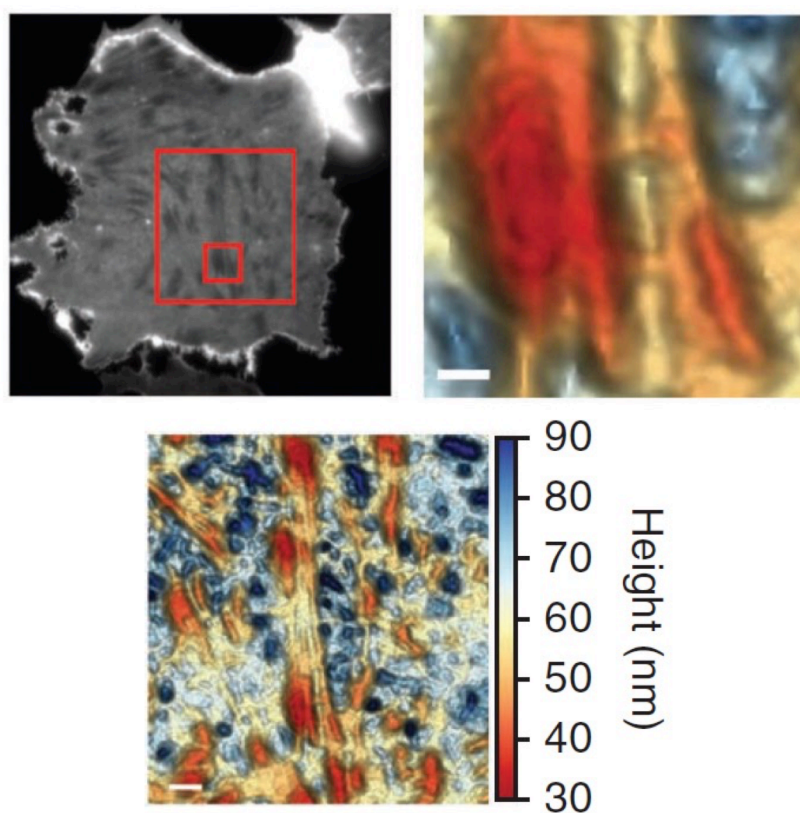


Fig 2.9 Validation of Scanning Angle Interference Microscopy (SAIM). Three-dimensional image reconstruction of an epithelial cell ventral membrane that was labeled with the dye DiO (top-left), imaged with SAIM (488 nm, s-polarized excitation), and fit to an optical model to obtain the height of the membrane above the oxide surface. Height is reported as the absolute distance of the membrane above the silicon oxide surface. ROI scale bar – 2 μm . Zoom scale bar – 750 nm.

The biological applicability of SAIM was demonstrated by imaging the vertical nanometer position or topography of three types of cellular structures: the plasma membrane (**Fig. 2.9**), the microtubule cytoskeletal network (**Fig. 2.10 A**), and molecules within the focal adhesion complex (**Fig. 2.10 B**). To visualize membrane architecture, we prepared dye-labeled ventral cell membrane “ghosts” by extracting the dorsal membranes of epithelial cells plated on silicon substrates, followed by paraformaldehyde fixation and lipid labeling with the green fluorescent lipophilic dye DiO (**Fig 2.9**).

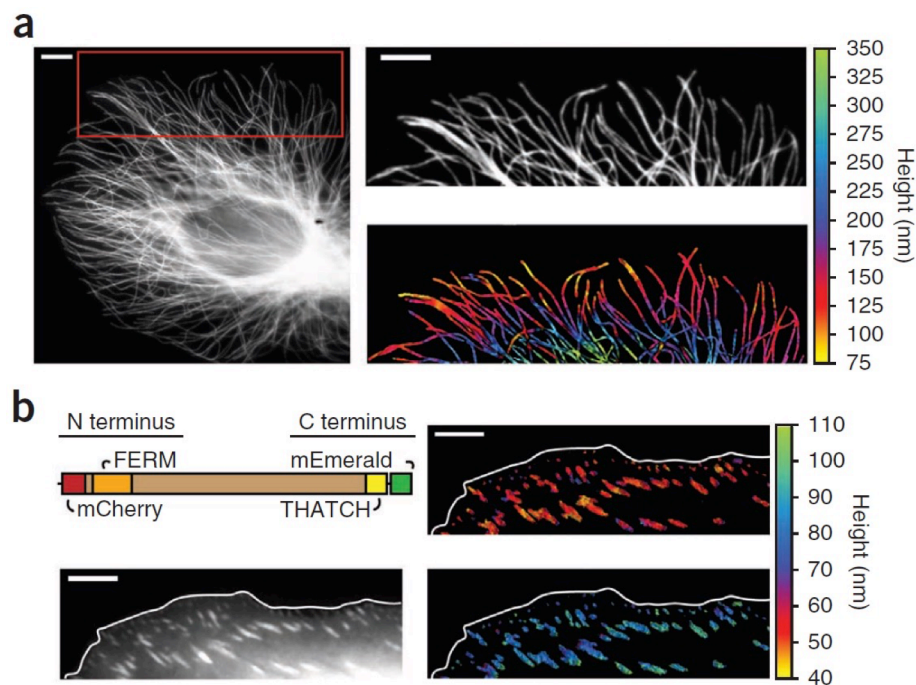


Fig 2.10 SAIM imaging of microtubules in a MCF10A mammary epithelial cell. **(a)** Conventional epifluorescence image of microtubules in a fixed epithelial cell labeled with monoclonal anti-tubulin and Alexa-488 secondary antibody. Enlarged, diffraction-limited immunofluorescence image of microtubules in the lamella (right) from the marked region in (left) is shown with corresponding SAIM image (bottom). **(b)** SAIM functions as a molecular ruler in a live cell by determining the axial distance between the N- and C-terminal ends of dual-labeled talin, an adhesion protein molecule (N-terminus mCherry and C-terminus mEmerald). Scale bars – 5 μ m.

Visual reconstructions from a sequence of scanned angle images revealed fine axial detail, displaying distinct topographical features corresponding to the sites of focal adhesion complexes, the cortical actin network proximal to the plasma membrane, and the variations in glycocalyx thicknesses ranging from 40 to 85 nm (**Fig. 2.9**). Cytoskeletal architecture in intact fixed cells was assessed by labeling microtubules with tubulin monoclonal antibodies coupled to Alexa488-labeled secondary antibody. The SAIM images had sufficient vertical resolution that they could show that the microtubule network in non-transformed mammary epithelial cells bends downward toward the substrate in the lamella (**Fig. 2.10 A**). Lateral resolution of the technique was close to diffraction limited (full width at half maximum $\sim$ 300 nm; **Fig 2.11 A,B**). Notably, the measured microtubule heights ranged from approximately 70 to 350 nanometers, which is

beyond the working range of other techniques that offer high axial precision, including variable incidence angle TIRF, interference-based PALM, and standard implementations of FLIC.

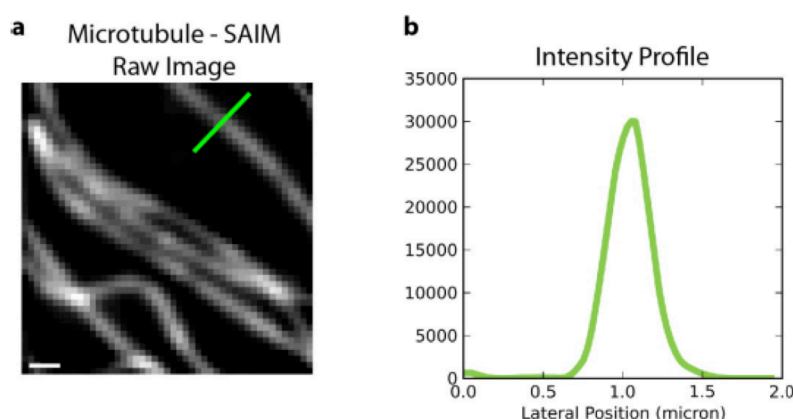


Fig 2.11 SAIM imaging of microtubules in a MCF10A mammary epithelial cell. **(a)** Raw epifluorescence image of Alexa488 labeled microtubules in fixed cells acquired with SAIM. **(b)** The intensity profile across the green line shown in **(a)**. The full width at half maximum of the microtubule cross-section is 300 nm, indicating that SAIM has approximately diffraction-limited lateral resolution.

2.3.5. Molecular Rulers with SAIM

We next determined if the axial precision of SAIM could be applied as a molecular ruler in biological samples, such as a living cell. Imaging of a genetically-expressed N-terminus mCherry and C-terminus mEmerald double labeled talin, an integral focal adhesion molecule, we found that SAIM could easily resolve a 37 nm difference in height between the two talin termini with a precision comparable to iPALM (**Fig. 2.10 B**). Moreover, SAIM has the distinct advantage that these measurements can be conducted in live cells, which affords the opportunity to conduct temporal sampling rates on the order of several seconds. The current implementation of SAIM does not incorporate vertical sectioning and is therefore ideally suited for the measurement of discrete structures with nano-scale thickness (< 150 nm) in the axial dimension, such as plasma membranes and associated proteins, cytoskeletal filaments, microtubules, and vesicles. For fluorophores distributed vertically in a diffraction-limited spot, SAIM provides an average height

of these molecules. For example, the measured height of talin double labeled with mCherry on its C- and N-termini was 72 nm, approximately the average height of the N- and C- termini positions measured using different color fluorophores (**Fig 2.11**).

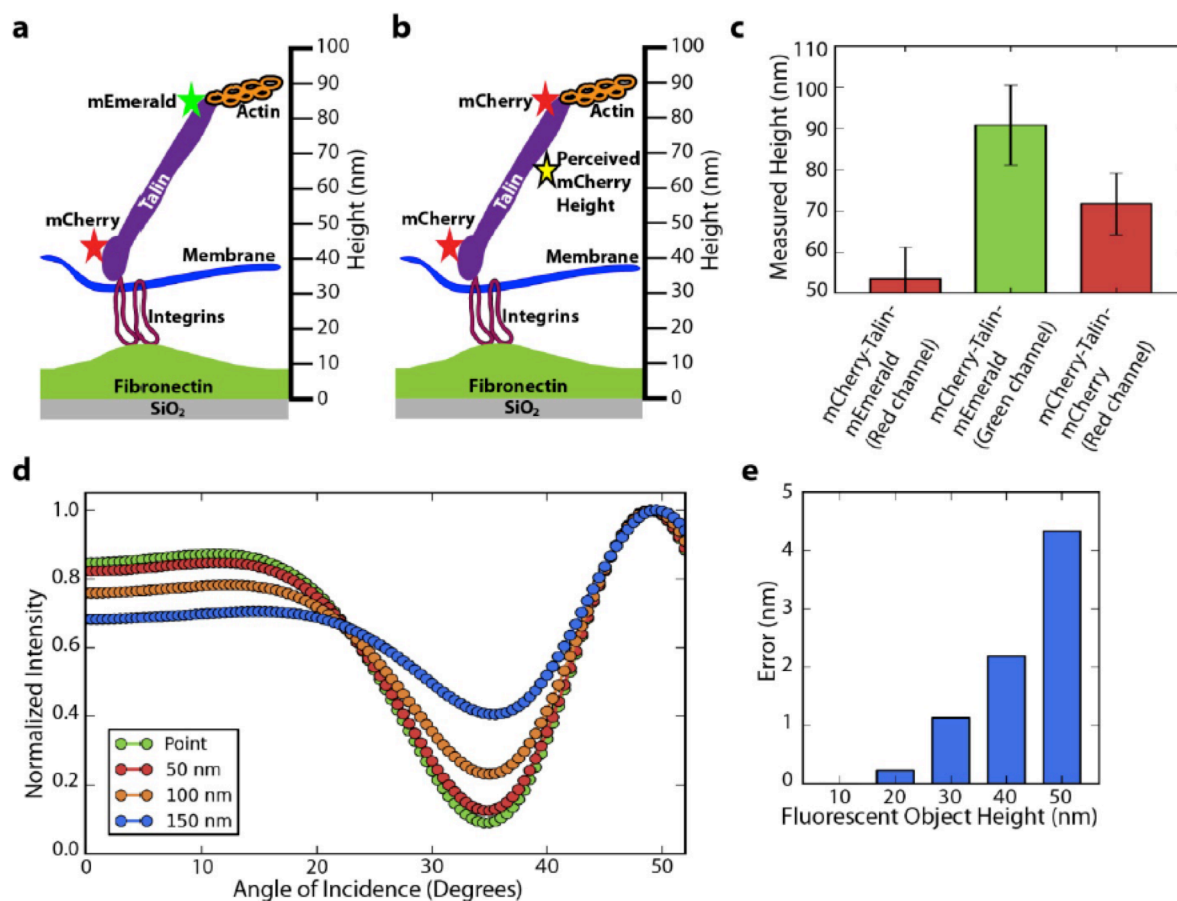


Fig 2.12 Measurement of vertically stacked fluorophores – Talin example. For the case where multiple fluorophores are stacked vertically within a diffraction-limited spot, SAIM provides an average height of the distributed fluorophores. To confirm the performance of SAIM when there are multiple layers of fluorophore within a diffraction limited spot, two double labeled conjugates of the focal adhesion protein talin were constructed. **(a)** In the first construct, mCherry and mEmerald were conjugated to the integrin-binding N-termini and the actin binding C-termini of talin, respectively (mCherry-talin-mEmerald). **(b)** In the second construct, mCherry was conjugated to each termini of talin (mCherry-talin-mCherry). **(c)** Measured height of the talin constructs imaged with the indicated color channel. Imaging the green and red color channels separately for mCherry-talin-mEmerald enabled the resolution of the N- and C- termini axial positions of talin. Measurement of mCherry-talin-mCherry with SAIM reported the average axial position of the talin termini. Averaging of heights for vertically distributed fluorophores was predicted by theoretical results. **(d)** Theoretical intensity profiles for fluorophores with a mean height of 100 nm, distributed randomly across the indicated axial range (point source to 200 nm). **(e)** Results of simulations demonstrating the expected error associated with calculating the height of the distributed fluorophores in **(d)** assuming in the optical model that fluorescence originates from a point source. Interference contrast is significantly reduced for fluorophores distributed randomly over a range greater than 150 nm, setting an upper limit on the thickness of structures that can be imaged by SAIM in its current implementation.

2.3.6. Novel Biological Applications of SAIM – Live cell adhesion dynamics

The unique combination of axial precision and dynamic capabilities of SAIM makes it particularly useful for studying the molecular mechanisms and topological arrangements underlying mechanotransduction, where nanometer strains on cellular structures activate signaling. We chose to image temporal changes in paxillin and vinculin, two key structural and signaling molecules located in focal adhesions, in live cells, where we could monitor in real time mechanical cycles of protrusion and retraction associated with cell migration and physical perturbations in plasma membrane/cytoskeletal/adhesion plaque contacts following cell-cell collision. We next simultaneously measured the heights of paxillin-mEmerald and vinculin-mCherry conjugates, two structural molecules localized to focal adhesions. We observed qualitatively that paxillin increased in height relative to vinculin in the adhesions that formed adjacent to sites of cell-cell contact following the collision of two motile cells (**Fig. 2.13 A,B** and **Supplementary Movie 1**).

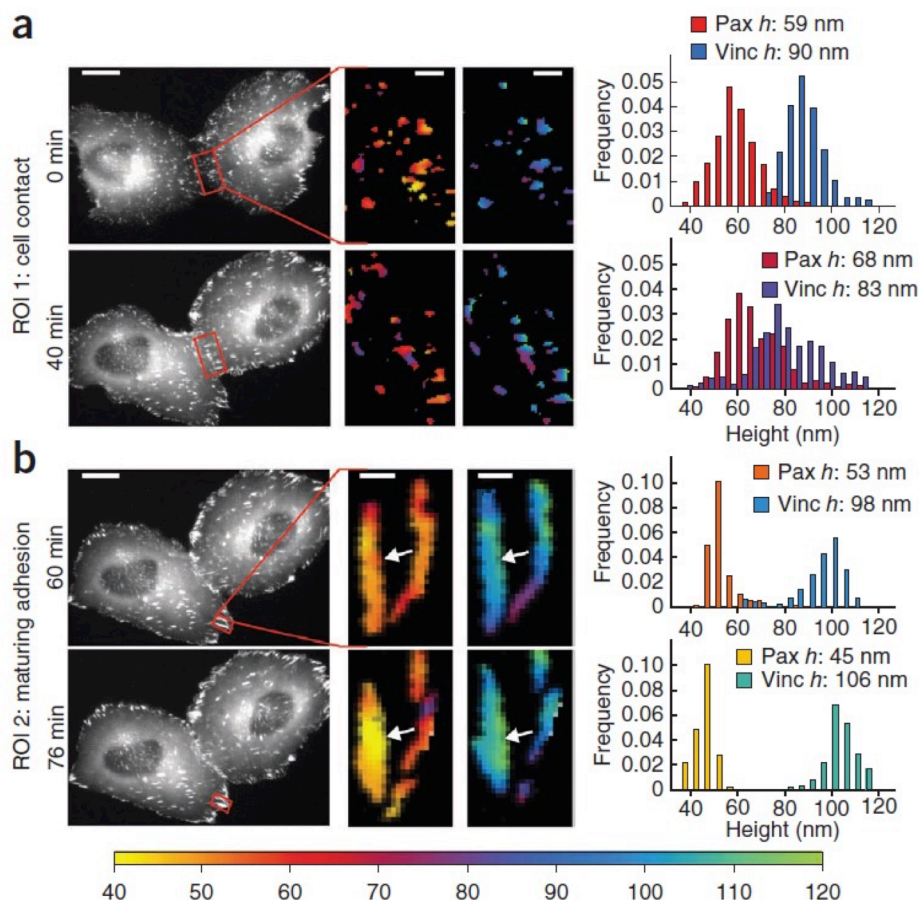


Fig 2.13 Live-cell time lapse multicolor SAIM imaging of MCF10A mammary epithelial cells (MECs) expressing the adhesion complex scaffolding proteins paxillin-mEmerald and vinculin-mCherry. (left) Paxillin epifluorescence image at the indicated time point, region of interest (ROI) boxed in red. (middle) Heights of paxillin and vinculin in ROI, see height color map at bottom of figure. (right) Distribution of paxillin and vinculin heights in ROI, mean height (H) above the oxide surface is calculated over 35 to 120nm. **(a)** ROI 1: Following cell collision, mean paxillin height increased 9nm and mean vinculin height decreased 7nm at the cell-cell interface. **(b)** ROI 2: Assembling adhesion at the site of cell-extracellular matrix interaction; histogram quantification is for adhesion indicated by white arrow.

2.3.7. Novel Biological Applications of SAIM – Nanoscale changes in adhesion architecture

In more quantitative measurements, we observed that paxillin and vinculin were vertically stratified in motile cells; paxillin was at an average height of 61 ± 6.5 nm, and vinculin was at an average height of 99 ± 7.2 nm ($P < 0.001$; $n > 20$ cells; **Fig 2.14**).

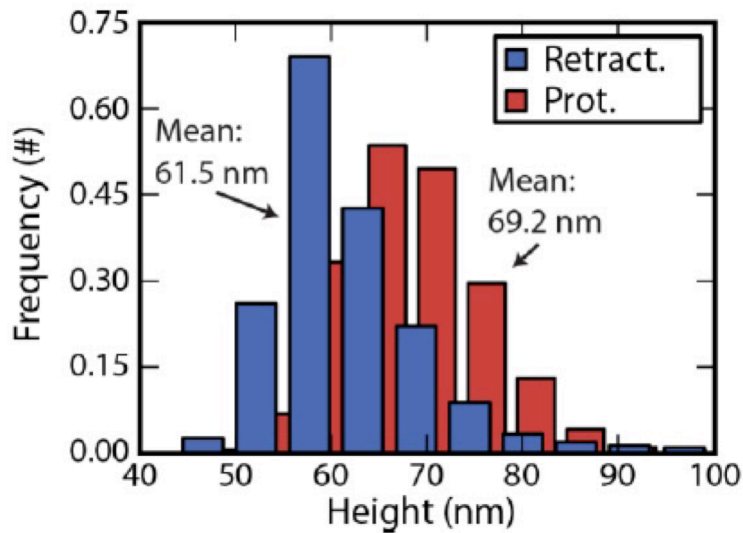


Fig 2.14 Histogram of paxillin-mCherry height in adhesions at the leading edge of live migrating cells during protrusion (Prot.) and retraction cycles (Retract.). The downward movement of paxillin during retraction cycles is significant ($P < 0.001$; $n = 5$ cells, > 100 adhesions).

Finally, we imaged paxillin and vinculin during cycles of cell retraction in the maturing adhesions of motile cells (**Fig. 2.13 A,B** and **Supplementary Movie 1**). Imaging randomly selected adhesions in motile cells revealed that the average height of paxillin in maturing adhesions was significantly lower than that in stable adhesions in nonretracting regions of the cells than that in small, newly formed adhesions in protruding regions ($n = 5$ cells; $P < 0.001$; **Fig 2.15 A,B**). We found that paxillin was typically lowered in maturing adhesions within 5–10 min of the start of cell retraction cycles. In contrast, axial movement of vinculin in maturing adhesions was more random (**Fig. 2.13 A,B** and **Fig 2.15 A,B**). These findings highlight the ability of SAIM to visualize the dynamics of focal adhesion architecture in response to force, and illustrate how proteins within this complex could transduce mechanical information through the spatial reorganization of its molecular components. Our findings indicate a mechanism through which force could activate mechanotransduction by changing the molecular organization of the adhesion complex, or by changing the relative position of molecular layers in response to applied tension¹⁹⁷.

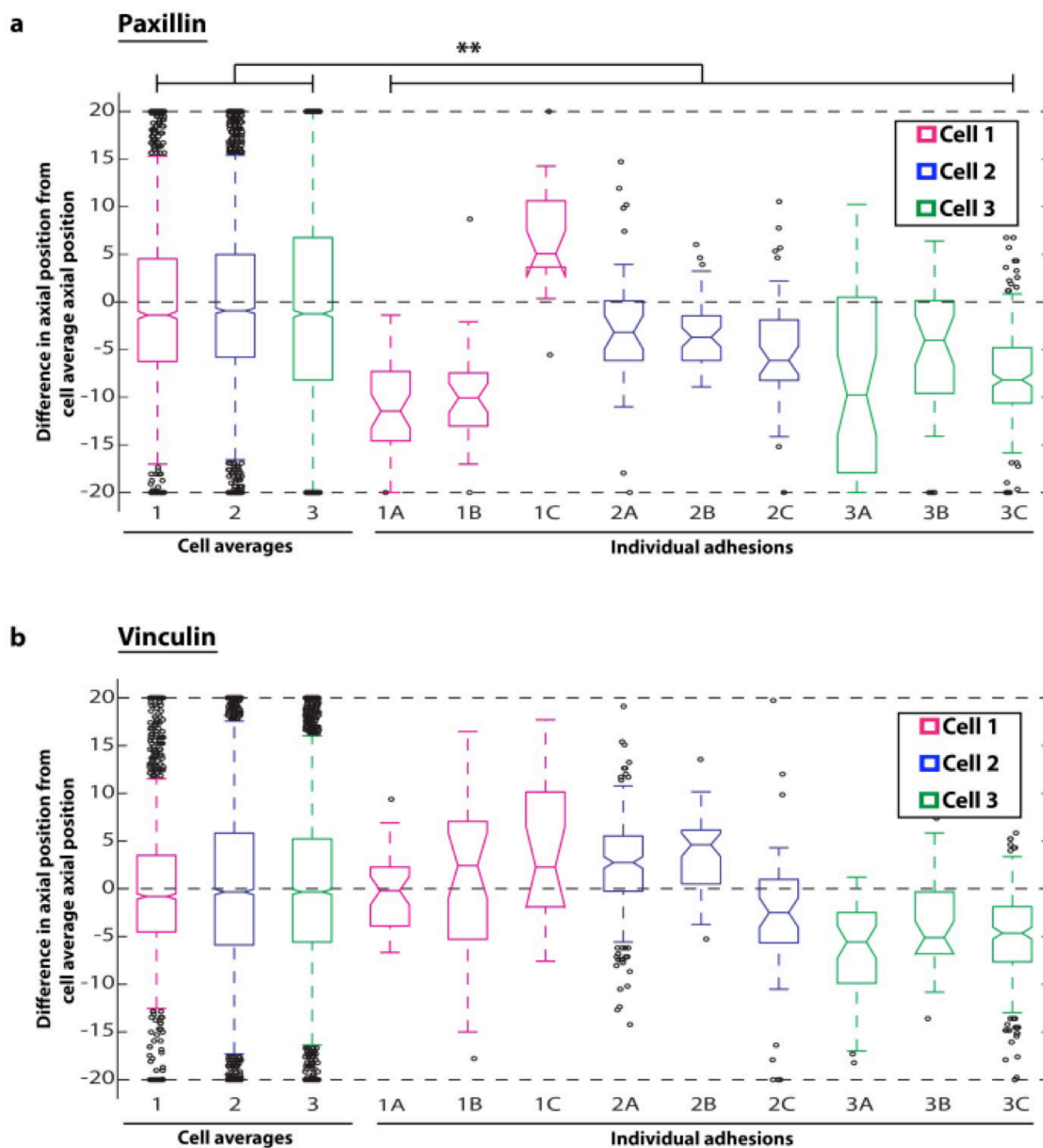


Fig 2.15 Analysis of paxillin-mCherry and mEmerald-vinculin axial position in live motile cells via SAIM measurements at 5 minute intervals. The average heights of paxillin and vinculin in three different cells were compared to the height of these proteins in individual adhesions on the leading edge of lamellar protrusions. Individual adhesions analyzed here were randomly selected from the population of adhesions that met the following criteria: initially larger than 300 nm^2 in the xy plane, growing in size (i.e. maturing), and undergoing retrograde motion from the cell edge. Whole cell average measurements consisted of all visible adhesions. A single boxplot represents the measurements at each pixel comprising an adhesion or set of adhesions; approximately 4000 pixels for a cell average box plot, and ~ 40 pixels per individual adhesion. Axial position is plotted as the difference in height from the respective cell average height at the same timepoint. Measurements of paxillin and vinculin are paired across cells and adhesions. **(a)** Paxillin cell average height did not vary significantly between cells, via ANOVA analysis. Paxillin axial position in individual adhesions were significantly ($p < 0.01$) different from paxillin axial position across an entire cell, via ANOVA and Holm's T-Test statistical methods. In 8 of 9 adhesions, paxillin was displaced downward towards the substrate. **(b)** Vinculin in individual adhesions had both significant downward and upwards movements. We did not observe a correlation of paxillin and vinculin movements in these three cells.

2.4. Discussion

2.4.1. SAIM – Future Applications

Here we have demonstrated that SAIM is capable of localizing biological structures with nanometer-scale precision along the optical axis in living cells on time scales suitable for observing fast dynamic processes. The combination of high axial and temporal resolution, extended depth of field, and the ability to image live cells distinguishes SAIM from existing super-resolution techniques. SAIM can be readily implemented on most *total internal reflection fluorescence* (TIRF) microscopes, and should be broadly applicable to addressing emerging questions related to the spatial organization and nano-dynamics of molecules at or near the membrane, cytoskeleton and extracellular matrix. In particular, SAIM is an excellent new tool for mechano-biology, where nanoscale dynamics and signal transduction have a long range spatial coordination in processes, such as motility and multicellular morphogenesis.

2.4.2. New approaches: Super-resolution microscopy from 2012-2015

Since the publication of SAIM in *Nature Methods* in 2012¹², super-resolution microscopy has become routine in biology labs around the world. Moreover, 3 scientists were awarded the Nobel Prize in Chemistry in 2014 for their contributions to super-resolved microscopy methods. These include Eric Betzig for his contributions to single-molecule microscopy methods including PALM, photoactivated localization microscopy. Additionally, Stefan Hell and William Moerner were awarded for their work in developing STED, stimulated emission depletion. The three of these scientists work were critical for the development of SAIM.

For future investigations into protein nanoscale position, there are several points to consider. For resolving molecules in live cells with <5nm resolution in the axial plane of the microscope objective, SAIM remains the best in the field. For lateral super-resolution of fixed samples, new techniques have emerged that allow both PALM and electron microscopy²²¹. For super-resolution imaging of live samples in three dimensions at high speed, Eric Betzig and his colleagues at Janelia Farms developed lattice light-sheet microscopy for ~20nm 3D resolution of 1x1x1mm space in ~1 second²²². However, it remains seen if this technique will be simplified for use in labs with less microscopy expertise and funding. In comparison, SAIM costs ~\$200,000 to implement, while lattice light-sheet microscopy >\$1,000,000.

2.4.3 Contributions

Matthew Paszek and Valerie Weaver conceived and initiated the project. Matthew Paszek, Christopher Dufort, Matthew Rubashkin, Kurt Thorn, and Valerie Weaver designed experiments. Matthew Paszek and Kurt Thorn developed the optical theory and C++ computational framework. Matthew Paszek, Christopher Dufort, Kurt Thorn and Matthew Rubashkin designed and implemented the imaging hardware. Matthew Rubashkin, Matthew Paszek and Christopher Dufort implemented the calibration methodology and microscopy macros for operation. Matthew Rubashkin and Matthew Paszek developed and implemented ImageJ and Python data analysis and visualization scripts. Matthew Paszek, Christopher Dufort and Matthew Rubashkin performed cellular experiments on fixed samples. Matthew Rubashkin, Christopher Dufort and Matthew Paszek performed live cell experiments. Christopher Dufort, Matthew Paszek and Matthew Rubashkin performed *in vitro* verification experiments including bead height measurements. Matthew Paszek performed *in silico* verification experiments. Michael Davidson

and Matthew Paszek designed and prepared plasmid constructs. Matthew Paszek, Christopher Dufort and Matthew Rubashkin prepared the figures with assistance from Valerie Weaver and Jan Liphardt. Matthew Paszek, Christopher Dufort, Matthew Rubashkin, and Valerie Weaver, wrote the manuscript with editorial assistance from all authors.

2.4.4. SAIM – Acknowledgements

I graciously thank the team that developed SAIM, Matthew Paszek, Christopher Dufort, Kurt Thorn, Mike Davidson, Jan Liphardt, and Valerie Weaver. We graciously thank J. Lakins for guidance on vector design and the construction and preparation of the talin lentiviral constructs and T. Onuta for assistance with the fabrication of silicon wafers at the Maryland Nanocenter FabLab. We also thank M. Krummel and S. Peck for helpful discussions. Image acquisition for this study was performed at the Nikon Imaging Center at the UCSF.

2.4.5. SAIM – Funding

This work was supported by BCRP DoD Era of Hope grant W81XWH-05-1-0330, NIH/NCI TMEN grant 1U54CA163155-01, NCI grant U54CA143836-01, and NIH/NCI R01 CA138818-01A1.

Chapter 3. ECM Stiffness Drives Vinculin

Mediated Adhesion Stabilization Enhancing

PI3K Signaling, Promoting Tumor Progression

3.1. Introduction

3.1.1. Abstract

Extracellular matrix stiffness induces focal adhesion assembly to drive malignant transformation and tumor metastasis. Nevertheless, how force alters focal adhesions to promote tumor progression remains unclear. Here, we explored the role of the focal adhesion protein vinculin, a force-activated mechano-transducer, in mammary epithelial tissue transformation and invasion. We found that extracellular matrix stiffness stabilizes the assembly of a vinculin-talin-actin scaffolding complex that facilitates PI3-kinase (PI3K) mediated phosphatidylinositol (3,4,5)-triphosphate phosphorylation (PIP3). Using defined two and three dimensional matrices, a mouse model of mammary tumorigenesis with vinculin mutants and a novel super resolution imaging approach, we established that ECM stiffness, per se, promotes the malignant progression of a mammary epithelium by activating and stabilizing vinculin and enhancing Akt signaling at focal adhesions. Our studies also revealed that vinculin strongly co-localizes with activated Akt at the invasive border of human breast tumors, where the ECM is stiffest and we detected elevated mechano-signaling. Thus, extracellular matrix stiffness could induce tumor progression by promoting the assembly of signaling scaffolds; a conclusion underscored by the significant association we observed between highly expressed focal adhesion plaque proteins and malignant transformation across multiple types of solid cancer.

3.1.2 Remodeling of the Extracellular Matrix in Tumor Progression

The extracellular matrix (ECM) is remodeled and stiffened in tandem with the malignant transformation of tissues^{223–227}. Enhanced collagen cross-linking stiffens the ECM *in vivo* and *in vitro*, increases phosphorylation of focal adhesion kinase (p^{397} FAK), and promotes mammary tumor progression; whereas preventing ECM cross-linking and decreasing tissue tension reduces FAK activity, and prevents tumor cell invasion and metastasis^{223,228}. Although these findings implicate force-induced focal adhesions in malignant progression the molecular mechanisms remain unclear. Moreover, ECM concentration and organization also influence focal adhesion assembly, and ECM density differs in tumors, while the invasive front of a transformed epithelium frequently contains tracts of perpendicularly oriented, collagen fibers^{7,72,223,229,230}. These data suggest that the altered ECM topology and density in tumor tissue could also promote integrin focal adhesion assembly to drive malignant progression. Along these lines Kubow and colleagues argue that adhesion-mediated cell migration in three dimensions (3D) is determined not only by myosin activity, but also by the architecture and density of the local ECM²³¹. Indeed, whether a cell can even assemble a focal adhesion in a 3D ECM and in tissues has recently been debated^{70,232,233}.

3.1.3 Focal adhesion mechanobiology

Focal adhesions are the conduits through which cells process extrinsic mechanical signals¹⁶⁴. Vinculin is a critical component of focal adhesions and has been shown to regulate cell spreading and stabilize focal adhesions^{182,234–236}. The phosphorylation and mechanical unmasking of a

cryptic auto-inhibited binding site in vinculin favors a conformational change that permits binding to talin and actin^{182,237–239}. Vinculin also mechanically couples actin retrograde motion and increases force transmission at focal adhesions^{235,240,241}. Thus, vinculin is a mechanically-activated mechano-transducer that is ideally posed to mediate force-dependent cell invasion. Consistent with this hypothesis, vinculin is up-regulated in primary invasive human cancers, mediates single cell invasion within a 3D collagen hydrogel, and its loss compromises cell migration during embryonic development^{242–244}. Nevertheless, whether and how vinculin mediates force-induced tumor cell invasion has yet to be determined.

ECM adhesion is critical for cell growth and survival and integrins can directly and indirectly enhance growth factor receptor signaling^{245,246}. Integrins are a major molecular constituent of focal adhesions and focal adhesions facilitate actin assembly and actomyosin stimulation and regulate growth factor-dependent ERK and phosphoinositide 3-kinase (PI3K) activation^{247,248}.

3.1.4 Overview of studies performed.

We showed that ECM stiffness promotes growth factor-dependent PI3K activation of Akt in culture²²³. Using a mouse model of HER2/Neu induced mammary cancer we also demonstrated that inhibiting collagen cross-linking and reducing tissue tension prevents malignant transformation and represses tumor cell invasion while simultaneously decreasing PI3K signaling through Akt²²³. These findings suggest that ECM stiffness could promote malignant progression and tumor cell invasion by enhancing growth factor receptor signaling. Here we asked whether vinculin, as a major mechanically sensitive focal adhesion molecule that binds actin and talin and transmits mechanical cues, could translate ECM stiffness to drive malignant

transformation by potentiating growth factor receptor signaling through PI3K. We found that ECM stiffness stabilizes a mechanosensitive vinculin-talin-actin adhesion complex that facilitates PI3K-mediated Phosphatidylinositol (3,4,5)-triphosphate (PIP3) production. Thus by driving the assembly of a molecular scaffold at the focal adhesion, ECM stiffness potentiates oncogenic signaling through PI3K to drive malignant transformation.

3.2. Methods

3.2.1. Cell Culture and Reagents

MECs including non-malignant MCF10A (ATCC, 2007; verified by epithelial cellular morphology in 2D, ability to form hollow polarized acini in 3D rBM gels, and expression of epithelial markers including E-Cadherin and cytokeratins 8 and 14) and pre-malignant Ha-ras transformed MCF10AT (Karmanos Cancer Institute, 2008; verified by epithelial cell morphology in 2D) were cultured (7) and 3D multicellular spheroids were generated in recombinant basement membrane (rBM; Matrigel) suspension cultures (32). MCF10A vinculin knockdown (KD) and wild-type recovered MECs were generated using vinculin shRNA and vinculin-GFP retrovirus (gifted by Dr. Kris DeMali 2013; verified by epithelial cell morphology in 2D) (33). Vinculin homozygous null mouse embryonic fibroblasts were cultured as previously described (gifted by Dr. Andreas Garcia, 2013; verified by mesenchymal cell morphology in 2D)(18). The following small molecule inhibitors were used: Y27632 ROCK inhibitor at 10 μ M (Cayman Chemical), FAK Inhibitor 14 at 1 μ M (Tocris), GDC-0941 PI3K inhibitor at 1 μ M (Selleckchem), NSC 23766 Rac1 inhibitor at 50 μ M (Cayman Chemical), ML7 MLCK inhibitor at 10 μ M (Sigma Aldrich), and U0126 MEK1/2 inhibitor at 10 μ M (LC Laboratories).

3.2.2. Expression Constructs

N-terminus GFP tagged vinculin (Gallus) constructs (gifted by Dr. Susan Craig)(24), including pEGFPC1/ Gg V1-258 WT, pEGFPC1/ Gg V1-1066 T12, pEGFPC3/ Gg V884-1066, pEGFPC1/ Gg V1-1066 A50I, pEGFPC1/ Gg V1-851. Vinculin tension sensor components 26019, 26020, and 26021 were acquired from Addgene. Other plasmid constructs used included N and C terminus tagged vinculin-mEmerald, paxillin-mCherry, farnesyl-mEmerald, farnesyl-mCherry. Transient transfection was performed using the Lonza Nucleofector Kit V, with program T024 for MECs and program U024 for fibroblasts. Lentiviral construct PGK-H2BeGFP was used in combination with Clontech mCherry-CAAX and mEmerald CAAX vectors and the pLV MCS vector to generate pLV hPGK mCherry-CAAX. The phosphatidylinositol 4,5-bisphosphate, PIP₂, (GFP-PH-PLC δ) and phosphatidylinositol (3,4,5)-triphosphate, PIP₃, (mKO2-PH-Grp1) activity probes were prepared as described (gifted by Keith Mostov)(34).

For constructing the pcDNA3.1 Vinculin T12 Tension Sensor, the C-terminal tail of pEGFPC1/ Gg V1-1066 T12, was amplified with PCR using a forward primer that introduced a 5'-SacII site (5'-GGCACGGCCGCGGAGTTCCCAGAGCAGAAAGCAGGA-3') and the reverse primer: 5'-CAAATGTGGTATGGCTGATTATGATC-3'. The PCR fragment was then cloned in place of the C-terminal tail of vinculin tension sensor (Addgene # 26019, Martin Schwartz depositing scientist) using the SacII and EcoRI restriction sites to make pcDNA3.1 Vinculin T12 Tension Sensor. Lentiviral construct PGK-H2BeGFP (Mark Mercola depositing scientist, Addgene #21210) was digested with BamHI and KpnI to remove H2B-eGFP and the WPRE. DNA encoding mCherry-CAAX and mEmerald-CAAX was PCR amplified from corresponding

plasmid vectors (Living Colors Series, Clontech) from the Davidson Laboratory, Florida State University, using the following primers: Forward GACTGGATCCGAATTCGCTACCGGTCGCCACCATG and Reverse GAGCGCGGCCGCATCAGGAGAGCACACTTGCAGCTC and the resulting products digested with BamHI and NotI. The WPRE was isolated from lentiviral vector pLV MCS as a NotI-KpnI fragment and ligated together with (BamHI-NotI) mCherry-CAAX or mEmerald-CAAX and KpnI-BamHI x to generate lentiviral constructs pLV hPGK mCherry-CAAX and pLV hPGK mEmerald-CAAX in which the farnesylated fluorescent protein is expressed from the human phosphoglycerate kinase promoter. For stable expression of paxillin-mEmerald, vinculin-mCherry, mCherry-Farnesyl, and mEmerald-Farnesyl recombinant lentivirus was prepared by transient transfection into HEK-293T cells and used to transduce MEC cells ²⁴⁹. Stable cell lines were selected in 200 ng ml⁻¹ G418, 380 ng ml⁻¹ hygromycin, or 1 ug/ml puromycin, and sorted on a FACS Aria II cell sorter (BD Biosciences) for mEmerald, mVenus or mCherry expression. Expression of paxillin-mEmerald or vinculin-mCherry was induced with 200 ng/ml doxycycline in growth medium for 1 d before experimentation.

3.2.3. Substrate and Hydrogel Preparation

Collagen gels were fabricated by diluting 3.8 mg/ml acid solubilized rat tail collagen (BD) in 1:1 DMEM-F12 medium (Invitrogen), neutralizing to pH 7.4 with 1M NaOH. SAP gels were fabricated by suspending cells in pre-sonicated 0.8mg/ml or 5.0mg/ml SAP (BD PuraMatrix™ Peptide Hydrogel), with 10% sucrose, 0.1% bovine serum albumin, human plasma fibronectin (Millipore), and growth medium ¹¹⁶. 2D polyacrylamide (PA) gels (height ~200um) were fabricated by varying the acrylamide and cross-linker concentration and assessing stiffness by

Atomic Force Microscopy or using a shear rheometer^{225,250}. N-type [100]-orientation silicon wafers with 1933nm silicon oxide (Addison Engineering; 1 cm x 1 cm) and borosilicate glass coverslips (~150µm thick) were cleaned by successive sonication in acetone and 1M KOH (20 min) followed by chemical activation with silane (0.5% (3-aminopropyl)trimethoxysilane) and glutaraldehyde (0.5% in water). The substrates were then UV and ethanol sterilized and incubated overnight (4°C) in extracellular matrix (human fibronectin; 10 µg/ml). Prior to use, substrates were washed in PBS (5 times; pH 7.4), treated with sodium borohydride (20mg/ml) and washed with PBS. SAIM calibration wafers were prepared by sonicating carboxylate-modified red fluorescent spheres (100nm; Invitrogen), following by bead deposition (5X10⁸ beads per ml) in NaCl (100mM) PBS solution^{12,251}.

3.2.4. Immunofluorescence

Cultures were fixed with 4% paraformaldehyde (2D: 20 minutes, room temperature; 3D: overnight, 4°C) and staining was performed as described (1). Primary antibodies against vinculin (hVIN-1, Sigma Aldrich; 700062, Invitrogen; V284, Santa Cruz Biotechnology), β 1 integrin (AIIB2, isolated from rat hybridoma), β 4 integrin (3E1, Millipore MAB1964), p397FAK (44625G, Invitrogen), p473Akt (Cell Signaling 9271S), Akt(pan)-Alexa488 (Cell Signaling C67E7), E-cadherin (610181; BD Transduction), β -catenin (610153, BD), pan-laminin (L9393, Sigma), laminin 5 (P3H9, isolated from mouse hybridoma), α 6 integrin (GoH3, eBiosciences), phospho-myosin light chain kinase 2 - Thr18/Ser19 (3674, Cell Signaling), talin (T3287, Sigma), zyxin (BD ,610521), and AlexaFluor phalloidin (633-conjugate, Invitrogen) were used. Secondary antibodies used include AlexaFluor goat anti-mouse, anti-rabbit, and anti-rat (488, 568 and 633 conjugates).

3.2.5. Microscopy and Analysis

Imaging was performed on a motorized TIRF inverted microscope system (Ti-E Perfect Focus System; Nikon) controlled by Metamorph software (Molecular Devices), equipped with 488nm, 561nm, and 640nm lasers, 350/50 epifluorescence, a CSU-X1 spinning disc confocal unit (Yokogawa Electric Company), electronic shutters, motorized stage, a scientific sCMOS camera (Zyla 5.5 megapixel, Andor), and an electron-multiplying charged-coupled device camera (QuantEM 512; Photometrics). Temperature and CO₂ was controlled by an environmental chamber and PID controlled heater (In-Vivo Scientific). Samples were imaged with a 100x-1.49NA TIRF oil-immersion, 40x-1.2NA Long-Working-Distance (LWD) water-immersion, 20x-0.75NA air, and 10x-0.45NA air objectives. For live-cell imaging, phenol red-free growth media with 10 mM Hepes (pH 7.4) was used. For 3D imaging, confocal image slices were taken at 0.2µm or 1.0µm slices, and the individual planes were maximum intensity z-projected for 2D visualization. A custom software package for image analysis was written in Python, using ImageJ and the Eclipse Development Environment in Linux. All images were subject to a Gaussian blur of 1.0 pixel to smooth background noise. DAPI images were subject to a local background subtraction of ~5µm to reduce fringing from epifluorescence imaging through the spinning disc unit. Intensity and colocalization measurements of single confocal planes were calculated on a pixel-by-pixel basis. 3D image rendering was performed in Imaris (Bitplane). Cell projections and adhesions were counted manually. Acini cross-sectional area was quantified with the freehand selection tool in ImageJ. Photobleaching FRET (pbFRET) and Scanning Angle Interference Microscopy (SAIM) were performed as described ¹², using data analysis and 3D visualization programs written in Matlab® and Python using libraries distributed by Enthought, Inc.

3.2.6. Förster resonance energy transfer (FRET) Microscopy

Imaging was performed on an inverted Ti-E Perfect Focus System (Nikon) controlled by NIS-Elements software (Nikon); equipped with a CSU-X1 spinning disk confocal unit, 60x-1.49NA objective, environmental control, controlled electronic shutters, motorized stage, and a charge-coupled device camera (Clara; Andor). For measurement of FRET efficiency, the acceptor photobleaching method pbFRET was implemented with live cells on the spinning disk confocal. mTFP1 was first imaged with 454 nm excitation and a 480/20 emission filter, mVenus was subsequently bleached using a 200 mW 515 nm laser for 10 seconds, and mTFP1 was imaged again following bleaching of mVenus. Microscope Z-focus was maintained during image acquisition using the Ti-E Perfect Focus System. Background images were constructed by imaging 10 unique cell-free regions on the coverslip and averaging the intensity at each pixel. The FRET efficiency was calculated on a pixel-by-pixel basis according to:

$$\text{FRET Efficiency (\%)} = \left[1 - \frac{I_{pre} - B_{pre}}{I_{post} - B_{post}} \right] * 100\%$$

where I_{pre} is the mTFP1 intensity prior to bleaching mVenus, B_{pre} is the mTFP1-channel background intensity prior to bleaching mVenus, I_{post} is the mTFP1 intensity after bleaching mVenus, and B_{post} is the mTFP1-channel background intensity after bleaching mVenus. Appropriate controls were implemented to account for inadvertent mTFP1 photobleaching and incomplete mVenus photobleaching.

3.2.7. Two-Photon-Microscopy

Second harmonic imaging was performed on an upright Olympus base microscope controlled by MicroManager software, with a 20× (NA .95) water immersion objective. The setup included a mode-locked 10W MaiTi Ti:Sapphire femtosecond laser (720–1050nm) and four separate channel simultaneous photomultiplier tubes for fluorescent detection. Paraformaldehyde fixed, hydrated samples were exposed to polarized laser light at a wavelength of 830 nm and emitted light was separated with a filter set (dichroic mirror 495nm, bandpass 400/40nm, and bandpass 645/75nm).

3.2.8. Mouse and Human Studies

Cohorts of PyMT (polyoma middle T) and FVB mice were maintained in accordance with University of California IACUC guidelines under protocol AN092125. Starting at 4 weeks of age, mice were treated with a LOX function-blocking polyclonal antibody (3mg/kg; OpenBiosystems) injected intraperitoneally twice per week (1). Mice were sacrificed at 80-95 days of age at which time the 4th mammary gland was paraformaldahyde fixed. Under protocol 10-05046 and in accordance with the UCSF Committee on Human Research, formalin-fixed and paraffin-embedded human breast cancer biopsies containing normal, DCIS (ductal-carcinoma-in-situ) and estrogen receptor and progesterone receptor positive invasive cancer tissue were obtained. Mouse mammary tissue (10µm) and human breast biopsy (6µm) sections were analyzed for histology (H&E) and parallel tissue was stained for the focal adhesion proteins vinculin, p397FAK, and β 1 integrin and for the PI3K downstream target p473Akt.

3.2.9. Atomic force microscopy of single collagen fibers.

Mica disks were glued to microscope slides and freshly cleaved prior to use with a piece of tape. A drop of collagen alignment buffer containing 150 mM KCl and 50 mM L-glycine adjusted to pH 9.2 with KOH was then added to the mica surface and allowed to incubate for 30 min. Rat tail collagen was added to the droplet at a final concentration of 0.1 mg ml⁻¹ and incubated overnight at room temperature. The next day the disks were gently washed with PBS to remove unbound collagen. Imaging and force measurements were performed on an Asylum Research MFP-3D (Santa Barbara, CA) atomic force microscope. Samples were imaged in air in non-contact AC-mode using Olympus AC160TS ($k = 42$ N/m) cantilevers, and were calibrated by the thermal tune method. The 3D image of the individual collagen fiber is the height with phase channel painted over the image to increase the contrast of the repeating subunits. Force measurements were collected at individual points along single fibers and set to a trigger force of 1.5 μ N.

3.2.10. Bioinformatics Assay

We analyzed immunohistochemical (IHC) data compiled by the Human Protein Atlas database²⁵². In this database, the abundance of 15,156 proteins has been estimated in 20 panels of tumor samples and in 83 normal cell types. Each normal cell type was assayed once, and the number of tumor samples assayed for each protein ranged between one and sixty tumor samples (median 12). The abundance of each protein's expression within each sample was classified as undetectable, low, moderate, or high expression levels. For each protein, we determined the most common expression level observed across all samples within each tumor type to generate composite profiles of “typical” protein expression levels for each tumor type. We then matched

these composite estimates to the protein abundance estimates derived from each normal cell type to identify the normal cell type whose protein expression patterns most closely match those observed within each tumor class. We estimated the similarity of expression profiles as the proportion of proteins whose abundance was identical in both samples, and assigned the normal cell types most similar to each tumor composite to be the paired normal tissue used in subsequent analyses.

To determine how the abundance of each protein differs between the assayed tumor samples and their paired normal cells, we tested the proportion of samples within each cancer type in which the protein's abundance was elevated relative to the level observed in the paired normal cell type. Using a defined set of adhesion proteins²⁵³ and oncogenic driver proteins²⁵⁴, we calculated the median proportion of tumor samples in which the expression of each adhesion protein was elevated, and compared these values to the distribution of similarly calculated medians derived from 10,000 sets of n randomly selected proteins, where n is the number of proteins assigned to the adhesion. We defined empirical P-values for each tumor type quantifying the evidence for a positive shift in adhesion protein expression as the proportion of permuted sets whose median increase equaled or exceeded that observed within the adhesion protein set. To control for biases related to the expression structure of the adhesion protein set (e.g., differences in the number of highly expressed or lowly expressed proteins in the adhesion relative to the full distribution) we performed this analysis separately within the sets of genes assigned to each expression class within the normal tissue.

We observed variability in the evidence that the adhesome is disproportionately upregulated in the specific tumor types assayed, and so to summarize the overall evidence for adhesome changes across tumor types we directly compared the distributions of tumor-specific empirical P-values estimated above using a one-sided Kolmogorov-Smirnov test.

3.2.11. Statistics

Statistical analysis for 2 groups was performed with an unpaired, two-tailed student's t-test. For multiple comparisons, an ANOVA test and Holm–Bonferroni t-test method were performed. Calculations were implemented in Python.

3.3. Results

3.3.1. ECM stiffness and ligand density regulate focal adhesions to promote tumor cell invasion in three dimensions

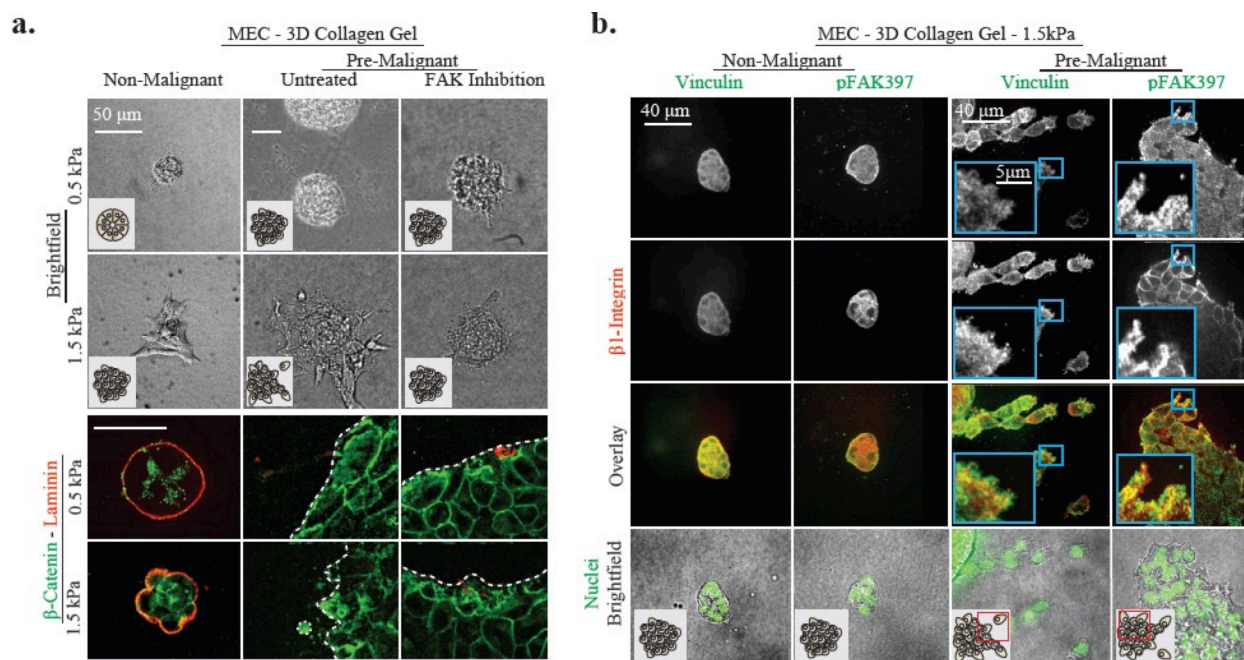


Fig 3.1 In 3D, ECM cues promote vinculin recruitment to focal adhesions, projection formation, and cell invasion. **A.** MEC spheroids composed of MCF10A cells in 0.5 kPa or 2 mg/ml 1.5 kPa 3D collagen gels for 24hrs. Invasion is increased by matrix stiffness and Ha-ras malignant transformation (MCF10AT cell line), and decreased via inhibition of integrin mediated FAK signaling (FAK Inhibitor 14 at 1μM). To document loss of normal 3D cell organization in stiff 3D collagen, cell polarity was studied 48 hours after seeding in collagen via immunostaining for β-catenin (cell-cell junctions) and pan-laminin (basal surface). Dotted white lines indicate spheroid edge. **B.** Pre-malignant and non-malignant MEC 1.5kPa 3D collagen gels for 48 hours, immunostained for integrin β1, vinculin and pFAK39. Images are maximum intensity z-projections of 1μm increment confocal image slices, and demonstrate focal adhesion formation at cell-ECM borders.

Most studies linking ECM stiffness to focal adhesion assembly and cell motility have been conducted using single transformed cells or mesenchymal fibroblasts on 2D substrates^{255,256}. Here we examined the importance of tension-induced focal adhesions in nonmalignant versus transformed epithelial cell invasion both as single cells and tissue-like structures in 3D. We first transplanted 5 day old, rBM pre-assembled, proliferating nonmalignant MCF10A and premalignant Ha-ras MCF10AT transformed mammary epithelial cell (MEC) spheroids into

collagen-rBM gels with compliances calibrated to match normal (0.5kPa, 0.5mg/ml), premalignant (1.5kPa; 2.0mg/ml) and malignant (2.5kPa; 5.0mg/ml) mammary tissue ^{7,223,225}. Phase contrast (**Fig 3.1A**, top 2 left panels) and confocal immunofluorescence imaging (**Fig 3.1A**, bottom left 2 panels) revealed that the transplanted nonmalignant mammary spheroids retained their integrity, even 24 hours after embedment within the compliant collagen/rBM gels, as indicated by the maintenance of spherical acini, intact adherens junctions, and tissue polarity, as revealed by basally-localized laminin and cell-cell localized b-catenin (**Fig 3.1A**, left column). However, immunostaining revealed that after 48 hours in the stiffer gels the basal polarity and cell-cell junctions in the nonmalignant structures were severely compromised (**Fig 3.1A**, center column; **Fig 3.2A**). Importantly, the nonmalignant cells at the periphery of the colonies in the stiffer gels probed the local ECM, as indicated by prominent cell protrusions ²⁵⁷, although they never invaded into the gel (**Fig 3.1A**, left column).

3.3.2. Role of oncogene activation in 3D cell adhesion and invasion

In contrast to the non-malignant MCF10a, the Ha-Ras premalignant mammary spheroids retained a semblance of tissue polarity when embedded within the softest collagen/rBM gels (**Fig 3.1A**, center column) indicated by retention of cell-cell localized β -catenin and minimal protrusions; in the stiffer gels the structures completely collapsed and the transformed MECs invaded both collectively and individually (**Fig 3.1A**, center column)²²³. Interestingly, two photon imaging revealed that Ha-ras transformed MCF10AT MECs invaded collectively and as single cells along collagen bundles that appeared to project perpendicularly from the colonies (**Fig 3.3A**), and atomic force microscopy illustrated that collagen bundles can be quite stiff under compression (**Fig 3.3B, 3.3C**) ^{223,258}. Thus, although these findings do not rule out the possibility that ECM

invasion into these collagen gels was also induced by the topological features of the collagen, the data do strongly implicate ECM stiffness.

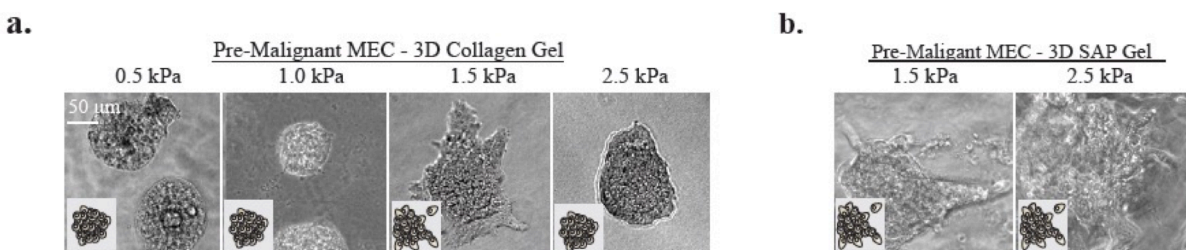


Fig 3.2 ECM cues, malignant transformation, and integrin signaling, promote cell spreading and invasion. **A.** 5 day pre-malignant MCF10AT spheroids in 0.5, 1.0, 2.0 or 3.0 mg/ml (0.5 kPa, 1.0 kPa, 1.5 kPa, 2.5 kPa) 3D collagen gels for 24hrs. Invasion from these spheroids into the surrounding gel is increased by matrix stiffness up to 1.5 kPa; and impaired at 2.5 kPa **B.** Pre-malignant MEC spheroids in 1.5 kPa and 2.5 kPa 3D SAP gels for 24 hours. Spheroids are invasive in 1.5 kPa and 2.5 kPa SAP gels.

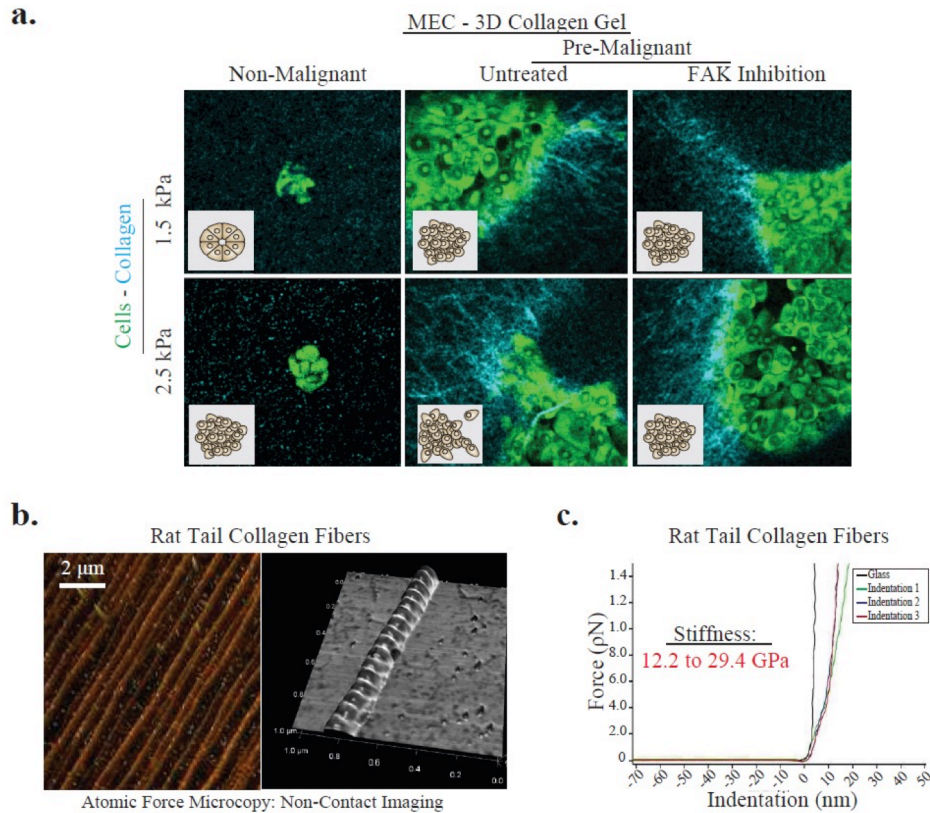


Fig 3.3 MECs remodel 3D collagen gels for cell invasion. Individual collagen fibers are stiff and $>500\text{nm}$ in diameter. **A.** MEC spheroids composed of MCF10A cells in 0.5 kPa or 1.5 kPa 3D collagen gels for 48hrs. Collagen fibers are imaged via two-photon second harmonic generation (SHG) and cells are stained with propidium iodide. Collagen fibrils bundling is induced by Ha-Ras malignant transformation (MCF10AT cell line), but not inhibited by FAK inhibition. Invasion is increased by matrix stiffness and malignant transformation, and decreased via inhibition of integrin mediated FAK signaling (FAK Inhibitor 14 at $1\mu\text{M}$). In stiff 3D gels, untreated malignant MEC spheroids invade both collectively and as single cells into the matrix along collagen fibrils. **B.** Atomic force microscopy images (non-contact mode) of rat tail collagen fibers aligned on a mica surface. **C.** Atomic force microscopy mechanical measurements of individual rat tail collagen fiber imaged in B. Compression measurements shown here were collected at individual points along a single fiber at a trigger point of $1.5\mu\text{m}$.

3.3.3. Oncogene transformation and tissue stiffness synergistically drive tumor cell invasion

To explore functional links among focal adhesions, ECM stiffness, and tumor cell invasion, we stained 3D multi-cellular structures for the transmembrane protein $\beta 1$ integrin which connects the ECM to cell adhesions, and the focal adhesion proteins vinculin and $p^{397}\text{FAK}$, as well as fluorescently labelled phalloidin to image filamentous actin. Confocal imaging revealed that MECs at the periphery of the Ha-ras premalignant colonies, as well as the individual transformed

MECs invading into the stiffer collagen/rBM gels, stained robustly for all three focal adhesion proteins (**Fig 3.1B**, right columns) as well as F-actin (data not shown). Moreover, treatment of the premalignant spheroids with 1 μ M FAK-Inhibitor 14 ablated the invasive behavior of these transformed mammary tissues within the stiffer gels (**Fig 3.1A**; right column). However, we also detected abundant levels of these adhesion proteins arranged as punctate structures reminiscent of focal adhesions in the nonmalignant MECs at the periphery of the spheroids where the cells interact directly with the stiff ECM (**Fig 3.1B**; left columns). Thus, although these findings suggest ECM stiffness activates focal adhesions to promote MEC invasion in 3D, the data also indicate that focal adhesions are not by themselves sufficient, and MEC invasion also requires the activation of pathways engaged by oncogenic transformation^{7,223}.

3.3.3. Cells respond to ECM stiffness and ligand availability in both traditional 3D collagen matrices as well as synthetic self-assembled-peptide gels with constant pore size

To directly test the role of ECM stiffness on tumor cell invasion in 3D, in the absence of changes in ECM pore size, topology, density or composition we used 3D RADA-16 self-assembling peptide (SAP) gels doped with saturating concentrations of laminin 111 or fibronectin¹¹⁶. By increasing polymer concentration, the compliance of these SAP gels can be varied across a range of stiffness (0.5-5kPa) appropriate for mammary and other epithelial tissues, with negligible effects on pore size and ECM topology, and we and others showed that cells embedded in these gels ligate the ECM protein(s) passively adhered to the peptides and respond to the stiffness of the gel^{116,259}. Consistently, we observed that MECs embedded in SAP gels with saturating concentrations of ECM ligand (fibronectin; 6.2 mg/ml) increased their protrusive activity in

proportion to the stiffness of the gel (**Fig 3.4A; 3.2B**); similar to what we observed in 3D collagen gels.

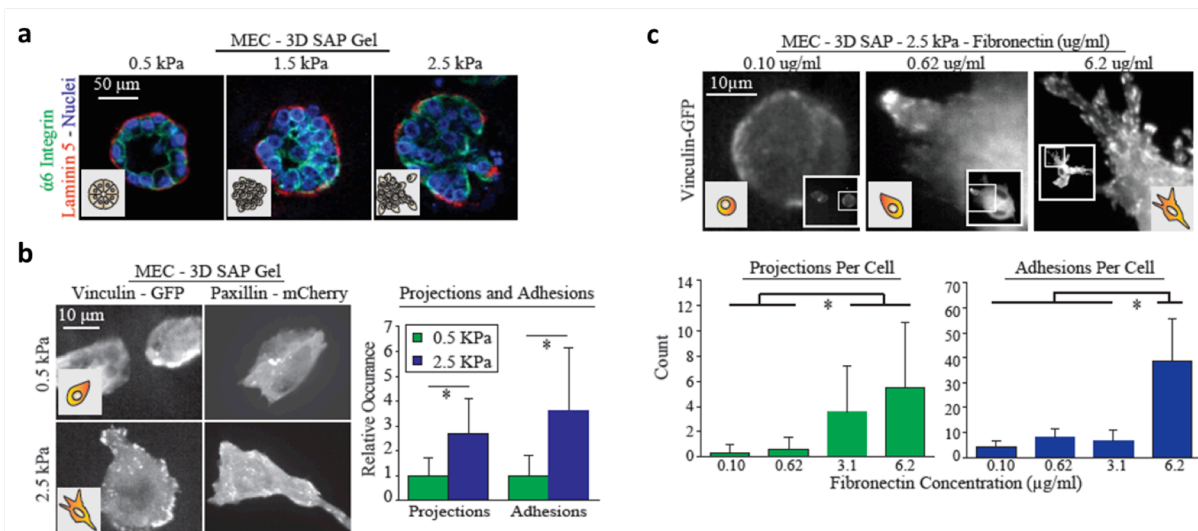


Fig 3.4 A. Non-malignant MEC spheroids in 3D SAP gels with 0.8, 2.2 or 5mg/ml RADA-16 polymers; stained for polarity markers $\alpha 6$ Integrin (cell-cell junctions) and laminin-5 (basal polarity). **B.** Non-malignant MEC adhesions and projections in soft and stiff 3D SAP gels; with quantification of projections and adhesions per cell ($p < .01$; $\pm$ SD, $n > 18$ cells per condition). **C.** Non-malignant MEC expressing vinculin-GFP in 3D SAP gels with controlled ECM fibronectin concentration. Images are maximum intensity z-projection of 1.0um confocal image planes. Cellular projections and adhesions per cell were significantly upregulated by ECM stiffness and fibronectin ($p < .01$; $\pm$ SD, $n > 12$ cells per condition).

Confocal imaging also revealed that MECs co-expressing GFP-tagged vinculin and mCherry paxillin assembled small punctae reminiscent of focal contacts in the softest SAP gels, and larger focal adhesion-like structures in the stiffer SAP gels (**Fig 3.4A**). Furthermore, the size and number of these adhesions as well as the number of cellular protrusions, which is a proxy for invasive behavior, increased in proportion to ligand concentration (**Fig 3.4B, 3.4C**). Importantly, after embedment into the stiffer SAP gels immunofluorescence imaging revealed that the integrity and polarity of the nonmalignant MEC organoids was severely compromised, as indicated by loss of basally-localized $\alpha 6$ integrin and basally deposited laminin 5 (**Fig 3.4A**). Moreover, in marked contrast to their behavior in the collagen/rBM gels where pore size limited

invasion (**Fig 3.2A**, 4th column), phase contrast imaging revealed that the invasive behavior of the premalignant mammary colonies increased further in the stiffest SAP gels (**Fig 3.2A**). These observations show that ECM stiffness and ligand density regulate focal adhesions to permit the invasion of an oncogenically-transformed epithelium in 3D.

3.3.5. Vinculin is a major focal adhesion plaque protein whose structure-function is exquisitely sensitive to mechanical force

It has been previously shown that vinculin is a mechanically sensitive protein that can act as a mechanical clutch to stabilize adhesions ^{235,240}. This prompted us to ask if ECM stiffness promotes tumor cell invasion by activating vinculin to stabilize focal adhesions. Consistently, we noted that MECs expressing a wild-type vinculin (vinculin WT) that were plated on a soft fibronectin-conjugated polyacrylamide gel (PA gel) assembled small focal contacts, showed only modest protrusive activity and failed to spread (**Fig 3.5A**, top left panel) ⁷. By contrast, parallel cultures of MECs plated on soft gels that expressed a constitutively active vinculin T12, which lacks the auto-inhibition domain, had increased adhesion area, exhibited robust protrusive activity and spread appreciably (**Fig 3.5A**, top right panel; **3.5B**).

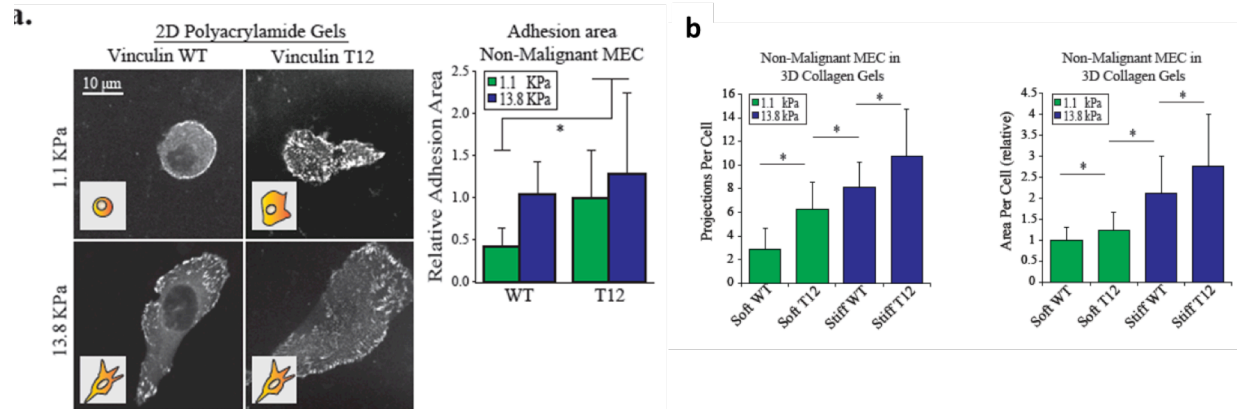


Fig 3.5 Vinculin activity is necessary for adhesion stabilization. **A.** Non-malignant MEC expressing vinculin-WT or vinculin-T12 TFP1-mVenus tension sensors on 2D polyacrylamide (PA) gels of varying stiffness coated with fibronectin and quantification of relative adhesion area per cell ($p < .01$; $\pm$ SD, $n > 10$ cells per condition). Vinculin T12 expression significantly increases cell adhesion area on soft matrices. **B.** Non-malignant MEC expressing vinculin-WT or vinculin-T12 TFP1-mVenus tension sensors on 2D polyacrylamide (PA) gels of varying stiffness coated with fibronectin, with quantification of cell projections and relative spread area. Vinculin T12 expression significantly increases cell spreading and projections on soft matrices as compared to vinculin WT expressing MECs ($p < .05$; $\pm$ SD, $n > 19$ cells per condition).

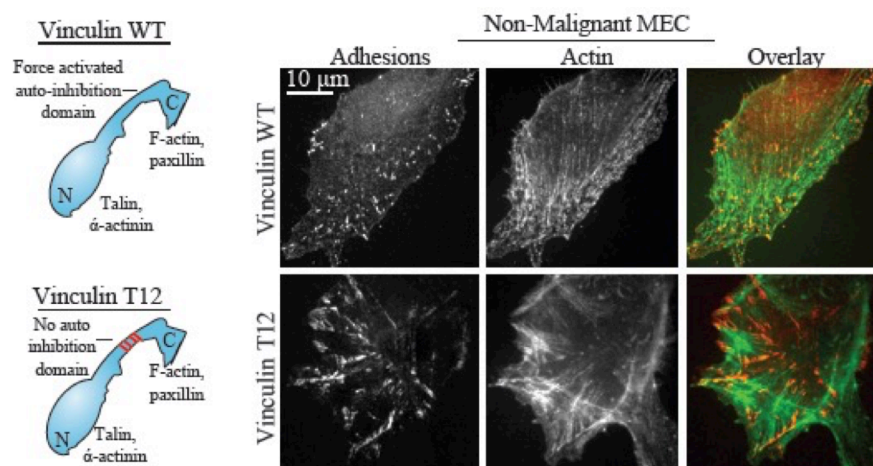


Fig 3.6 Diagram of vinculin WT and vinculin T12 activity mutant prominent binding domains, and confocal images of N-terminus GFP tagged vinculin and filamentous actin stained with phalloidin in non-malignant MEC on 2D glass coated with fibronectin. MECs expressing the activated vinculin T12 show prominent stress fibers and localized more vinculin at the focal adhesions. Vinculin T12 expression significantly increases cell spreading and projections on soft matrices as compared to vinculin WT expressing MECs ($p < .05$; $\pm$ SD, $n > 19$ cells per condition).

MEC expressing vinculin T12 on stiff substrates had prominent stress fibers and localized more vinculin at the focal adhesions (**Fig 3.6**)¹⁸². Moreover, MECs in which vinculin levels were reduced using shRNA had significantly reduced protrusive activity, reflecting invasive behavior,

even when the cells were embedded within a stiff, fibronectin-saturated, SAP gel (**Fig 3.7A**). By contrast the protrusive activity of these MECs was fully restored following re-expression of an RNAi resistant vinculin (**Fig 3.7A**). In this regard, we observed that the ability of vinculin to restore the protrusive activity in vinculin null murine fibroblasts in response to ECM stiffness required a critical level of cellular vinculin, where the greatest protrusive activity was noted in cells with the highest vinculin expression (**Fig 3.7B**). Thus, fibroblasts expressing high amounts of vinculin assembled punctate adhesive-like structures analogous to focal adhesions, and increased their protrusive activity in response to a stiff SAP gel (**Fig 3.7B**)²⁴⁴. These data demonstrate that ECM-induced invasion requires the engagement of a critical threshold of vinculin that stabilizes focal adhesions.

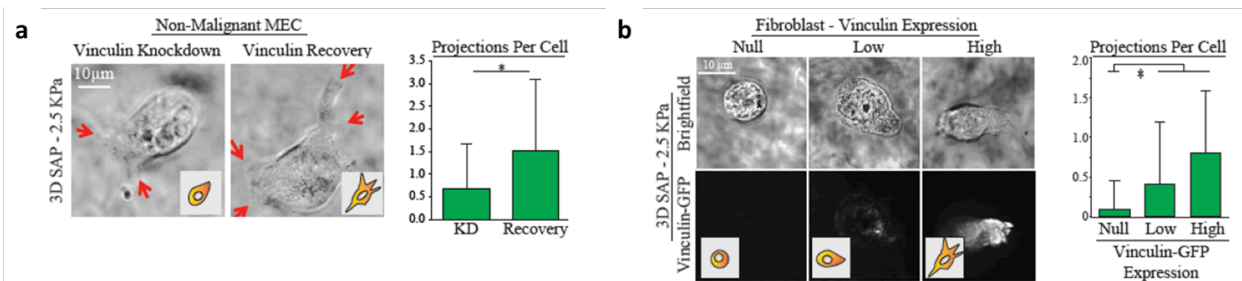


Fig 3.7 A. Non-malignant MEC projections (indicated by red arrows) in 3D 2.5 kPa SAP gels with 6.2ug/ml fibronectin after vinculin shRNA knockdown and recovery via vinculin-GFP expression, and quantification of projections ($p < .01$; $\pm$ SD, $n > 27$ cells per condition) **D.** Vinculin homozygous null mouse fibroblasts transiently expressing different levels of vinculin-GFP in 3D 2.5 kPa SAP gels with 6.2ug/ml fibronectin and quantification of projections ($p < .01$; $\pm$ SD, $n > 9$ cells per condition). Vinculin significantly increases cell projections in stiff 3D SAP gels in MEC and fibroblasts.

3.3.6. Relationship of vinculin nanoscale position and activity to cell intrinsic forces

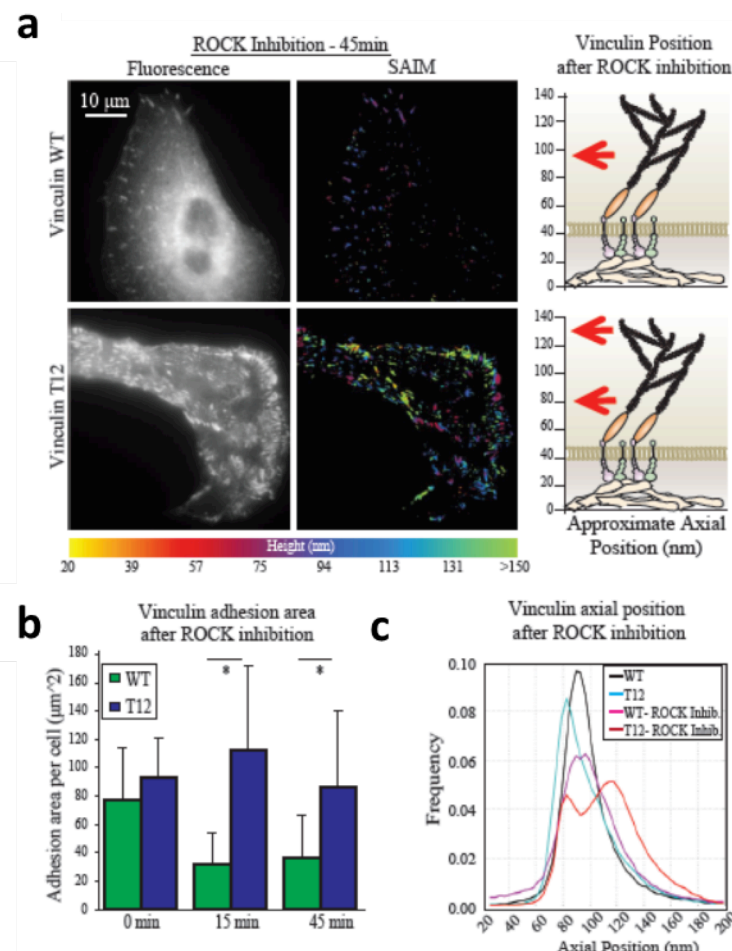


Fig 3.8 A. Non-malignant MEC plated on 2D fibronectin-coated glass, 45 minutes after ROCK inhibition (10 μM Y27632) Left: representative image of vinculin WT and T12 fluorescence, Middle: axial position of vinculin WT and T12 via SAIM. Right: diagram of vinculin WT and T12 after ROCK inhibition.

B. Quantification of adhesion area per cell after ROCK inhibition ($p < .01$; $\pm\text{SD}$, $n > 18$ cells per condition).

C. Axial distribution of vinculin and vinculin T12 after ROCK inhibition. Histogram constructed from >3500 single pixel ($0.012 \mu\text{m}^2$) SAIM measurements per condition ($n > 18$ cells per condition). ROCK inhibition reduced the size of vinculin WT positive adhesions but did not perturb axial position; while vinculin T12 positive adhesions were not reduced in size, but localized to axial positions of 80nm and 120nm

We next explored the relationship between force, vinculin activation, and focal adhesion stabilization. We first demonstrated that 15-45 minutes following ROCK inhibition (Y27632; 10 μM), the size and number of the vinculin positive focal adhesions was significantly decreased in the non-malignant MECs expressing a GFP-tagged vinculin WT (**Fig 3.8**, bottom left graph). By contrast, no quantifiable change in either the size or the number of adhesions was observed in the ROCK inhibitor treated MECs expressing the GFP-tagged vinculin T12 (**Fig 3.8**, bottom left

graph). These findings agree with prior data obtained in fibroblasts which established a role for actomyosin tension in vinculin-mediated focal adhesion stabilization^{182,236}.

3.3.7. Focal adhesion protein architecture after inhibition of actomyosin contractility

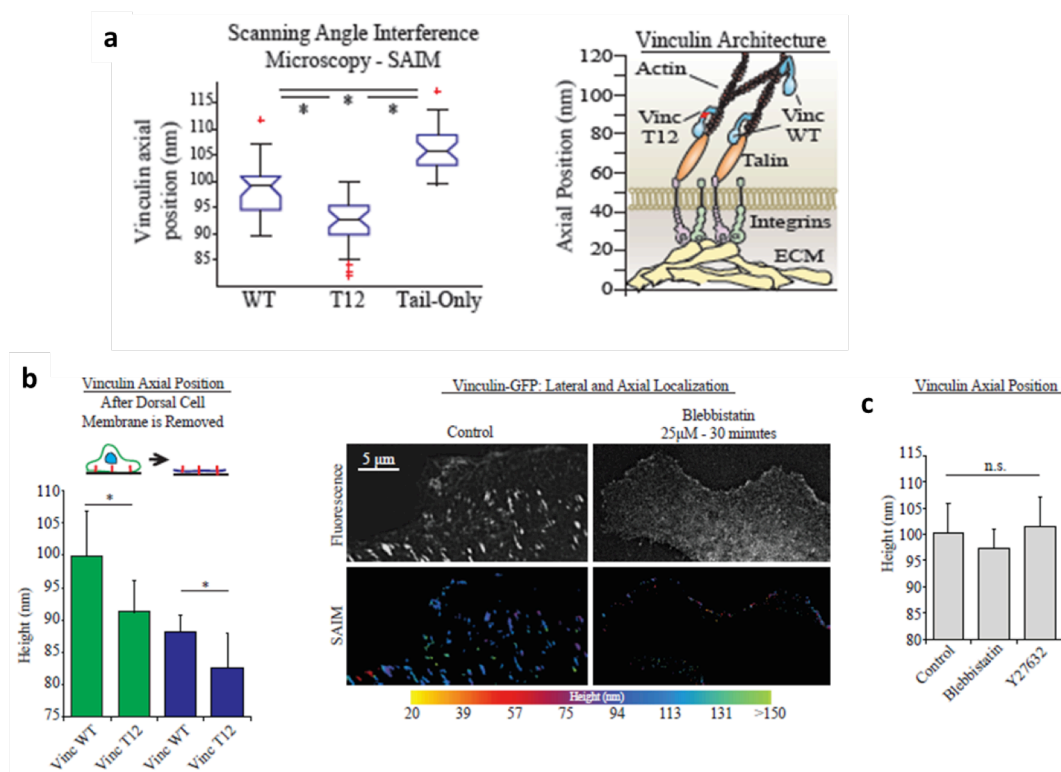


Fig 3.9 A. Vinculin nanoscale axial position in non-malignant MEC plated on a 2D glass surface via SAIM measurements of whole cell averages ($p < .01$; $\pm$ SD, $n > 26$ cells per condition), and diagram of vinculin axial position relative to other adhesion proteins. Vinculin T12 is at a height (95nm) indicating talin-actin binding, vinculin WT is at a height of (100nm) indicating both talin-actin and actin only interactions. **B.** SAIM measurements of vinculin in the ventral surface of non-malignant MECs on 2D glass with or without the removal of the dorsal membrane and cytoplasmic vinculin. Vinculin T12 is localized 5-10nm beneath vinculin WT with or without the cytoplasmic vinculin fraction, indicating vinculin T12 is simultaneously engaged with talin and actin, while vinculin WT has both talin-actin and actin only interactions¹². Mean and standard deviation are calculated from all adhesions over several cells ($p < .05$; $\pm$ SD, $n > 2000$ pixels per condition). **C.** MEC on 2D glass after 25 minutes of myosin II inhibition (Blebbistatin; 25μM) or control treatment. Top: representative image of vinculin WT fluorescence; Bottom: axial position of vinculin via SAIM; Right: Vinculin axial position after Myosin II or ROCK (Y27632; 10μM) inhibition, mean and standard deviation are calculated from all adhesions over several cells (no significant difference between treatments).

Focal adhesions are composed of over 200 proteins which are segregated into stratified functional layers^{218,253}. Yet, scanning angle interference microscopy (SAIM), which accurately records the nanoscale position of the molecular constituents of focal adhesions, indicates that the spatial organization of many of adhesion plaque proteins, including the scaffolding molecule paxillin change dramatically and dynamically during focal adhesion assembly and cell invasion¹². Therefore, to understand the interplay between extrinsic and intrinsic force and vinculin-mediated stabilization of focal adhesions and cell invasion we monitored vinculin dynamics during focal adhesion assembly and disassembly using SAIM. Imaging showed that vinculin WT localized at 100 nm (**Fig 3.9A**), which is a composite of the talin-actin and actin only bound molecules. Indeed, we observed that a constitutively active vinculin T12, all of which is bound to a talin-actin complex, resides at 95 nm and a vinculin tail only mutant, which binds entirely to actin, localized to a height of 105 nm (**Fig 3.9A**).

To rule out the effect of cytoplasmic vinculin, we removed the dorsal cell membrane and cytoplasm in MEC and were then able to measure a significant change in vinculin T12 axial position compared to vinculin WT in the ventral cell surface (**Fig 3.9B**). We also observed similar behavior of several vinculin mutants between *vinc*^{-/-} mouse fibroblasts and MEC, and were able to measure the separation of vinculin's N and C terminus (**Fig 3.10A, 3.10B**).

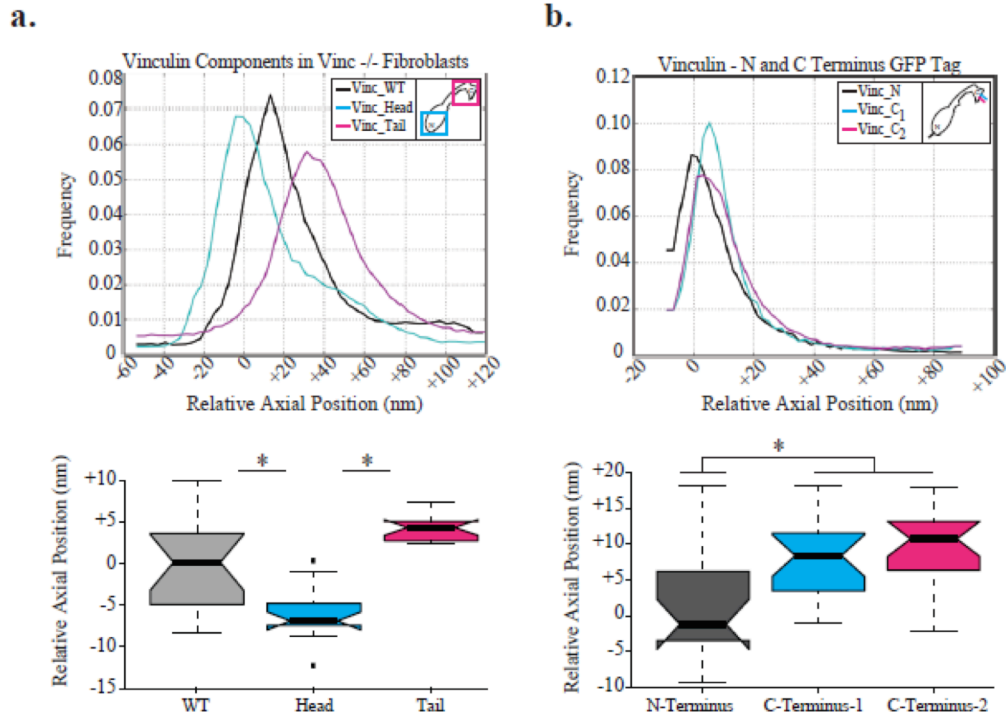


Fig 3.10 A. Vinculin null mouse fibroblasts on 2D fibronectin coated reflective glass substrates transiently expressing N-terminus EGFP tagged vinculin components: Amino acids 1-1066 Vinc_WT, 1-851 Vinc_Head, and 884-1066 Vinc_Tail. Axial position was measured per pixel via SAIM, and data from all cells are included in histogram and boxplots (created with the BoxPlotR webtool)⁴²⁰ of relative vinculin component height. There were significant differences between Vinc_WT, Vinc_Head and Vinc_Tail ($p < .01$; $\pm$ SD, $n > 14$ cells per condition). **B.** Vinculin null mouse fibroblasts on fibronectin coated reflective substrates transiently expressing N and C-terminus EGFP tagged vinculin. Two C-terminus clones from different sources were tested. Axial position was measured per pixel via SAIM, and data from all cells is included in histogram and boxplots of relative vinculin component height. There was a significant difference in height between N- and C-terminus tagged clones ($p < .01$; $\pm$ SD, $n > 9$ cells per condition).

3.3.8. Paxillin protein architecture after inhibition of actomyosin contractility

Ablating actomyosin intrinsic tension in the MECs, by treatment with a ROCK inhibitor (**Fig 3.8**) shifted the bulk of the vinculin WT to the actin associated fraction, while vinculin T12 remained localized to the focal adhesions with altered axial localization (**Fig 3.8**). The vinculin WT that remained at the adhesions after ROCK or myosin II inhibition (Blebbistatin; 25 μ M) was bound at a stable height of 100nm, which is a composite of talin-actin bound and actin-bound (**Fig 3.8**; **Fig 3.9B**). In contrast, a different adhesion plaque protein, paxillin, shifted from 60nm to a height of 90nm following myosin inhibition (**Fig 3.11**). The focal adhesion architecture of paxillin is further investigated in **Chapter 4**. These findings illustrate that once engaged; the vinculin-talin-actin complex is remarkably stable.

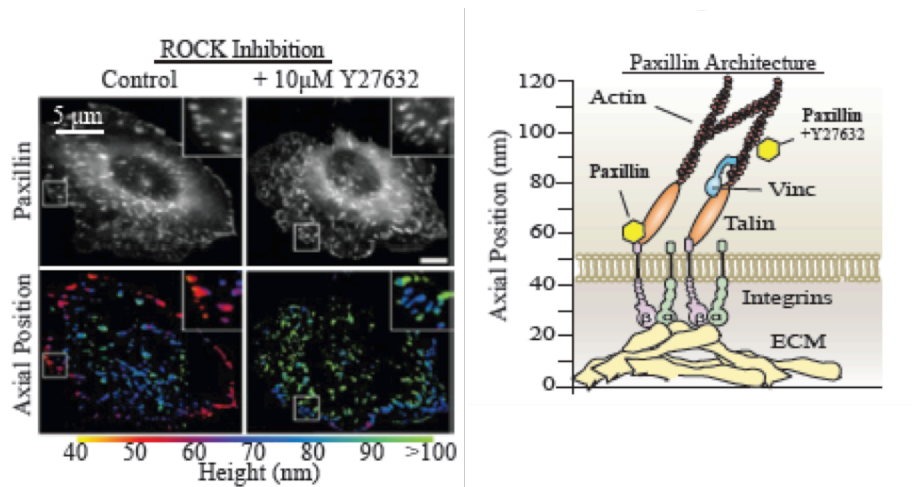


Fig 3.11 Paxillin-GFP fluorescence and SAIM measurements in MEC plated on a 2D glass surface. Paxillin is localized to focal adhesions and is anchored at a low height in peripheral cell adhesions and higher in internal fibrillar and sub-nuclear adhesions. After 5 minutes of actomyosin contractility inhibition via 10 μ M Y27632, paxillin axial position is increased from 60 to 100nm.

3.3.9. Intramolecular force over vinculin is impacted by vinculin activity levels and cellular response to ECM stiffness

To determine if ECM stiffness promotes cell invasion by stably increasing cell-generated force over the vinculin-actin-talin complex we used a vinculin intramolecular tension sensing probe²³⁸

with the photobleaching Förster resonance energy transfer (pbFRET) method (**Fig 3.12A**). We then examined the response of vinculin WT and mutant expressing cells to ECM stiffness using fibronectin-conjugated PA gels. We found in MECs on either soft (1.1kPa) or stiff (13.8kPa) fibronectin-conjugated PA gels, that vinculin WT incorporated into focal adhesions was under the same tension (**Fig 3.12B**). However, the tension on the auto-activated vinculin T12, which forms a stable talin-actin-vinculin complex, increased significantly in MECs on the stiffer gels as compared to level on the softer gels, presumably because more force-activated talin becomes available to bind (**Fig 3.12A,B**). Thus, force activates vinculin to facilitate its assembly into a highly stable talin-actin complex that in turn applies actomyosin-mediated tension on the ECM that is proportional to the level of recruited vinculin.

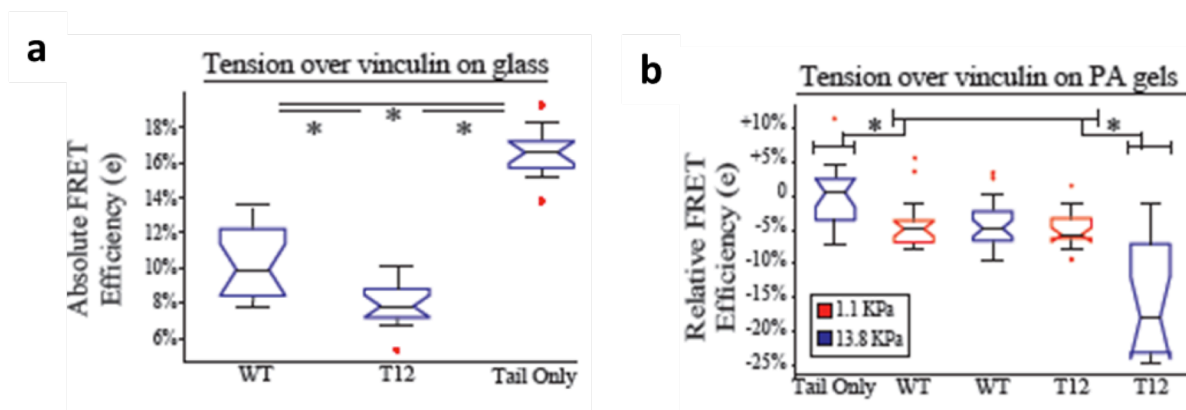


Fig 3.12 A. Tension over vinculin activity mutants in MECs plated on a 2D glass surface via an intramolecular vinculin FRET sensor using the photo-bleaching FRET method, measurements of all pixels in adhesions averaged over whole cell ($p < .05$; $\pm$ SD, $n > 17$ cells per condition). **B.** Tension over vinculin on soft and stiff PA gels ($p < .05$; $\pm$ SD, $n > 10$ cells per condition). Tension over vinculin is increased through a T12 activity mutation and ECM stiffness.

3.3.10. ECM stiffness drives increases in vinculin localization to adhesions as well as FAK and Akt signaling

Tumor cell invasion *in culture* and *in vivo* requires PI3K activity^{223,260,261} Moreover, ECM stiffness enhances epidermal growth factor receptor dependent PI3 activity *in culture* and

inhibiting ECM cross-linking and stiffening reduces PI3K activity and tumor cell invasion²²³. We therefore asked whether ECM stiffness promotes PI3K-dependent tumor cell invasion by increasing the amount of the vinculin-actin-talin complex at the focal adhesion. Consistently, we observed that more vinculin was recruited to adhesion sites when the ECM was progressively stiffened (**Fig 3.13 A,B**). ECM stiffness also increased the quantity of activated ^{p473}Akt and to a lesser extent ^{p397}FAK to the sites of adhesion (**Fig 3.13 A**), where we were able to quantify substantial co-localized vinculin to ^{p473}Akt and some vinculin and ^{p397}FAK (**Fig 3.13 B**).

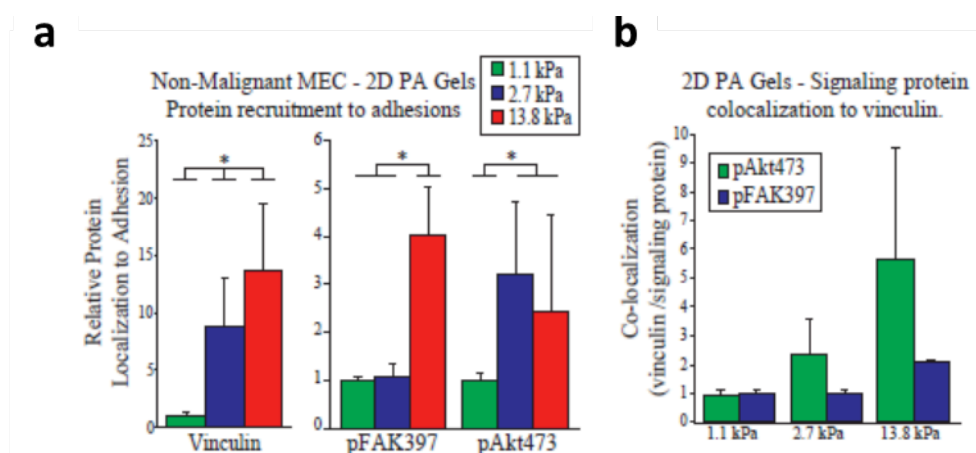


Fig 3.13 Vinculin, ^{p473}Akt, and ^{p397}FAK recruitment to adhesions in non-malignant MEC on 2D PA Gels of different stiffness, coated with fibronectin. Left: Protein recruitment measured by quantitative immunofluorescence at single pixels (0.011 μm^2) and averaged over whole cell ($p < .01$; $\pm$ SD, $n > 18$ cells per condition) Right: Quantification of signaling protein colocalization to vinculin per pixel cell, averaged over whole cell ($p < .01$; $\pm$ SD, $n > 18$ cells per condition).

3.3.11. ECM stiffness mediated vinculin stabilization promotes signaling via Akt and FAK phosphorylation at adhesions

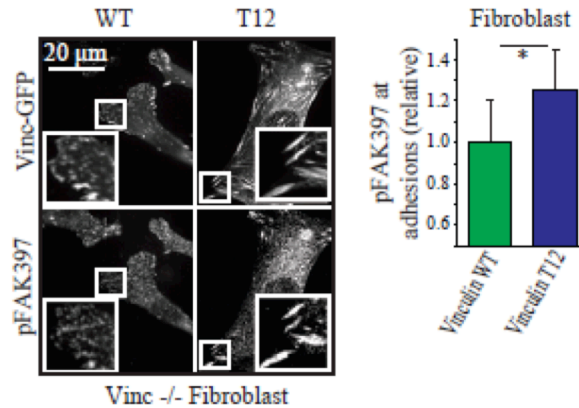


Fig 3.14 p^{397} FAK recruitment to adhesions in vinculin homozygous null mouse fibroblasts expressing vinculin WT or T12 plated on a 2D PA gel. Vinculin T12 significantly increases p^{397} FAK; measurements made at single pixels and averaged over whole cell ($p < .05$; $\pm$ SD, $n > 9$ cells per condition).

The quantity of signaling molecule recruited to the adhesion was significantly enhanced in vinculin null fibroblasts expressing the vinculin T12, which fosters the assembly of a stable talin-actin complex, as indicated by enhanced p^{397} FAK and vinculin as compared to the lower levels measured at the adhesions in the WT vinculin expressing fibroblasts (**Fig 3.14**). These findings suggest that ECM stiffness promotes the assembly of a stable vinculin-actin-talin complex at the focal adhesion that then permits the nucleation and subsequent efficient activation of signaling molecules critical for cell invasion. We observed that 10 minutes after EGF stimulation Akt was recruited to adhesions on both soft and stiff 2D PA matrices in MECs, but importantly, we were only able to document a significant increase in activated p^{473} Akt in vinculin-enriched focal adhesions in MECs on a stiffer substrate (**Fig 3.15**).

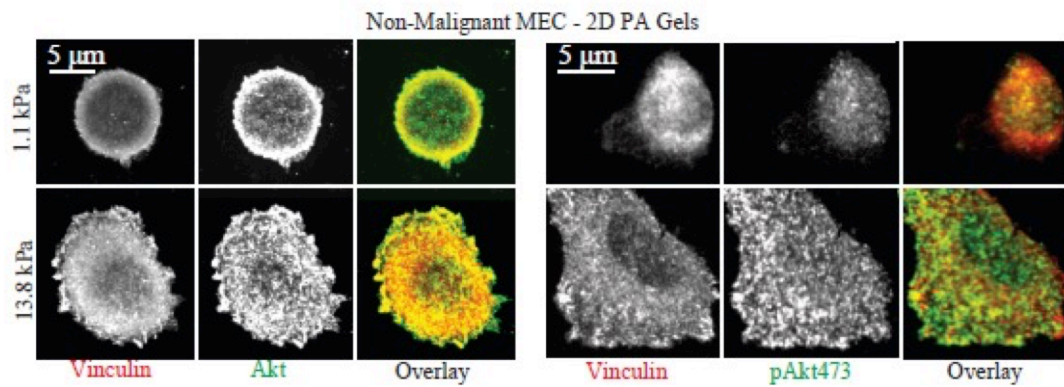


Fig 3.15 Vinculin, Akt, and p^{473} Akt recruitment to adhesions in non-malignant MEC on 2D PA Gels. Akt is recruited to adhesions on soft and stiff gels, while p^{473} Akt is dramatically increased in adhesions on stiff PA gels.

3.3.12. ECM stiffness and vinculin promote tumor cell invasion in 3D, but this invasion requires both PI3K and FAK dependent signaling

Treating MCF10AT premalignant mammary colonies with the PI3K inhibitor GDC-0941 (1 μ M) repressed their protrusive activity and invasion into a stiffened 3D collagen/rBM gel (1.5kPa) (Fig 3.16, rightmost panel), while there was no reduction in vinculin and b1 integrin positive focal adhesions, even after 48 hours of treatment (Fig 3.16). These data indicate that ECM stiffness stimulates tumor cell invasion by activating vinculin to assemble a stable complex with talin and actin that enhances PI3K signaling.

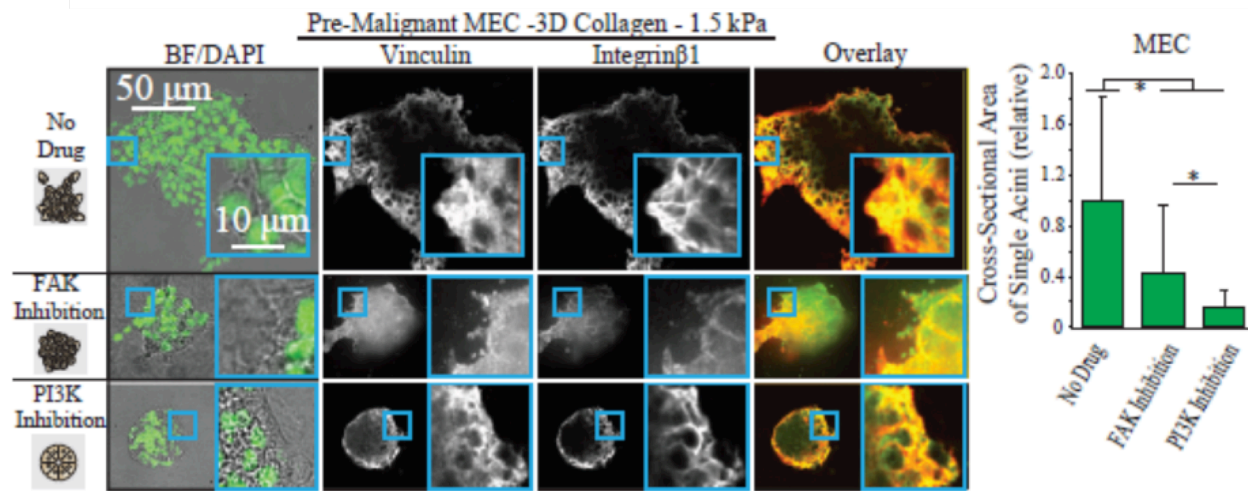


Fig 3.16 Pre-malignant MEC spheroids in 1.5 kPa 3D collagen gels for 48 hours, with FAK (1 μ M FAK Inhibitor 14) or PI3K (1 μ M GDC-0941) inhibition. Before fixation, the cross-sectional area of spheroids was measured to quantify 3D cell invasion ($p < .01$; $\pm$ SD, $n > 26$ spheroid per condition). Spheroids were then cryosectioned and immunostained for vinculin and integrin $\beta 1$ to demonstrate that focal adhesions are not

3.3.13. ECM stiffness drives altered microscale membrane topology and vinculin positive adhesions correspond to areas of altered nanoscale membrane architecture

We next asked how the force-induced assembly of a stable vinculin-talin-actin complex potentiates PI3K signaling. In this regard, recent findings by Wang and colleagues showed that Phosphatidylinositol (3,4,5)-triphosphate (PIP3) clusters into distinct nanodomains in the plasma membrane²⁶². We therefore hypothesized that vinculin-adhesions could enhance the ability of PI3K to convert PIP2 to PIP3 in the plasma membrane. Consistently, in response to ECM stiffness, MECs expressing an inert membrane probe mEmerald-farnesyl assembled more and longer membrane protrusions (**Fig 3.17 A**). To directly examine if force-induced vinculin activation alters the nanoscale topography of the plasma membrane, MECs expressing vinculin WT or a vinculin T12 together with an inert membrane probe (mCherry-farnesyl) were seeded on fibronectin coated silicon wafers and SAIM was performed to visualize the nanoscale position of

vinculin and the associated ventral plasma membrane (**Fig 3.17 B**). Imaging studies revealed that while the height of the plasma membrane varied widely across the cell (up to ± 70 nm), the height of the membrane significantly decreased by approximately 20nm at sites of vinculin-rich focal adhesions, where vinculin remained constant at 100 nm (**Fig 3.17 C**).

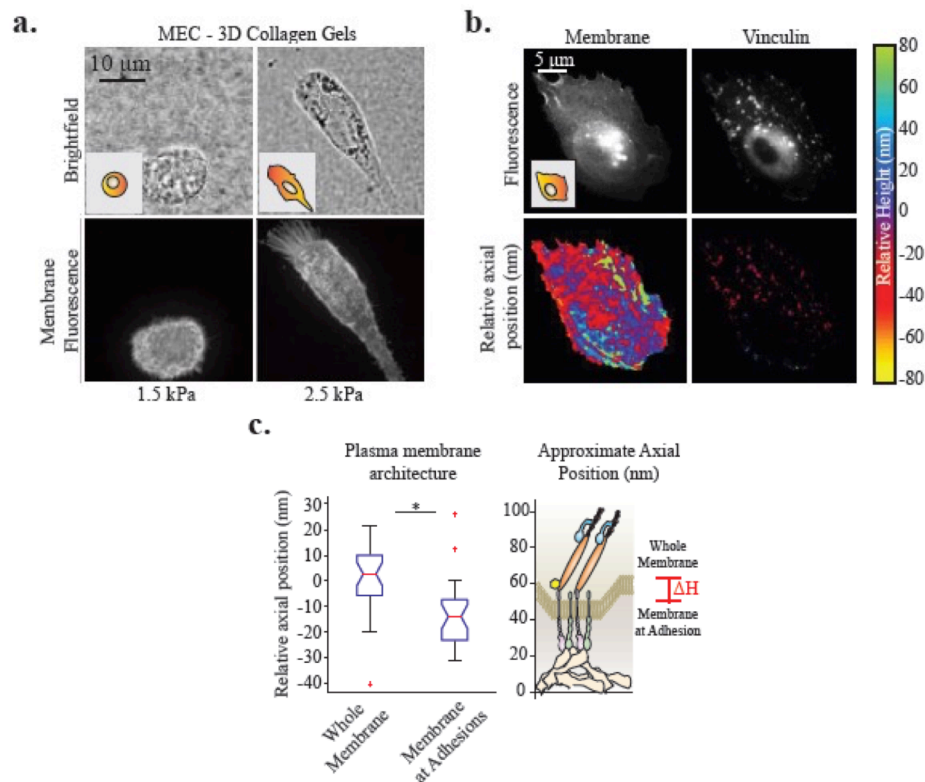


Fig 3.17 Vinculin positive focal adhesions are colocalized to areas of altered membrane topography and composition. **A.** Non-malignant MEC in 3D collagen gels for 24hrs. Membrane protrusions are dependent on high (2.5 kPa) matrix stiffness; visualized via mEmerald-farnesyl membrane probe. **B.** Organization of vinculin and the ventral plasma membrane of MEC plated on a 2D glass surfaces, via SAIM measurements of vinculin-GFP and mCherry-farnesyl. Axial position is plotted relative to the mean height of the membrane and vinculin, respectively. r FAK inhibition. **C.** Quantification of the significant decrease in membrane axial position at focal adhesions ($p < .01$; $\pm$ SD, $n > 18$ cells per condition).

3.3.14. Force activates vinculin to increase membrane protrusions and nucleate PIP3

As vinculin positive focal adhesions correlate to areas of altered membrane topography, we conclude that the assembly of a stable vinculin-talin-actin complex likely modifies membrane topology, and hypothesize that these areas of distinct membrane architecture correspond to changes in the lipid makeup of the plasma membrane in those areas.

To directly determine whether a vinculin stabilized focal adhesion facilitates PIP3 accumulation to enhance Akt activation, MECs expressing a probe for PIP3 (mKO2-PH-Grp1) with either a probe for PIP2 (PLC δ -PH-EGFP), GFP vinculin WT, or GFP vinculin T12 were seeded on fibronectin-coated coverslips and imaged. Following growth factor stimulation, we observed that PIP2 was decreased and PIP3 was increased via PI3K activity (**Fig 3.18A**). Data revealed that enhancing the assembly of the vinculin-talin-actin complex in MECs, by expressing activated vinculin T12, significantly increased the levels of PIP3 at the focal adhesion site, as compared to MECs expressing vinculin WT (**Fig 3.18B**). These results build upon previous findings that PIP3 is localized to the cell-ECM basal border in epithelial acini embedded within a 3D ECM²⁶³, and suggest the force-induced assembly of a stable vinculin-talin-actin complex potentiates Akt activation via PI3K by facilitating membrane changes that favor the accumulation of PIP3 at the adhesion complex. Given that many oncogenes enhance PI3K activity; this mechanism may explain why ECM stiffness specifically induces invasion in a transformed cell.

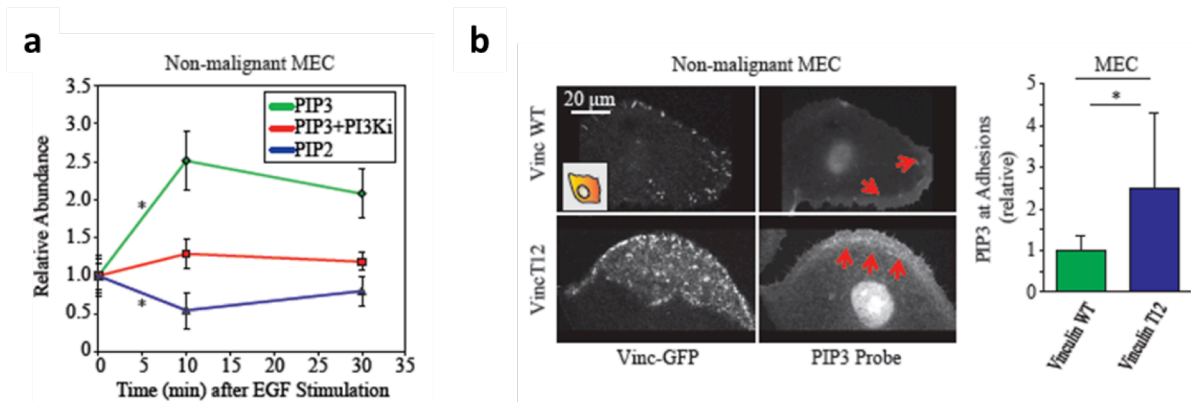


Fig 3.18 A. Line graphs showing relative abundance of PIP2 and PIP3 in the ventral membrane of non-malignant MEC between 0-30 minutes following EGF stimulation. 10 minutes after stimulation PIP2 significantly decreases and PIP3 significantly increases. However, inhibiting PI3K activity (1 μ M GDC-0941) is able to abrogate this increase in PIP3 ($p < .05$; $\pm$ SE, $n > 25$ cells per condition). **B.** Confocal images of pre-malignant MEC plated on 2D PA gels of increasing stiffness expressing vinculin-GFP activity mutants and a probe for PIP3 activity (mKO2-PH-Grp1), where red arrows indicate areas enriched in PIP3 activity. Measurements of all pixels in adhesions were averaged over whole cell; PIP3 was significantly enriched in focal adhesions of cells expressing activated vinculin T12 ($p < .05$; $\pm$ SD, $n > 7$ cells per condition).

3.3.15. ECM stiffness regulates vinculin and p^{473} Akt activity in experimental mouse tumors and human breast cancers

We and others have shown that p^{473} Akt and p^{397} FAK co-localize at the invasive front of experimental mammary tumors and we found that this phenotype is lost if ECM cross-linking and stiffening is prevented^{223,264}. Here we determined that ECM stiffness mediates the localization and levels of these signaling molecules at the invasive front by activating vinculin. Thus, while confocal immunofluorescence imaging revealed strong co-localization of vinculin and p^{473} Akt at the invasive front of PyMT mammary tumors where the ECM is stiffest, in tissue from mice that had been treated with either a pharmacological lysyl oxidase inhibitor (data not shown) or a function-blocking antibody to inhibit lysyl oxidase activity (**Fig 3.19**) to prevent collagen cross-linking and stiffening, p^{473} Akt and vinculin levels were greatly reduced and little to no co-localization of these molecules was observed.

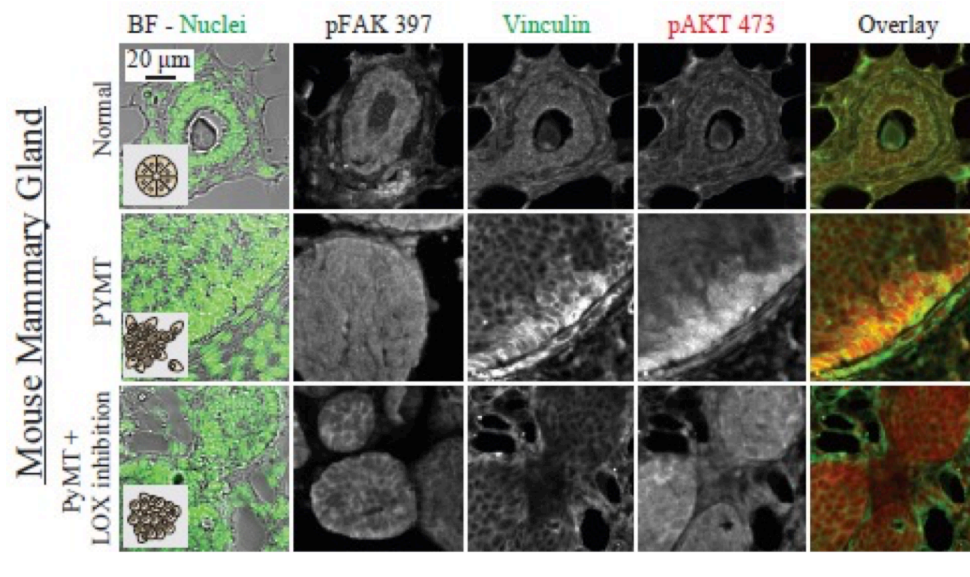


Fig 3.19 . Vinculin, ³⁹⁷FAK and ⁷³Akt immunostaining of mammary gland tissue from FVB (normal), and PyMT background mice +/- LOX cross-linking inhibition. Inhibition of cross-linking via BAPn reduces vinculin and ⁴⁷³Akt recruitment to cell-cell borders, indicative of less vinculin recruitment to adhesions and decreased signaling through Akt.

3.3.16. Vinculin co-localizes with vinculin-rich adhesions in invasive human breast cancer;

The clinical relevance of these findings was illustrated by showing a progressive increase in the levels and co-localization of vinculin and ^{p473}Akt as well as b1 integrin and ^{p397}FAK (**Fig 3.20A, 3.20B**) in normal, DCIS and ER/PR positive invasive human breast cancer. This work was done in collaboration with Yunn-Yi Chen at UCSF Parnassus, a clinical pathologist who scored tissues and assisted in determining the cancer status of various sections and sub-section locations.

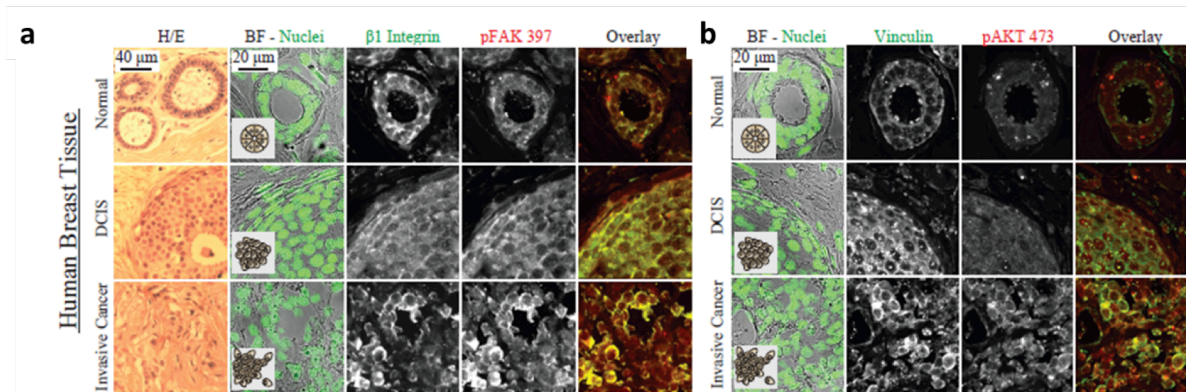


Fig 3.20 A. p^{397} FAK and $\beta 1$ integrin immunostaining of an ER/PR positive human breast cancer tumor biopsy and representative H/E staining of these tissues; **B.** Vinculin and immunostaining of an ER/PR positive human breast cancer tumor biopsy. p^{397} FAK, $\beta 1$ Integrin, Vinculin and 473 Akt are enriched at the cell-ECM border in invasive tissue.

3.3.17 Adhesome proteins are up-regulated in solid tumors, as calculated in an analysis of the human proteome via the Human Protein Atlas

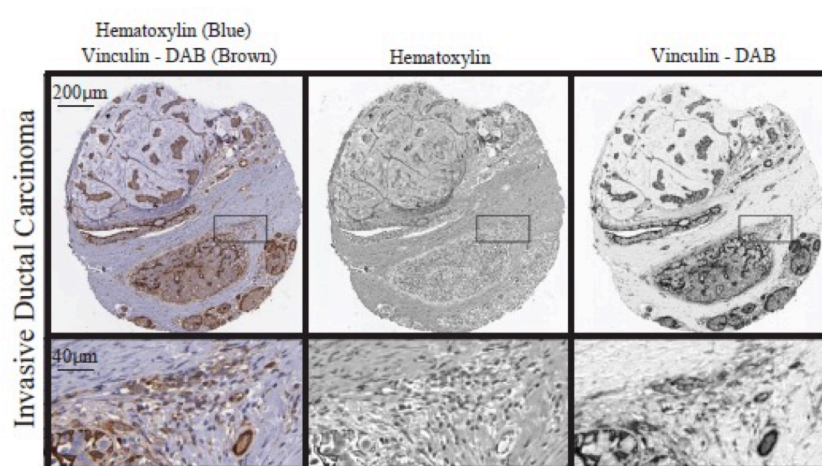


Fig 3.21 Vinculin is localized to cell-ECM borders in invasive breast cancer and adhesome proteins are upregulated across human cancers. **A.** Histology of normal tissue and invasive ductal carcinoma stained with hematoxylin (blue) and vinculin-DAB (brown) from the Human Protein Atlas ²⁵², with grayscale color deconvolution of blue and brown channels to determine vinculin localization to cell-cell or cell-ECM borders. Color deconvolution performed as described in ⁴²¹.

Having implicated force-regulated vinculin in tumor invasion, we next asked if other adhesion proteins might also be similarly involved in malignant progression. We used a bioinformatics approach that involved the analysis of pathologically-scored immunohistochemistry data

compiled by the Human Protein Atlas database (**Fig 3.21**)²⁵². Using an unbiased approach to pair each tumor type to its corresponding normal cell, we determined the most common expression level observed across all samples within each tumor type to generate composite profiles of "typical" protein expression levels for each tumor type. Using this approach we observed that focal adhesion proteins²⁵³ that were normally expressed at low to negligible levels in healthy tissues were significantly likely to be over expressed in their corresponding cancer tissue (**Fig 3.22 A**). Interestingly, we also found that many of the oncogenes implicated in cancer were also similarly up regulated (**Sup Fig 3.22 B**).

These bioinformatics results indicate that adhesome proteins as an aggregate are up regulated at the protein level across many different solid cancer types, in a manner that is analogous to known oncogenes, suggesting they may play a more significant role in malignant progression than previously appreciated.

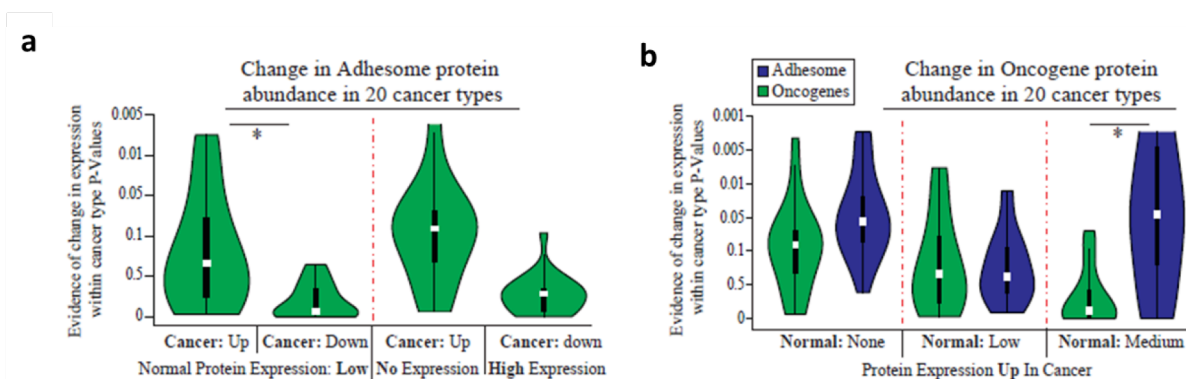


Fig 3.22 A. Changes in integrin adhesome protein expression in 20 cancer types as compared to normal tissue, and quantified from pathologically-scored immunohistochemical data compiled by the Human Protein Atlas database. Adhesome proteins that were normally expressed at a low or negligible level in healthy tissues were significantly likely to be overexpressed in respective cancer types; and adhesome proteins normally expressed at a high level, were significantly unlikely to be downregulated. **B.** Upregulation of oncogene and adhesome proteins as compared to expression in normal tissue, as quantified from Human Protein Atlas database. Oncogenes expressed at negligible or low levels in normal tissue were significantly upregulated in a similar manner to adhesome proteins.

3.4. Discussion

3.4.1. Role of Vinculin in Promoting Tumor Progression

The tumor microenvironment and specifically the extracellular matrix is an important regulator of malignant progression^{7,223,225,227,228,265}. Using defined 2 and 3D matrices, mouse models and human biopsies together with a series of molecular mutants and a novel super resolution imaging approach, we demonstrate for the first time in 3D and in tissues that ECM stiffness regulates the activation of vinculin and its nanoscale organization at focal adhesions. By stabilizing the vinculin-talin-actin complex our data provide the first detailed molecular mechanism to explain how tumor associated ECM cross-linking and stiffening is able to promote malignant transformation and invasion in tissue (**Fig 3.23 A, B**)^{223,231,244,266}. Our data illustrate how the induction and stabilization of the talin-vinculin-actin scaffolding complex facilitates oncogenic signaling through pathways such as growth factor receptor induced PI3K generation of PIP3 (**Fig 3.23 B**). Thereby our results emphasize vinculin's role as a nucleator of cell signaling and highlight a vinculin-dependent signaling circuit through which cancer cells both gather information about their altered environment and alter intracellular signaling in response to this input^{235,236,240,267}. Indeed, by demonstrating a central role for force-regulation of a vinculin-talin-actin complex we provide the first definitive evidence for why malignant transformation and tumor cell invasion require the cooperative interplay between oncogenic transformation and a stiffened ECM (**Fig 3.23 A**)^{7,223,225,268}.

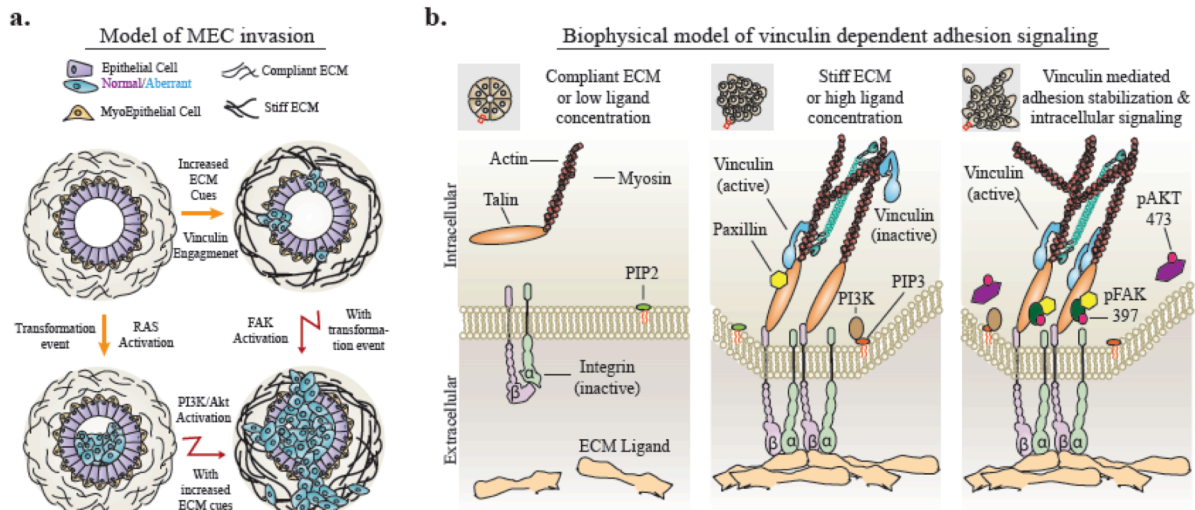


Fig 3.23 Model: MEC invasion is dependent on ECM cues, vinculin engagement and malignant transformation. **A.** Model of mammary duct organization, which can be perturbed by malignant transformation or changes in ECM stiffness. Together, these events lead to cancer cell invasion through PI3K, FAK and vinculin activation. **B.** Biophysical model of vinculin dependent adhesion signaling. Extracellular cues drive integrin activation, talin binding, and vinculin activation. Vinculin-talin-actin stabilization facilitates PI3K conversion of PIP2 to PIP3, activation of FAK and Akt, and intracellular signaling that leads to cancer cell invasion.

proteins as compared to expression in normal tissue, as quantified from Human Protein Atlas database. Oncogenes expressed at negligible or low levels in normal tissue were significantly upregulated in a similar manner to adhesome proteins.

3.4.2. Future Directions: *In Vitro* and *In Vivo*

While most available work on focal adhesions has been performed in 2D *in vitro* systems, our studies link and extend existing knowledge to cell behavior in a physiologically relevant context. We combine nanoscale characterization with 3D and *in vivo* analysis to provide a holistic view of how ECM stiffness changes the context in which intracellular signaling takes place. Our data show that by driving molecular scaffolding at focal adhesions, increased ECM stiffness promotes signaling in pathways that are important in both growth and invasion. On the molecular level, our data establish vinculin as a versatile adhesion molecule involved in many aspects of cell-ECM interactions. We confirm that vinculin's auto-inhibition domain reacts in a force-dependent manner and operates as a mechanical clutch connecting the actomyosin network with the ECM. This tantalizingly suggests similar mechanisms of regulation for other plaque proteins containing

cryptic binding sites, including talin and α -actinin at cell-ECM junctions, cadherins and PECAM at cell-cell junctions, and ECM proteins such as fibronectin may also play an equally important role in regulating malignancy^{190,269–272}.

At the tissue level, our data suggest that cells migrating as a collective also likely employ vinculin as a force sensor at cell-cell junctions, and force responsive actin cross-linking proteins; including CAS and FERM family proteins^{260,273}. More broadly, our results confirm the existence of focal adhesions and focal adhesion activity in 3D and *in vivo* and demonstrate their functional importance by showing that they are instrumental in regulating growth factor receptor signaling^{231,232,274,275}. Indeed, our data showed that neither matrix stiffness nor oncogenic transformation are sufficient for malignant transformation and tumor cell invasion, suggesting ECM stiffness collaborates with key oncogenic pathways to exacerbate the potentiating mutations found in cancer. Indeed, our findings highlight vinculin in particular at the intersection of extrinsic mechanical properties and intracellular growth factor signaling²⁷⁶. Moreover, our bioinformatics findings, which show amplification of adhesome molecules at the protein level across 20 solid tumor types suggests that further scrutiny of the role of up regulated adhesion proteins may be another method by which cancers may be categorized and screened^{252,277,278}.

3.4.2. Clinical Relevance

The clinical relevance of our culture studies was demonstrated by our findings that there is a progressive increase in co-localized vinculin and p⁴⁷³Akt at the invasive tumor border in human breast cancer where the ECM is stiffest and mechanosignaling is elevated, as revealed by increased p³⁹⁷FAK²⁷⁹. Functional links between tissue mechanics and the vinculin-PI3K

phenotype was illustrated by our studies in the PyMT mouse model of mammary carcinogenesis by the loss of vinculin-^{p473}Akt association and ^{p397}FAK when collagen cross-linking and ECM stiffening were prevented by inhibiting lysyl oxidase activity ^{223,280}. It is therefore feasible that the concentration of vinculin at the invasive front tumors also affects the signaling of other growth and survival enhancing pathways, including enhanced Src-dependent growth or PI3K/PIP3/Akt dependent activation of mTOR and altered cell metabolism or apoptosis resistance through Bcl-2 activation of Bit1 ^{10,281–283}. Accordingly, strategies to target vinculin's scaffolding function may offer a new therapeutic approach to treat pre malignant lesions and may even help to identify high risk, non-invasive lesions ²⁸⁴.

3.4.4 Contributions

Matthew Rubashkin and Valerie Weaver conceived and initiated the project. Matthew Rubashkin, Valerie Weaver, and Luke Cassereau designed experiments. Russell Bainer and Matthew Rubashkin performed the data analysis on the human bioinformatics data. Luke Cassereau, Yoshihiro Yui and Matthew Rubashkin performed the 3D multicellular experiments of tumor invasion. Matthew Rubashkin and Christopher Dufort performed the 2D cell adhesion and spreading experiments. Matthew Rubashkin, Christopher Dufort, and Matthew Paszek performed the SAIM super-resolution microscopy and analysis. Matthew Rubashkin and Christopher Dufort performed the vinculin FRET sensor experiments and analysis. Yunn-Yi Chen provided and scored the pathological samples for immunohistochemistry. Michael Davidson, Jonathan Lakins, Matthew Rubashkin and Matthew Paszek designed and produced plasmid constructs and biosensors. Matthew Rubashkin and Luke Cassereau performed immunohistochemistry experiments of cells and tissue samples. Matthew Rubashkin and Luke

Cassereau prepared the figures with assistance from Valerie Weaver. Matthew Rubashkin, Valerie Weaver and Guanqing Ou wrote the manuscript with editorial assistance from all authors.

3.4.5. Acknowledgements

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3.4.6. Funding

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Chapter 4. Cellular Mechanosensing in Invasive Breast Cancer is controlled by Mesenchymal-like Behavior and Paxillin

4.1. Introduction

4.1.1. Abstract

Cellular mechanosensing is required for focal adhesion assembly and signaling, which in turn drives mesenchymal-like cell invasion, malignant transformation and tumor metastasis. Nevertheless, how cells interpret the mechanical tumor microenvironment to promote mesenchymal-like cell invasion remains unclear. Here, we explored the role of the focal adhesion protein paxillin, a previously identified mechanotransducer, in breast cancer migration, transformation and invasion. Using human bioinformatics data, single and multicellular models of tumor invasion with paxillin mutants, super-resolution analysis of focal adhesion architecture via SAIM and 3B microscopy, as well as cellular mechanical measurements via traction and atomic force microscopy we find that epithelial to mesenchymal transition (EMT) induces alternative paxillin splicing that enhances cell plasticity in tumor-like ECM, driving cancer cell invasion. We establish that the mesenchymal paxillin alpha isoform is required for EMT mediated cell plasticity through a Src-RhoA-Erk signaling axis, indicated by increased force per integrin, traction on tumor-like matrix, and cellular invasion. These findings reveal that EMT could induce tumor progression by promoting the alternative splicing of the mechanotransducer paxillin; a conclusion underscored by paxillin mesenchymal splicing observed in human invasive

tumors and cell lines, and paxillin over-expression significantly increasing patient mortality in human breast cancer.

4.1.2 Background on Breast Cancer Cell Invasion

Breast cancer metastasis requires changes in a cancer cell's ability to invade through heterogeneous local and distal tissues^{17,285,286}. This invasion requires specific motility phenotypes that are dependent on the properties of the extracellular matrix (ECM)^{20,287}. In cancer the tumor stroma is remodeled and stiffened, presenting stiff collagen fibers to invading tumor cells that in turn can drive oncogene signaling^{7,8,288–290}. Moreover, the tumor microenvironment has been shown to enable efficient mesenchymal-like single cell motility phenotype as well as growth associated collective cell migration^{291,292}. Recent work has implicated mesenchymal-like, ECM-adhesion-dependent motility of single or leader basal cells with increased stromal penetration, intravasation and metastasis^{293–295}. However, it is not known how cells sense their external environment to specifically transduce basal or mesenchymal-like cell behaviors, and why some specific cancers which employ this migration phenotype including mesenchymal-like triple-negative breast cancers have worse clinical prognosis and greater risk of metastasis^{30,296}. Furthermore, there are not adequate biomarkers and likewise targets for effective treatment of breast cancers which present mesenchymal-like motility and increased mortality²⁹¹.

4.1.3 Mesenchymal Cell Invasion, Cell Signaling and Paxillin

Mesenchymal like motility entails integrin positive adhesions to facilitate migration and invasion, and requires myosin contractility based force on the external environment ^{54,297}. Cell-ECM adhesion proteins have been implicated in force transmission from integrins to actin ^{21,170}. Proteins involved in connecting the cell cytoskeleton to the ECM include talin and vinculin for force transduction and signaling scaffold molecules including p130cas and focal adhesion kinase (FAK) ^{13,165,171}. An outstanding question in the field is how do cells employ adhesions molecules in normal or disease like ECMs, and are some of these proteins responsible for biphasic changes in cell behavior in soft and stiff environments ^{172–175}. Interestingly, paxillin has been implicated in cell adhesion mechanics and spreading as well as serving as a signaling scaffold that can possibly drive FAK, Src, Erk, Rac1 and Akt activity ^{200,298–300}. Nevertheless, it is unclear how cells couple increased ECM stiffness to mesenchymal like adhesions and changes in cell signaling events; and if multifunctional proteins like paxillin are responsible for changes in protein recruitment, cellular mechanosensing of rigidity or ligand density sensing, or signaling mechanotransduction ²⁰.

4.1.4. Epithelial to Mesenchymal Transition and Focal Adhesion Biology

Focal adhesions are the means through which cells receive and interpret mechanical signals from the extracellular matrix, and serve a critical role in mesenchymal-like cell motility ^{20,164}. Notably, mesenchymal like cell motility can be induced by epithelial-to-mesenchymal transition (EMT), a developmental process known that is often hijacked in cancer ^{17,301}. Canonical EMT involves loss of apical basal cell polarity, reduction in cell-cell adhesion including cadherin and tight junctions, as well as increased cell-ECM adhesion ³⁰². EMT can be triggered through chemokine

exposure including transforming growth factor beta 1 (TGF β) or hepatocyte growth factor (HGF), as well as being associated with stiffened microenvironments found in chronic wounds or tumor stroma³⁰³. Interestingly, this mesenchymal like behavior is a hallmark of triple negative breast cancers, and it has been shown that blocking EMT *in vitro* and *in vivo* can lead to decreased cell invasion and metastasis and increased survival^{296,304–306}. EMT is often accompanied by epigenetic changes in SMAD and SLUG, driving PAI1 and SMAD7, concordant decreases in E-cadherin and increases in N-cadherin expression, and changes to the spliceosome^{307,308}.

4.1.5. Paxillin Splicing and Known Binding Partners

Like the SMAD family of transcription factors, many focal adhesions proteins including β 1 integrin, paxillin and FAK and are critical for EMT and mesenchymal-like cell motility^{309–311}. Interestingly, after EMT or in mesenchymal like cells, paxillin has increased phosphorylation at tyrosines 31 and 118, and reduced splicing at the internal protein translation site at methionine-133²³. Previous studies have indicated that truncated paxillin-delta transcribed at the internal 133 translation site would be missing residues critical for interactions with binding partners including FAK and Src^{202,298}.

4.1.6. Predictions of Paxillin Splicing and Behavior

We predicted that mesenchymal paxillin-alpha compared to the epithelial paxillin-delta could drive an altered mechanosensing response in cancer by altered interactions with binding partners responsible for EMT signaling and behavior. Thus we hypothesized that alternative scaffolding of mesenchymal paxillin alpha could lead to changed forces over integrins, traction force and

mesenchymal invasion by driving enhanced cellular mechanosensing at disease-like tissue stiffness.

4.1.6 Overview of studies performed

Here we explain the interconnection between force and mesenchymal-like cell migration in metastatic cells. We demonstrate that *in silico* and *in vitro* that metastatic cells have altered invasive behavior, biophysical properties and adhesion architecture that lead to increased cell adhesion area and force per integrin. Consistent with our hypothesis that paxillin splicing regulates mesenchymal-like cell adhesion and invasion, we observed that paxillin alternative splicing regulates cellular traction, mechanosensing and invasion through a FAK/Src/RhoA/Erk signaling axis, observed paxillin mesenchymal splicing in human invasive tumors, and show that paxillin over-expression significantly increasing patient mortality in human breast cancer. Therefore mesenchymal cancer cells can employ alternate paxillin splicing to enhance oncogenic signaling to drive malignant behavior and our data suggests that paxillin could be used as a potent biomarker for negative outcomes in human breast cancer.

4.2. Methods

4.2.1. Cell Culture and reagents

Mammary epithelial cells (MECs) including non-malignant MCF10A (ATCC, 2007; verified by epithelial cellular morphology in 2D, ability to form hollow polarized acini in 3D rBM gels, and expression of epithelial markers including E-Cadherin and cytokeratins 8 and 14) and pre-malignant Ha-ras transformed MCF10AT (Karmanos Cancer Institute, 2008; verified by epithelial cell morphology in 2D) were cultured as described¹³. Tumorigenic PYMT Flox3 and metastatic Met1 cell lines (kind gift of the Moses laboratory) were verified by epithelial and mesenchymal morphology in 2D, and cultured as described³¹². 4T1 cell line (kind gift of the Werb laboratory) were verified by epithelial morphology in 2D and cultured in DMEM high glucose medium, supplemented with 10% fetal bovine serum, and penicillin–streptomycin; and cultured at 37 C in humidified 5% CO₂. KD2 paxillin homozygous null fibroblasts with HIC5 knockdown (referred to as KD2 cells) were originally isolated from T17 paxillin null embryonic stem cells, infected with HIC5 shRNA, and verified by mesenchymal cell morphology and staining for paxillin expression; and were cultured as described²⁹⁸. KD2 cells (kind gift of the Vande Pol laboratory) expressing retrovirally transduced paxillin and Hic5 expression constructs were generated as described²⁹⁸.

3D multicellular spheroids were generated in recombinant basement membrane (rBM; Matrigel) suspension cultures as described¹³. MCF10a and Met1 cells were flow sorted on a FACSAria II cell sorter (BD Biosciences) via primary and secondary antibody staining for top ~20% or bottom ~20% expression of $\alpha 5$ integrin, $\beta 1$ integrin or $\alpha V\beta 3$ expression. The following chemokines were used: Human TGF β at 10nM (PeproTech) for 3 days to induce mesenchymal

behavior and Epidermal Growth Factor (EGF) at 10nM (PeproTech) to induce cell migration. Small molecule inhibitors that were used included: Y27632 ROCK inhibitor at 10 μ M (Cayman Chemical), ML7 MLCK inhibitor at 10 μ M (Sigma Aldrich), and Dasatinib at 10nM (Cayman Chemical),

4.2.2 Expression constructs

Transient plasmid constructs used included clonotech vectors N and C terminus tagged vinculin-mEmerald, paxillin-mCherry, paxillin-mEmerald, farnesyl-mEmerald, farnesyl-mCherry, FAK-mEmerald, Talin-mEmerald, Talin-mCherry, Zyxin-mEmerald, Tensin-mEmerald. Piggybac transposable plasmids paxillin WT (alpha isoform), and paxillin F (Y $\rightarrow$ F at 31 and 118 residues) were synthesized and cloned (by DNA 2.0) into BsaI digested pHULK piggyBac Mammalian Expression Vector – CometGFP (pJ509-02 construct from DNA 2.0). Rac1 Raichu biosensor with GFP-mRFP donor/acceptor (kind gift of Anne Ridley and Tony Ng) was used as described

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Stable cell lines inducibly expressing BirA* (R118) Paxillin derivatives were prepared using a custom built piggyBac transposable vector; pPB puro Tet. Briefly pPB puro Tet features the heptamerized TetO minimal CMV promoter driving expression of a transgene with 5' chimeric intron and polyadenylation signal from bovine growth hormone and an expression cassette for puromycin phosphotransferase in opposite orientation between piggyBac inverted terminal repeats. BirA* was engineered with three tandem repeats of the myc 9E10 epitope at the N terminus and a 22 amino acid C terminal glycine and serine rich flexible linker in frame with either full length paxillin-alpha isoform, or paxillin F31/118 with tyrosine to phenylalanine

mutations at residues 31 and 118. Truncated paxillin-delta isoform which is expressed via initiation at the downstream methionine-133 ATG, was prepared via PCR truncation at the N terminus. All paxillin isoforms were expressed as C-terminal in frame fusions with mEmerald. A C terminal fusion of mEmerald to myc tagged BirA* served as control.

Transient transfection was performed using either Lipofectamine 2000 reagent or the Lonza Nucleofector Kit V, with program T024 for all epithelial cell lines, and program U024 for KD2 fibroblasts. Recombinant lentivirus of the TET-promoter RTTA-neo was prepared by transient transfection into HEK-293T cells, generating lentivirus titer used to transduce cells as previously described ¹³. For stable expression of TET-responsive lentiviral introduced genes including Muc1-ΔTR-venus membrane probe ¹⁴, α5 integrin, paxillin-mEmerald, paxillin-mCherry, and vinculin-mEmerald, HEK-293T cells were also used as described ¹³. Target cells constitutively expressing the synthetic reverse Tet transcriptional transactivator rtTAs-M2 were prepared by transduction with a G418 selectable lentiviral vector. These cells were transiently transfected with pPB puro Tet constructs and an expression construct for a hyperactive piggyBac transposase pCMV-HAhyPBase (generous gift of Dr. Alan Bradley) ³¹⁴.

Stable cell lines were selected in 200 ng ml⁻¹ G418, 380 ng ml⁻¹ hygromycin, or 1 ug/ml puromycin, and sorted on a FACS Aria II cell sorter (BD Biosciences) for mEmerald, mVenus or mCherry expression. Expression of inducible plasmid was induced with 200 ng/ml doxycycline in growth medium for 1 d before experimentation.

4.2.3 Hydrogel and Substrate Preparation

Collagen gels were fabricated by diluting 3.8 mg/ml acid solubilized rat tail collagen (BD) in 1:1 DMEM-F12 medium (Invitrogen), neutralizing to pH 7.4 with 1M NaOH. For 3D collagen gels, a base layer of ~250 μm was cast at 37 C for 30 minutes, followed by a second layer that included the cell suspension at 37 C for 30 minutes. 2D polyacrylamide (PA) gels (height ~200 μm) were fabricated by varying the acrylamide and cross-linker concentration and assessing stiffness by Atomic Force Microscopy or using a shear rheometer²⁵⁰. Red fluorescent beads (100nm; Invitrogen) were incorporated in the polyacrylamide gels at 1:300 and centrifuged at 200 RCF to the top surface of the gels for traction force microscopy.

N-type [100]-orientation silicon wafers with 1933nm silicon oxide (Addison Engineering; 1 cm x 1 cm) and borosilicate glass coverslips (~150 μm thick) were cleaned by successive sonication in acetone and 1M KOH (20 min) followed by chemical activation with silane (0.5% (3-aminopropyl)trimethoxysilane) and glutaraldehyde (0.5% in water). The substrates were then UV and ethanol sterilized and incubated overnight (4°C) in extracellular matrix (human fibronectin; Millipore 10 $\mu\text{g/ml}$). Prior to use, substrates were washed in PBS (5 times; pH 7.4), treated with sodium borohydride (20mg/ml) and washed with PBS. SAIM calibration wafers were prepared by sonicating carboxylate-modified red fluorescent spheres (100nm; Invitrogen), following by bead deposition (5×10^8 beads per ml) in NaCl (100mM) PBS solution.

4.2.4 Immunofluorescence

Cultures were fixed with 4% paraformaldehyde (2D: 20 minutes, room temperature; 3D: overnight, 4°C) and staining was performed as described⁸. Primary antibodies against paxillin

(610620, BD; and P1093, Sigma Aldrich), phosphor-paxillin-tyrosine 118 (2541, Cell Signaling) $\alpha 5$ integrin (BIIG2, isolated from rat hybridoma), $\alpha 2$ integrin (AB1944, Millipore), AVB3 (MAB1976, Millipore), $\beta 1$ integrin (AIIB2, isolated from rat hybridoma), $\beta 4$ integrin (3E1, Millipore MAB1964), vinculin (hVIN-1, Sigma Aldrich; 700062, Invitrogen; V284, Santa Cruz Biotechnology), phosphor-FAK-tyrosine397 (44625G, Invitrogen), phospho-myosin light chain kinase 2 - Thr18/Ser19 (3674, Cell Signaling), talin (T3287, Sigma), zyxin (610521, BD), Vimentin (D21H3, Cell Signaling), and AlexaFluor phalloidin (633-conjugate, Invitrogen) were used. Secondary antibodies used include AlexaFluor goat anti-mouse, anti-rabbit, and anti-rat (488, 568 and 633 conjugates).

4.2.5 Fluorescence, Confocal and SAIM Microscopy and Analysis

Imaging was performed on a motorized TIRF inverted microscope system (Ti-E Perfect Focus System; Nikon) controlled by Metamorph software (Molecular Devices), equipped with 488nm, 561nm, and 640nm lasers, 350/50 epifluorescence, a CSU-X1 spinning disc confocal unit (Yokogawa Electric Company), electronic shutters, motorized stage, a scientific sCMOS camera (Zyla 5.5 megapixel, Andor), and an electron-multiplying charged-coupled device camera (QuantEM 512; Photometrics). Temperature and CO₂ was controlled by an environmental chamber and PID controlled heater (In-Vivo Scientific). Samples were imaged with a 100x-1.49NA TIRF oil-immersion, 40x-1.2NA Long-Working-Distance (LWD) water-immersion, 20x-0.75NA air, and 10x-0.45NA air objectives. For live-cell imaging, phenol red-free growth media with 10 mM Hepes (pH 7.4) was used. For 3D imaging, confocal image slices were taken at 0.2 μ m or 1.0 μ m slices, and the individual planes were maximum intensity z-projected for 2D

visualization. A custom software package for image analysis was written in Python, using ImageJ and the Eclipse Development Environment in Linux. All images were subject to a Gaussian blur of 1.0 pixel to smooth background noise. Intensity and colocalization measurements of single confocal planes were calculated on a pixel-by-pixel basis. Single cell invasion, acini and cell projections and cell adhesions were counted manually. Acini cross-sectional area was quantified with the freehand selection tool in ImageJ. Photobleaching FRET (pbFRET) and Scanning Angle Interference Microscopy (SAIM) were performed as described^{12,13}, using data analysis and 3D visualization programs written in Matlab® and Python using libraries distributed by Enthought, Inc.

4.2.6 Superresolution 3B Analysis

The 3B algorithm uses a Bayesian analysis technique to determine the positions of fluorophores based on patterns of blinking and bleaching events.^{315,316} We recorded the data on an EMCCD camera (Andor iXon) for ~200-300 frame movies at 20 Hz. The images were then processed with the 3B algorithm on virtual machines hosted on Amazon's EC2 cloud computing service.³ Each calculation ran on the compute-optimized c3.large servers for approximately 40 iterations. The positions and intensities of the fluorophores as determined by the 3B analysis were plotted on a coordinate system scaled up by a factor of three, representing a threefold increase in resolution.

4.2.7 Integrin Force Sensor

The molecular tension sensors (MTSs) used here are shown in Fig 4.3.3, and were previously developed by the Dunn Group at Stanford University^{170,317}. Integrins bind to the MTSs via an arginine-glycine-aspartate (RGD) sequence derived from fibronectin. Previous versions of MTSs relied on biotin-avidin conjugation for attachment to the glass coverslip. However, cell-generated forces applied over 1–2 h can break the biotin-avidin connection. Therefore, MTSs were re-engineered to covalently attach to PEG-functionalized glass coverslips via a modified, cysteine-free HaloTag domain. The covalent attachment of the MTSs prevents cell traction-mediated MTS detachment from the coverslip surface and thus facilitates time-lapse imaging. Mechanical stretch is detected via changes in FRET between the FRET donor Alexa 546 and the FRET acceptor ATTO 647N, which are connected by an extensible (GPGGA)₈ peptide linker. This FRET pair uses the red portion of the visible spectrum, allowing us to simultaneously visualize GFP-labeled proteins inside the cell. The FRET donor and acceptor are site-specifically

attached to the MTSs via a unique cysteine residue and an unnatural amino acid (4-azido-L-phenylalanine) at positions that flank the (GP₃GGA)₈ spring. This site-specific labeling strategy allows for a short resting distance for the FRET pair, resulting in a higher no-load FRET efficiency compared to first generation MTSs (71% vs 49% resting FRET). As a result, the dynamic range of the sensor is expanded.

4.2.8 Traction Force Microscopy

Cells were seeded overnight on polyacrylamide gels with 100nm fluorescent red beads. 20X images of cells in brightfield and beads were taken before adding 0.01% sodium dodecyl sulfate to lyse the cells from the surface. Afterwards a second picture of the beads was taken that was later aligned via the ImageJ (<http://rsb.info.nih.gov/ij>) registration plugin StackReg. To assess bead movement, a particle image velocimetry (PIV) program was implemented in ImageJ as described previously³¹⁸. The iterative scheme included a 1st pass at 32/64 (i.e. interrogation and search window size in pixels), followed by a 2nd pass at 16/32; all at a correlation threshold of 0.60. For PIV post processing a dynamic mean test was implemented as described³¹⁸. The traction force was calculated by the fourier transform traction cytometry (FTTC) method with a poisson ratio of 0.5 and no regularization factor. Total and maximum traction force was calculated using a mask of cell area from the brightfield image overlayed onto a per pixel magnitude plot of the traction vectors. The PIV and FTTC microscopy software for ImageJ was made kindly available by Qingzong Tseng at <https://sites.google.com/site/qingzongtseng/tfm>.

4.2.9 Biochemistry

Total RNA was isolated using the mirVana miRNA isolation kit (Life Technologies AM1560), according to manufacturer's instructions. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express kit (Active Motif 5300), according to instructions. For mRNA qPCR analysis, total RNA was reverse-transcribed using random primers (Amersham Biosciences), and RPL20 was used to control for cDNA concentration in a separate PCR reactions for each sample. LightCycler Fast Start DNA Master SYBR Green Mix (Roche) was added to each PCR reaction along with cDNA and 1 pmol primer in a total volume of 10 μ l.

Western blotting was performed as previously subscribed ²⁵⁰. Antibodies used included pFAK (1:1000, Invitrogen 44625G), Paxillin (1:1000, Sigma PXC-10), pSRC (1:1000, Cell Signaling 2101), β -actin (1:2000, Sigma A228), pMYPT (1:1000, Millipore ABS45), pAKT (1:1000, Cell Signaling 4060), pSMAD2 (1:1000, Cell Signaling 3101), E-cadherin (1:1000, Cell Signaling 3195), and pERK1/2 (1:1000, Cell Signaling 4377).

4.2.10 Cellular Mechanosensing Assays

MCF10a, MCF10aT, PYMT, Met1 and 4T1 cell lines were used. Cells expressing either Paxillin-WT-GFP, Paxillin 31/118F-GFP or no exogenous paxillin were cultured with +/- TGF β then seeded. For PA gels of different stiffness and ECM coated glass coverslips of different ligand concentration, human fibronectin (Millipore) was used at concentrations of 0.01, 0.1, 1.0 and 10 μ g/ml, with all PA gels conjugated with 10 μ g/ml fibronectin. Transwell assays were modified from ³¹⁹. Cells were seeded on 8.0um inserts in a 24 well plate (Falcon) in serum free

media, above a reservoir with epidermal growth factor (EGF) positive media and imaged after 24 hours.

Soft agar assays were modified from ³²⁰. 12 well tissue culture dishes were coated with 0.5mL containing 1% low melting point agarose (AllStar Scientific) diluted 1:1 in 2X growth media. Then 0.5mL containing 10,000 cells per well were added in growth medium with 0.7% agarose cooled to 40°C. Gels were polymerized in the incubator for 30 minutes, and 0.5mL of growth media was added to the well and replaced every 2 days. After 14 or 21 days, colonies were fixed with 4% PFA, and stained with 0.5% crystal violet in 25% methanol for 30 minutes.

4.2.11 Human Bioinformatics Studies

To analyze survival as a function of transcriptomic data in human breast cancer patients, we used the online www.kmplot.com tool ³²¹. Briefly, the KMplot tool employs gene expression data, relapse free and overall survival information from GEO, EGA and The Cancer Genome Atlas (TCGA); a total of over 4000 patients. To analyze the affect of specific gene in survival, patient samples are split into two groups by predefined metrics for calculating quantile expression. Affymetrix transcriptome data of a user-selected probe was analyzed in KMplot by auto-selecting best cutoff of gene expression and computing the median of expression over the entire database, and analyzing all patient subtypes as long as they were lymph node positive in the 2014 TCGA dataset. All suggested quality controls were implemented.

Paxillin proteomics data in human cell lines and tumor samples was obtained from ³²². Briefly, an integrative mass spectrometry approach was used to characterize over 12,000 peptides of the

human proteome in 20 cell lines and 4 triple negative breast cancer tumor samples³²². Paxillin alpha splice form corresponding peptides (F5H836) and paxillin delta splice form corresponding peptides (E7EMK8 and) were analyzed by ratiometric comparison. Cell lines were separated into invasive and non-invasive designations by transwell boyden invasion potential³²³.

Reverse phase protein array (RPPA) data from TCGA patients was analyzed using the online www.cbioportal.org tool³²⁴. Patients were separated into patients with paxillin gene amplification, mRNA upregulation, or protein upregulation; and patients without significant changes in paxillin at the gene, mRNA or protein level. These two patient groups were then compared for differences in activated and phosphorylated signaling proteins (including Src phosphorylation at tyrosine 416) via RPPA arrays.

4.2.12 *In-Silico* Motor Clutch Model of Cell Traction - Overview

In order to understand how paxillin splicing controls cell traction forces and spreading on hydrogel substrates, we modified an existing ‘motor-clutch’ model of cell traction forces generated by neuron growth cone filopodia on compliant hydrogel substrates.³²⁵ In this model, a cell contains n_{motor} myosin motors, each capable of generating a stall force of F_{motor} , with the product of these terms representing the total force generation of the cell. Motors pull an F-actin bundle with an unloaded sliding velocity (v_{unloaded}), resulting in a retrograde flow of F-actin towards the cell center. Molecular clutches (number of clutches given by n_{clutch}) represent adhesion complexes (e.g. integrin-mediated adhesions) that are coupled to the substrate through extracellular matrix proteins, and bind actin with a pseudo-first order association rate (k_{on}). Bound clutches are modeled as elastic springs with stiffness κ_{clutch} . This model has been implemented in previous publications in varying complexity^{175,326}

Clutches initially bind at the current substrate position ($x_{\text{substrate}}$) and are extended by retrograde flow from that position. Force on an individual clutch increases its off-rate from the basal first-order value (k_{off}^*) through a Bell's law³²⁷ model

$$k_{\text{off}}^* = k_{\text{off}} e^{\left(\frac{F_{\text{clutch},i}}{F_{\text{bond}}} \right)} \quad (1).$$

In this expression, $F_{\text{clutch},i}$ is the force on an individual clutch, and F_{bond} is the characteristic break force that scales the unbinding rate with force.

Bound clutches transmit motor-based forces to the cell substrate, also modeled as a single elastic spring with stiffness $\kappa_{\text{substrate}}$, which slows the actin sliding velocity from its unloaded rate by the following force-velocity relationship

$$v_{\text{retrograde}} = v_{\text{unloaded}} \left(1 - \frac{\kappa_{\text{substrate}} x_{\text{substrate}}}{n_{\text{motor}} F_{\text{motor}}} \right) \quad (2)$$

where $x_{\text{substrate}}$ is the extension of the substrate spring. Experimentally, both myosin II motors and actin polymerization exhibit force-velocity relationships³²⁸. The substrate position is calculated through a force balance between the substrate and bound clutches (n_{bound}), as given by the following

$$x_{\text{substrate}} = \frac{\kappa_{\text{clutch}} \sum_{i=1}^{n_{\text{bound}}} x_{\text{clutch},i}}{\kappa_{\text{substrate}} + n_{\text{bound}} \kappa_{\text{clutch}}} \quad (3).$$

The product of $x_{\text{substrate}}$ and $\kappa_{\text{substrate}}$ yields the traction force transmitted to the substrate.

4.2.13 *In-Silico* Motor Clutch Model of Cell Traction – Model Assumptions

As described previously³²⁵, we ignore inertial forces because the Reynolds number is low for clutch molecules ($Re \ll 1$) and we ignore the effects of viscous relaxation within the gel, considering it to be a purely elastic material. We also assume that the actin bundle and myosin motors are stiff compared to the clutches and compliant substrate, which will preferentially deform under load³²⁵. This model is agnostic to the specific identity of the clutch – integrin-mediated adhesions contain several putative clutch molecules necessary for efficient transmission of force – as well as the point of failure within the adhesion. Instead, it only requires the existence of a failure point somewhere between the extracellular matrix and the F-actin bundle³²⁵. In addition, we assume that clutches function as “slip bonds”, where unbinding rate increases exponentially with force. Although, more complex force-dependent unbinding rate models such as “catch-slip bonds” may be implemented, resulting in only slight qualitative changes to model behavior³²⁹. Last, to calculate spread area, we ignore the effects of stall force that slow polymerization of individual filaments against a rigid membrane³³⁰, and assume that membrane tension loads are shared by a large number of filaments.

4.2.14 *In-Silico* Motor Clutch Model - Calculation of traction stress and cell spread area

Since simulations model the compliant substrate as an elastic spring (units in terms of piconewtons per nanometer), the model outputs cell traction in units of 1D forces. By contrast, experimental measurements of cell traction that rely on fluorescent beads embedded in a gel substrate yield units of traction stress over a 2D area. To draw comparisons between simulation and experiment, we used a previously described approach to derive a relationship between 1D forces and 2D stresses on a continuous substrate³²⁹, as has been previously employed with stochastic simulations of 1D cell traction³³⁰. We assume that the cell-substrate contact area is a circle radius r_{adhesion} , so the 2D stress generated by the cell is

$$T_{cell} = \frac{F_{substrate}}{\pi r_{adhesion}^2} \quad (4),$$

where $F_{substrate}$ is the 1D traction force from simulation output. The effective Young's modulus is given by

$$E_{substrate} = \frac{9\kappa_{substrate}}{4\pi r_{adhesion}} \quad (5).$$

We implemented a second set of rules to the model to simulate an effective cell area. Spread area of adherent cells is governed by two antagonistic processes: ³²⁵ leading edge protrusion rate driven by actin polymerization, and ¹⁷⁵ actin network retraction by retrograde flow. An expression for the spreading velocity of a point along the cell radius (v_{edge}) is given by

$$v_{edge} = v_{protrusion} - v_{retrograde} \quad (6).$$

At steady state ($v_{edge}=0$), the area of a disk-shaped cell is calculated as $A=\pi r_{cell}^2$, where r_{cell} is the steady state radius of the cell, as was previously employed by Chaudhuri et al. ³³¹, although they did not consider steady state conditions. In order to implement a pseudo-steady-state solution for cellular dimensions, we constrained $v_{protrusion}$ by an actin mass balance. Actin subunits that are partitioned into a soluble pool of G-actin subunits and an F-actin bundle, giving

$$[Actin]_{Total} = [G-actin] + [F-actin] \quad (7)$$

as an expression for the total actin in the cell. Cell radius is then calculated as the length of the F-actin bundle (scaled by the length of an actin subunit, $L_{actin}=4nm$), plus the radius of the cell nucleus ($L_{nucleus}=5000nm$).

F-actin that is drawn inward from the leading edge (retrograde flow) is disassembled into individual subunits by severing and depolymerization, in turn generating new assembly-competent G-actin. The following expression was used to calculate the rate of G-actin turnover in the cell:

$$\frac{\partial[G-actin]}{\partial t} = [G-actin]_{in} - [G-actin]_{out} = \frac{v_{retrograde}}{L_{actin}} - \frac{v_{protrusion}}{L_{actin}} \quad (8).$$

The F-actin mass balance contains these two terms, but with opposite signs. In turn, the available pool of G-actin subunits influences $v_{protrusion}$ by the following relationship

$$v_{protrusion} = v_{polymerization} \left(\frac{[G-actin]}{[Actin]_{Total}} \right) \quad (9).$$

4.2.15 *In-Silico* Motor Clutch Model – Kinetic rate for clutch reinforcement

Clutch reinforcement adds a single kinetic rate and three new parameters to the model – a first-order clutch addition rate constant (k_{add}), a threshold force for clutch reinforcement signaling ($F_{threshold}$), and a maximum clutch pool ($n_{clutch,max}$) that represents the total number of available clutches in the cell. At any given simulation step, the number $n_{threshold}$ represents the number of clutches above a force threshold

$$n_{threshold} = \sum_{i=1}^{n_{clutch}} (F_{clutch,i} > F_{threshold}) \quad (10)$$

where $F_{clutch,i}$ is the force on a given clutch. This number is initially zero while clutches are unbound or bear low force, but increases as loaded clutches begin to unbind, leaving a large load shared between few clutches. For simplicity, we assume thresholded clutches are simple “on/off” switches and each thresholded clutch contributes equally to the strength of the reinforcement signal, independent of force. The available fraction of clutches is given by the following

$$k_{add}^* = k_{add} n_{threshold} f_{available} \quad (11).$$

In this expression, $n_{clutch}(t)$ is the clutch ensemble size at time t . These two terms scale the addition rate for clutch incorporation into the ensemble (k_{add}), such that at any given time t , the clutch addition rate is

$$k_{add}^* = k_{add} n_{threshold} f_{available} \quad (12).$$

Parameter	Description	Values and References
Motors		
n_{motor}	Number of myosin II motors	Adjustable; set to 75-150 to match measured cell traction stresses
F_{motor}	Stall force for myosin II motors	2 pN; measured <i>in vitro</i> via optical trap ^{325,332}
V_{unloaded}	Unloaded sliding velocity of myosin II motors on F-actin	120 nm/s; within range of previously published values ^{325,330}
Clutches		
n_{clutch}	Initial number of clutches in adhesions	Adjustable; set to 75-150 to match cell traction stress and area
k_{on}	Pseudo-first order binding rate for clutches to F-actin bundle	0.3 s^{-1} ; adjustable, set to match measured cell traction stresses ¹⁷⁵
k_{off}	Basal first-order unbinding rate for bound clutches (slip bonds)	0.1 s^{-1} ; estimated from Fluorescence Recovery after Photobleaching (FRAP) experiments ^{325,333}
F_{bond}	Characteristic bond rupture force for individual clutches	2 pN; experimentally estimated for integrin-fibronectin bond strengths ^{325,334}
κ_{clutch}	Molecular clutch spring constant	0.8 pN/nm; within published range of moduli for globular proteins ^{325,335} , model is insensitive to clutch stiffness ³²⁶
Substrate		
$\kappa_{\text{substrate}}$	Substrate spring constant	0.1-100 pN/nm; set to span range of gel Young's moduli used in this study
r_{adhesion}	Cell-substrate contact radius	0.55 μm ; scale of focal adhesion sizes ($\sim 1 \mu\text{m}^2$ circular contact area)
Actin		
L_{actin}	Length of an actin subunit	4 nm; size of actin subunit within F-actin filament (6)
[F-actin]	Initial subunits in F-actin bundle	2000; set to match cellular radius
[G-actin]	Initial subunits in G-actin pool	2000; set to match cellular radius
$V_{\text{polymerization}}$	Maximum leading edge actin polymerization velocity	200 nm/s; set to match maximum retrograde flow velocity ³³¹
Reinforcement		
$F_{\text{threshold}}$	Threshold force for clutch reinforcement signaling	$>10 \text{ pN}$; within range of measured values for talin unfolding ¹⁹²
k_{add}	First-order addition rate for clutch reinforcement	$0.1\text{-}1 \text{ s}^{-1}$; adjustable, set to match observed traction stress and cell area
$n_{\text{clutch,max}}$	Maximum number of clutches in cell	Adjustable; set to 750 to match observed traction stress and cell area trends

Table 4.1. Parameters for motor-clutch *in-silico* model of cell adhesion and traction

4.2.16 *In-Silico* Motor Clutch Model – Rationale for Clutch Reinforcement

Previous versions of the motor-clutch model predict a biphasic response to substrate stiffness - an optimum value for maximal force transmission (and corresponding minimal retrograde flow) that is bounded by regions of inefficient “slippage” and poor force transmission at higher and lower substrate stiffness values ^{325,326}. We did not observe a strong biphasic trend in traction stress or spread area for either epithelial or transformed MCF10A cells. Transformed cells had nearly uniformly higher traction stress and spread area on gels with a Young’s modulus above disease stiffness, while behaving similarly to non-transformed cells on 1.1kPa gels (**Fig 4.3.5**).

We hypothesized that adhesion reinforcement – represented by changing the size of the clutch ensemble in response to substrate stiffness – could explain the elevated traction stress and cell spread area in transformed cells. Elosegui-Artola et al. ³³⁰ recently proposed the “integrin-fibronectin clutch” model (based on the motor-clutch model), which incorporated integrin reinforcement and demonstrated either monotonic increase or multi-phasic behaviors in response to substrate stiffness. Their model assumes the cell expresses two types of integrins with different binding rates that compete for the same fibronectin molecules on the cell substrate. As reinforcement events occur, the integrin density (and, correspondingly, the binding rate) increases. At higher substrate moduli, this leads to stalled cells and a monotonic increase in traction force (and decrease in retrograde flow) above a threshold substrate modulus. In our model, we do not explicitly assume the identities of the clutches, only that there is a pool of available clutches to be recruited as thresholding events occur, representing the total number of clutch components in the cell.

4.2.17 *In-Silico* Motor Clutch Model – Model Implementation

Stochastic simulations of the motor-clutch model were written and run in Matlab version 2013b or 2014b (TheMathWorks, Natick, MA) using a direct Gillespie stochastic simulation method ³³⁶. The direct approach only generates two random variables at each simulation step, instead of a separate random

variable for each event (the indirect approach), as was used previously³²⁶. Briefly, at each simulation step, rates for all possible events are calculated based on the current state of the system. The time to the next event (Δt) is calculated using the summed rates of all events

$$\Delta t = \frac{-\log(URN)}{\sum_{i=1}^{N_{event}} k_i} \quad (14)$$

where URN is a uniform random variable between 0 and 1 (generated using the Matlab *rand* function), k_i is the rate of the i^{th} event at the current simulation step, and N_{event} is the total possible number of events at the current time step. N_{event} varies at each time step, depending on the current number of clutches – each clutch in the ensemble may bind or unbind, or a new clutch may be added. New clutches join the ensemble at the current position of the substrate ($x_{substrate}$) and are initially unbound. Once the event time is calculated from the summed event rates, a second random variable (URN) is generated and used to select a new event based on the relative rate for each event – i.e. events with a larger rate constant are more likely to be selected, because their contribution to the summed rate is larger. A single event is selected and executed, and the system is updated for the next time step.

The event time is also used to calculate the positions of clutches and the cell edge, and the net flux of G-actin into the pool of assembly-competent subunits. Before solving the force balance (Equation 3), engaged clutches are extended by a distance equal to the product of $v_{retrograde}$ (Equation 2) and Δt . Similarly, the product of $v_{protrusion}$ (Equation 10) and Δt determines the protrusion distance during the simulation step³³¹. The F-actin bundle length and G-actin pool are updated by a number of subunits as given by the mass balance in Equation 8. Each simulation begins with all clutches unbound, and was run at a single substrate stiffness value, over a range of values of $\kappa_{substrate}$ (**Table 1**), for 10,000 seconds of simulated time, to ensure that the simulations reached steady state.

4.2.18 Statistics

Statistical analysis for 2 groups was performed with an unpaired, two-tailed student's t-test. For multiple comparisons, an ANOVA test and Holm–Bonferroni t-test method were performed. Calculations were implemented in Python.

4.3. Results

4.3.1. Metastatic cells have altered invasive behavior and biophysical properties.

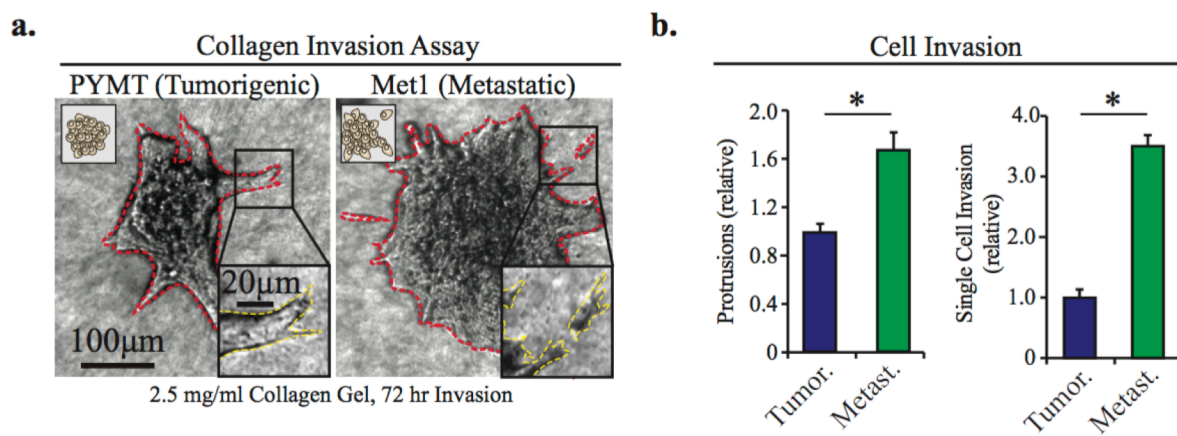


Fig 4.1 **A.** 3D collagen invasion assay of multicellular spheroids composed of tumorigenic PYMT cells or metastatic Met1 cells in 2.5mg/ml stiff 3D collagen gels for 72 hours. Metastatic cells are more invasive than tumorigenic cells. **B.** Quantification of invasion parameters. Metastatic cells have significantly increased protrusive fronts and single cell invasion ($p < 0.05$, $n > 30$ spheroids).

Most previous studies connecting tumor cell invasion to metastasis have employed 2D migration scratch assays, or multicellular 3D environments including Matrigel™ that do not adequately resemble the heterogeneous tumor ECM^{13,285,337}. Here we examined the importance of cellular metastatic potential in 3D mesenchymal-like invasive behavior of multicellular tumor cell aggregates. Tumorigenic carcinoma cell line PYMT Flox3 cell line were derived from primary tumors of MMTV-PyMT mice, and the metastatic Met1 cell line was derived from MMTV-PyMT tumor cells transplanted into syngeneic FVB/N mammary fat pads and isolated after multiple rounds of *in vitro* culture and *in vivo* transplantation as described³¹². Using a collagen invasion assay of multicellular spheroids previously developed in our lab³³⁷, we composed tumorigenic PYMT cells versus metastatic Met1 cells in 2.5mg/ml stiff 3D collagen gels for 72 hours (**Fig 4.1A**). We found that metastatic cells are significantly more invasive than

tumorigenic cells via several parameters including increased protrusive fronts and single cell invasion (**Fig 4.1B**).

4.3.2. Functional links among metastatic potential, ECM stiffness and cell adhesion

To explore functional links among metastatic potential, ECM stiffness, and cellular adhesion, we assayed tumorigenic and metastatic cells on polyacrylamide (PA) gels of either normal tissue stiffness (~1.0 kPa) or cancer tissue stiffness (~2.7kPa)²⁸⁸. Tumorigenic PYMT and metastatic Met1 cells seeded on 2.7 kPa polyacrylamide (PA) gels conjugated with fibronectin and embedded with fluorescent beads for traction force microscopy. We calculated the displacement of beads before and after cell lysis, which was then used to calculate traction forces. Similar to previous findings in mammary epithelial cells treated with TGF β to induce mesenchymal behavior^{172,173}, we found that at stiffness comparable to tumor ECM (~2.7kPa), compared to untreated tumorigenic PYMT, metastatic Met1 cells or tumorigenic PYMT cells treated with TGF β to induce mesenchymal behavior are more spread and exert more traction forces on the environment (**Fig 4.2 A,B**).

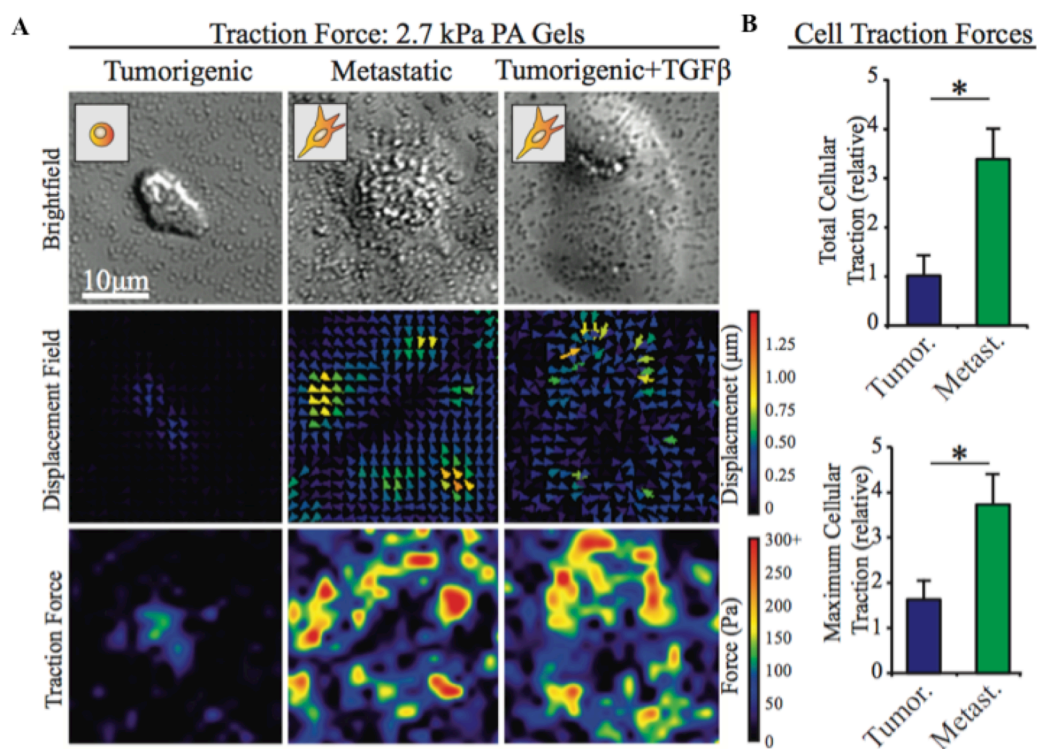


Fig 4.2 A. Tumorigenic and metastatic cells seeded on 2.7 kPa polyacrylamide (PA) gels conjugated with fibronectin and embedded with fluorescent beads for traction force microscopy. Displacement field is of beads before and after cell lysis, which is used to calculate traction forces. At stiffness comparable to tumor ECM (~ 2.7 kPa), metastatic cells or tumorigenic cells treated with TGF β to induce mesenchymal behavior are more spread and exert more traction forces on the environment. **B.** Quantification of cell traction parameters. Metastatic cells have significantly increased total cellular and maximum traction forces ($p < 0.05$, $n > 15$ cells).

4.3.3. Forces over integrin increase with cell metastatic potential

As cell-ECM adhesion proteins have been implicated in force transmission from the cell interior to the ECM^{21,170}, we investigated integrin force sensing in cells of different metastatic potential. The molecular tension sensors and the calculations of FRET efficiency used to deduce force per integrin used here are shown in **Fig 4.3 A,B,C** below^{170,317}.



Fig 4.3 A. Mechanical stretch is detected via changes in FRET between the FRET donor Alexa 546 and the FRET acceptor ATTO 647N, which are connected by an extensible (GPGGA)₈ peptide linker. Specifically, the FRET efficiency is equal to the ratio of intensities of the two probes, divided by the measured FRET efficiency weighted by the percentage of sensors that contain both probes, and the ratio of quantum yields of the dye. **B.** The force versus FRET calibration curve was implemented from previously measured properties of a (GPGGA) spring⁶⁸ **C.** The histogram relationship between FRET efficiency and count, wherein signal is considered true if it is offset by 3-sigma (three times larger than the standard deviation) of the noise.

Using this integrin force sensing assay employing tumorigenic PYMT or metastatic Met1 cells **Fig 4.4A**, or MCF10a +/- TGFB **Fig 4.4B**, we found that mesenchymal Met1 or MCF10a cells had significantly higher average force per integrin than epithelial PYMT or epithelial (untreated with TGFB) MCF10a respectively.

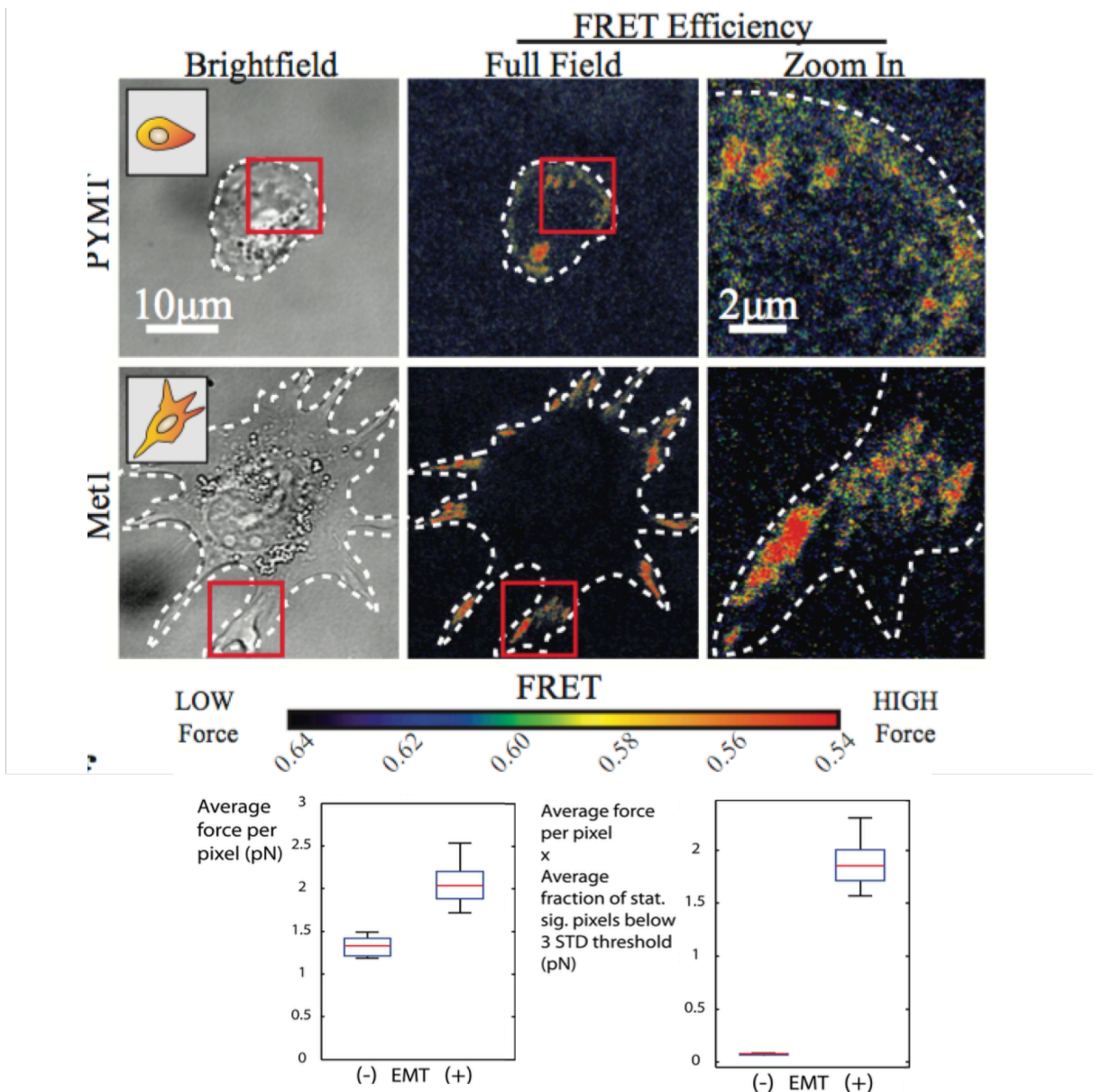


Fig 4.4 A. Integrin force sensing assay employing cells seeded on cover slips tethered with integrin FRET probes. Brightfield and FRET intensity of integrin probes are shown, where lower FRET signal (towards red) is associated with higher force. **B.** Quantification of integrin force sensor. Mesenchymal cells have significantly increased average adhesion FRET intensity and Force ($p < 0.05$, $n > 15$ cells).

4.3.4. Mesenchymal behavior of metastatic cells *in silico* increases cell adhesion area and force per integrin.

The mechanical properties of the ECM exert strong influence on cell behavior including adhesion formation and migration, but it is unclear how the mesenchymal behavior of metastatic cells could directly affect these cell properties^{17,175,331}. Furthermore, because different cell types can have different responses to the same ECM, we were curious what role cell plasticity played in allowing specific tumor cells to undergo mesenchymal-like cell invasion at disease like tissue stiffness¹⁷³. In order to study cell adhesion area and force per integrin we first turned to an *in silico* motor-clutch model of cell traction that included myosin contractility, actin assembly/tread-milling/disassembly, clutches with constant k_{on} and variable k_{off} rates (dependent on clutch strain x_{clutch}), and a compliant substrate (**Fig 4.5**). This model improved upon existed models developed by the Odde and Mooney lab by incorporating G and F-actin turnover for calculation of cell size, clutch (i.e. integrin) reinforcement upon a force threshold, as well catch and slip bond simulations

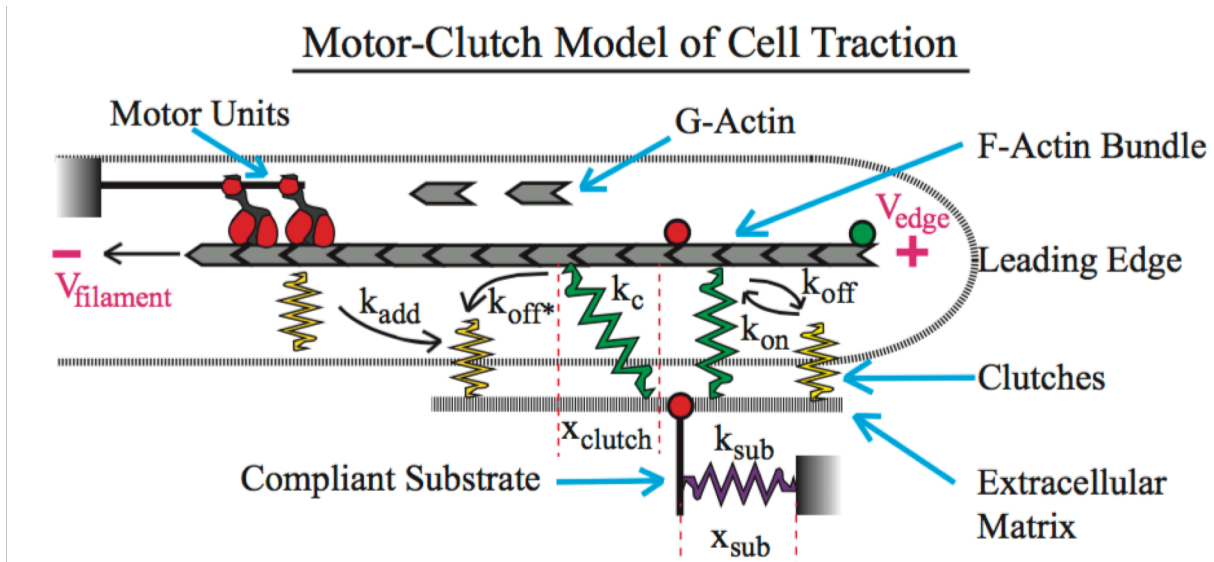


Fig 4.5. The base motor-clutch model contains eight parameters that describe numbers and physical properties of myosin motors and molecular clutches (e.g. integrin-mediated adhesion complexes), as well as kinetic rates for binding and unbinding of molecular clutches. The model also includes calculation of an effective spread area for a disk-shaped cell through explicit modeling of F-actin bundle length and material balance on total actin, and kinetic rates for molecular clutch reinforcement when a threshold force is reached by engaged clutches. Events in simulations were executed using a Gillespie stochastic simulation algorithm (kinetic Monte Carlo simulation)

With increasing integrin reinforcement^{330,338}, number of motors as hypothesized in mesenchymal and metastatic cells, or increased spring constant seen in tumor microenvironments⁸, our *in silico* model predicts increased traction force on the external environment (**Fig 4.6A**). Moreover, when cell adhesion area is extrapolated from length of the assembled actin filament, which in turn is dependent on substrate spring constant and number of motors and clutches, the model predicts that there will be more adhesion area with increased integrin reinforcement or increased substrate stiffness (**Fig 4.6B**). Our *in-silico* modeling experiment confirmed that cells with increased myosin contractility or integrin clutches, as seen in mesenchymal like cells³³⁹, would have increased ability to adhere and generate traction at disease level stiffness.

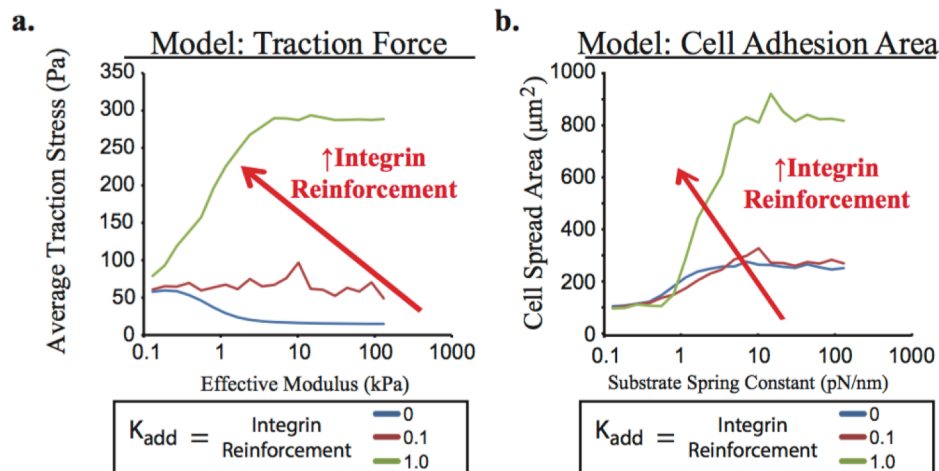


Fig 4.6. A. Motor-clutch model of cell traction, including myosin contractility, actin assembly/tread-milling/disassembly, clutches with constant k_{on} and variable k_{off} rates (dependent on clutch strain x_{clutch}), and a compliant substrate. **B.** Model of force per clutch with variable substrate spring constant, as well as number and ratio of motors:clutches. With increasing number of motors as hypothesized in metastatic cells, or increased spring constant, there is increased force per engaged clutch.

4.3.5. Mesenchymal behavior of metastatic cells *in vitro* increases cell adhesion area

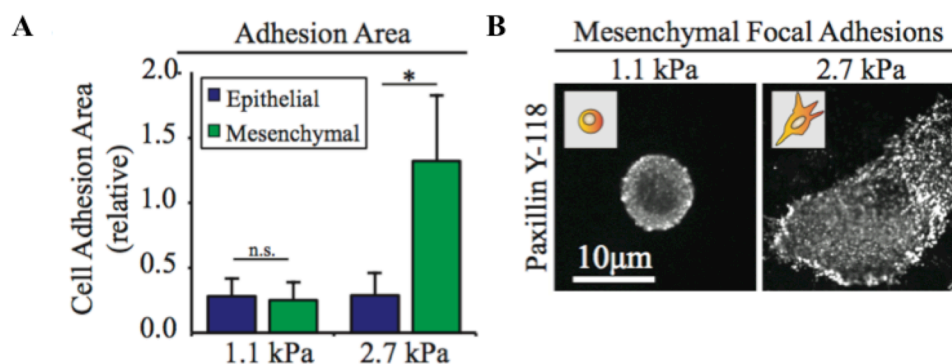


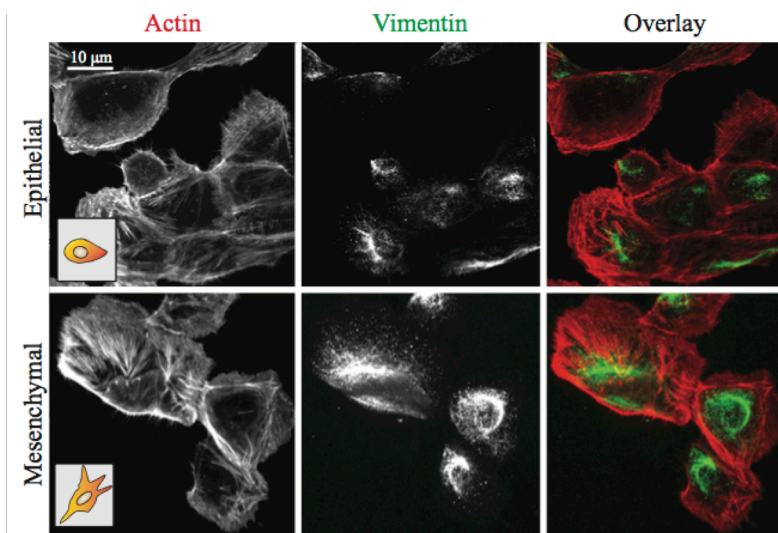
Fig 4.7. A. MCF10a epithelial cells treated +/- TGF β to induce mesenchymal behavior, seeded on PA gels of 1.1kPa (normal tissue stiffness) or 2.7 kPa (cancer tissue stiffness). At 2.7kPa, mesenchymal-like cells spread significantly more than epithelial cells at 1.1kPa or either phenotype at 1.1kPa ($p < 0.05$, $n > 20$ cells). **B.** Immunofluorescent staining of phosphorylated activated paxillin at tyrosine 118 (Y118) in mesenchymal-like cells at either 1.1kPa or 2.7kPa.

As mesenchymal behavior has been previously shown to increase myosin activity and RhoA dependent integrin recruitment^{340,341}, we treated epithelial MCF10a with the chemokine TGF β to induce a mesenchymal-like phenotype to see if increased myosin contractility or integrin

reinforcement could account for the adhesion and invasion behavior of mesenchymal-like cells *in vitro*. To evaluate the role of cell phenotype and validate our motor-clutch model, the epithelial or mesenchymal-like MCF10a cells were then seeded on PA gels of normal tissue stiffness (1.1kPa) or cancer tissue stiffness (2.7 kPa). At 2.7kPa, mesenchymal-like cells spread significantly more than epithelial cells at 1.1kPa or either phenotype at 1.1kPa (**Fig 4.7A**). Immunofluorescent staining of paxillin phosphorylation at Y118 in adhesions revealed an increase in activated paxillin in mesenchymal-like cells at either 1.1kPa or 2.7kPa (**Fig 4.7B**).

4.3.6. Mesenchymal behavior of metastatic cells *in vitro* increases filamentous actin formation and force per integrin.

A.



B.

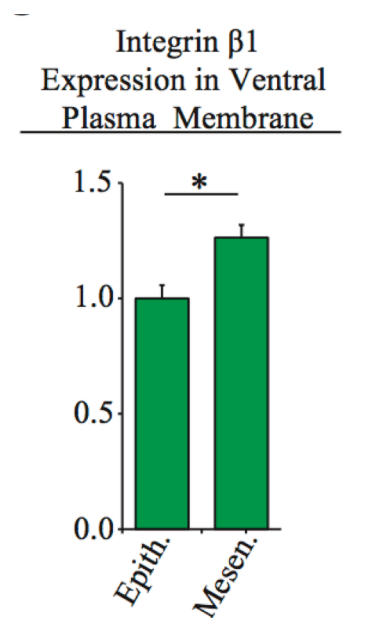


Fig 4.8. A. MCF10a +/- TGF β to induce a mesenchymal phenotype seeded on fibronectin coated glass, fixed and stained for actin and vimentin. Mesenchymal like cells have increased vimentin and fibrillar actin. **B.** Quantification of MCF10a +/- TGF β on fibronectin coated cover slips, stained for $\beta 1$ integrin in the ventral plasma membrane. Mesenchymal like cells have significantly more $\beta 1$ integrin than epithelial like cells ($p < 0.05$, $n > 20$ cells).

As reported previously in the literature ¹⁷, EMT increased filamentous actin in the mesenchymal like cells, accompanied by increased integrin $\beta 1$ at adhesions and vimentin cytoskeletal structures (**4.8A,B**). As predicted by *in silico* experiments we found that mesenchymal-like MCF10a exerted significantly more force per integrin than untreated epithelial MCF10a cells, similar to the differences previously seen in epithelial PYMT and mesenchymal-like and metastatic Met1 (**Fig 4.9**).

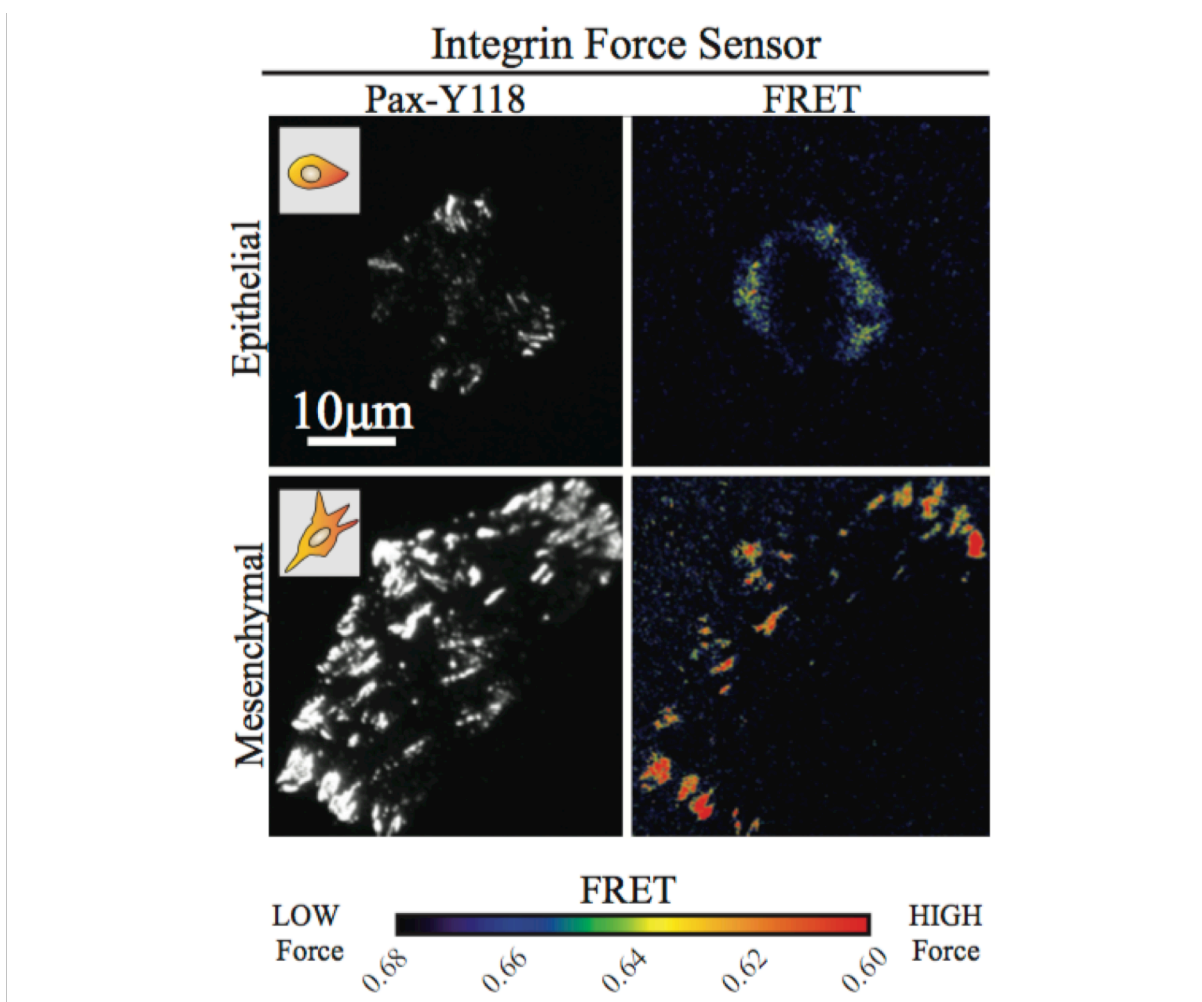


Fig 4.9 Integrin force sensing assay of MCF10a epithelial cells treated +/- TGF β . Mesenchymal-like MCF10a TGF β -treated cells have increased force per integrin, enlarged adhesion area, and increased cell spreading; as predicted by earlier experiments comparing PYMT to Met1.

4.3.7. Role of increased integrin expression on force per integrin

We next tested the role of increased cellular clutches, i.e. integrin expression, could effect cell adhesion behavior on tumor-like-microenvironments. As previous studies and our *in silico* motor-clutch model predicted that elevated integrin ligation can drive increased cell spreading and mechanosensing³³⁰. Moreover, we observed up to 2-fold increases in $\alpha 5$, $\alpha 2$, $\beta 3$, and $\alpha 5\beta 3$ integrin surface expression via flow cytometry in MCF10a cells +/- TGF β . Therefore we studied if $\alpha 5$ integrin overexpression could be responsible for changes in cellular mechanosensing. To test the role of integrin overexpression in cellular mechanosensing, MCF10a cells expressing Muc1- Δ TR-mVenus membrane probe¹⁴ +/- $\alpha 5$ integrin overexpression were seeded on coverslips of different fibronectin concentration and assayed for total adhesion area (**Fig 4.10A**). Surprisingly, we found that $\alpha 5$ integrin overexpression alone does not significantly increase cell adhesion and spreading at any of the fibronectin concentrations tested (**Fig 4.10B**). Additionally, we did not see increases in integrin force generation in metastatic and mesenchymal Met1 cells flow sorted for high $\alpha 5$ integrin expression. These results indicate that unlike paxillin-alpha overexpression, $\alpha 5$ integrin clutch manipulation alone is not sufficient to drive changes in cellular mechanosensing.

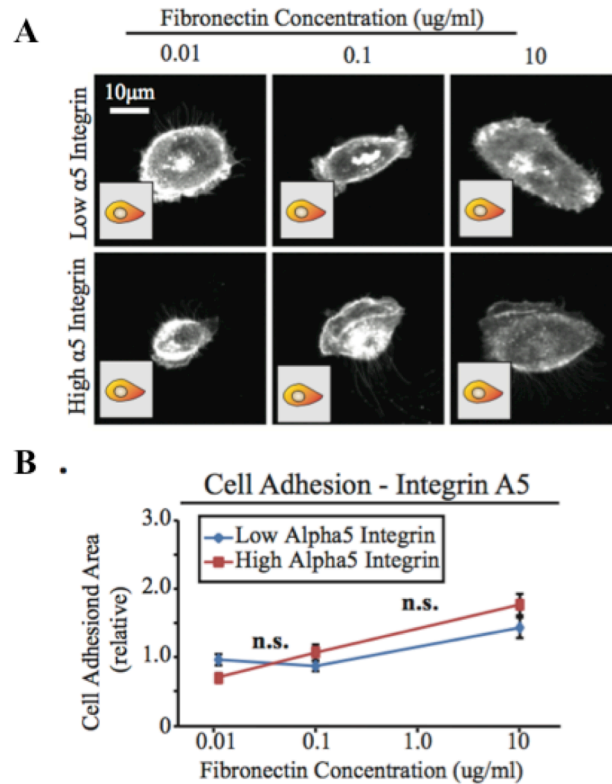


Fig 4.10 A. MCF10a cells +/- $\alpha 5$ integrin overexpression in fibronectin cellular mechanosensing assay. Cells are expressing Muc1- Δ TR-mVenus membrane probe. **B.** Quantification of MCF10a cells +/- $\alpha 5$ integrin overexpression. $\alpha 5$ integrin overexpression does not significantly increase cell adhesion.

4.3.8 Mesenchymal phenotype alters the micro-scale organization architecture of adhesions

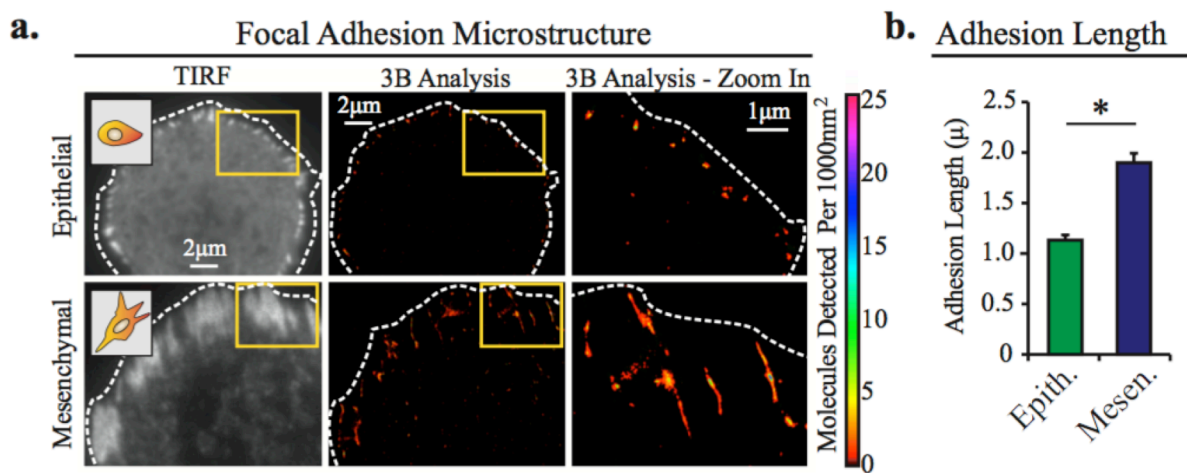


Fig 4.11 A. Focal adhesion microstructure of MCF10a +/- TGF β (+ mesenchymal, - epithelial) expressing paxillin-GFP, imaged with TIRF and 3B super-resolution microscopy. **B.** Quantification of mean adhesion length. Mesenchymal-like cells have significantly longer adhesions ($p < 0.05$, $n > 200$ adhesion over > 20 cells).

Focal adhesions are critical for mesenchymal-like cell motility seen in metastatic cells and their structure function has been postulated to control mechanical force transmission^{13,21,164}. This prompted us to ask if the mesenchymal phenotype alters the organization of focal adhesions, including changes in adhesion protein signaling scaffolds, to drive increased cellular mechanotransduction. We first characterized the focal adhesion microstructure of epithelial and TGF β induced mesenchymal MCF10a expressing paxillin-GFP via super-resolution bayesian analysis of blinking and bleaching (3B)(**Fig 4.10A**). We found that not only were mesenchymal-like adhesions larger in area, but they had significantly longer adhesions (**Fig 4.11B**).

4.3.9 Mesenchymal phenotype alters the turnover of focal adhesions

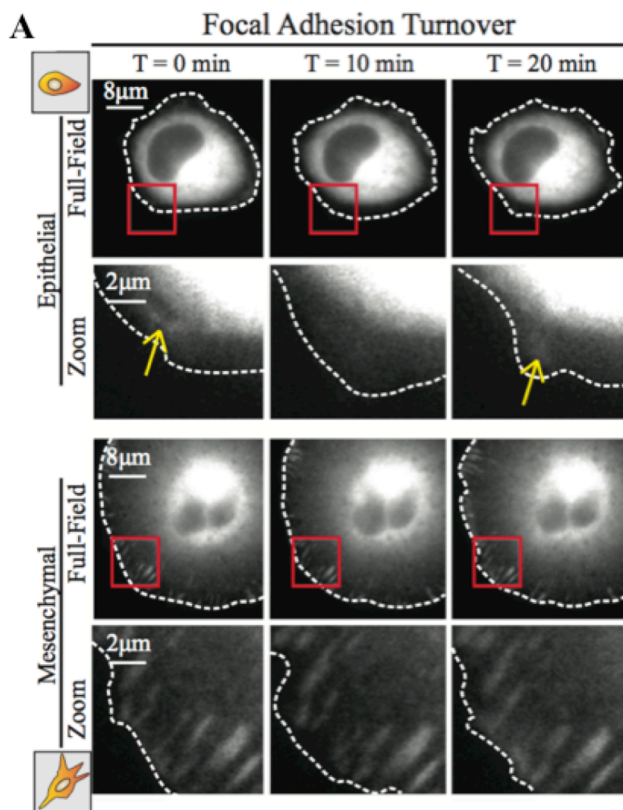


Fig 4.12 Live imaging of paxillin-GFP to visualize focal adhesion turnover in MCF10 +/- TGF β . Yellow arrows indicate adhesions that turnover rapidly in less than 10 minutes, seen primarily in epithelial-like cells.

As static single images of adhesions are insufficient for understanding dynamic adhesion properties, we next analyzed adhesion turnover via in live cells. We found that epithelial adhesions turn over much more rapidly than mesenchymal-like adhesions (**Fig 4.12**), suggesting that mesenchymal like adhesions have increased time to assemble mechanical or biochemical signaling scaffolds before turnover. To confirm that mesenchymal like adhesions were more mature (**Parsons 2010**), we studied tensin positive fibrillar-like adhesion formation in MCF10a +/- TGF β expressing paxillin-GFP and Tensin-RFP (**Fig 4.13A**). We found that mesenchymal-like cells have significantly more paxillin-tensin co-localization, indicating increased adhesion maturity (**Fig 4.13B**).

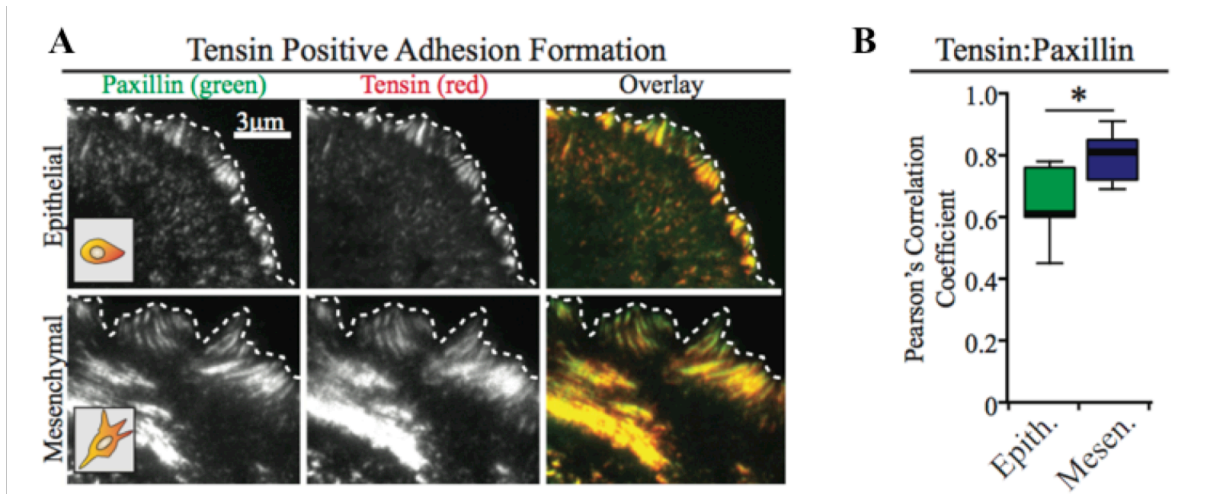


Fig 4.13 A. Tensin positive fibrillar-like adhesion formation in MCF10a +/- TGF β expressing paxillin-GFP and Tensin-RFP. **B.** Quantification of correlation between tensin and paxillin in adhesions. Mesenchymal-like cells have significantly more paxillin-tensin co-localization ($p < 0.05$, $n > 20$ cells).

4.3.10. Paxillin expression and phosphorylation is altered in mesenchymal cells

Through immunofluorescent analysis of epithelial and mesenchymal-like MCF10a, we found that paxillin expression and phosphorylation increased significantly in mesenchymal-like adhesions (**Fig 4.14**).

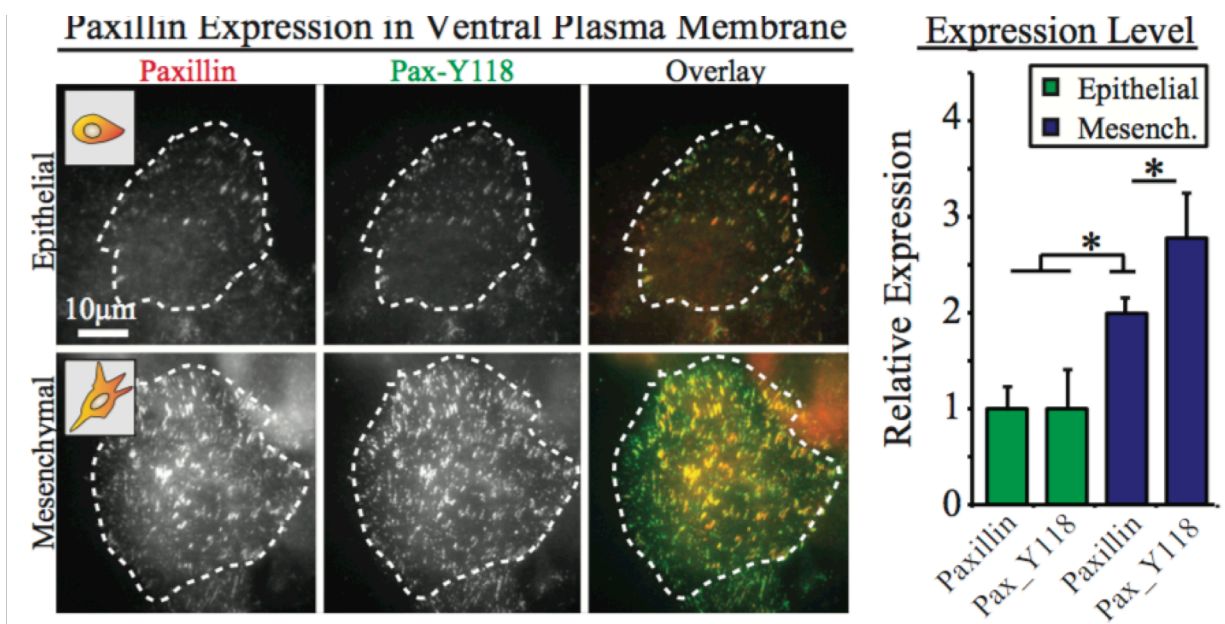


Fig 4.14 Paxillin expression in ventral plasma membrane of MCF10a +/- TGF β , via immunofluorescent staining and confocal imaging of paxillin and activated paxillin Y118. Quantification of paxillin expression level in ventral membrane. Mesenchymal-like cells have significantly more paxillin and activated paxillin ($p < 0.05$, $n > 15$ cells).

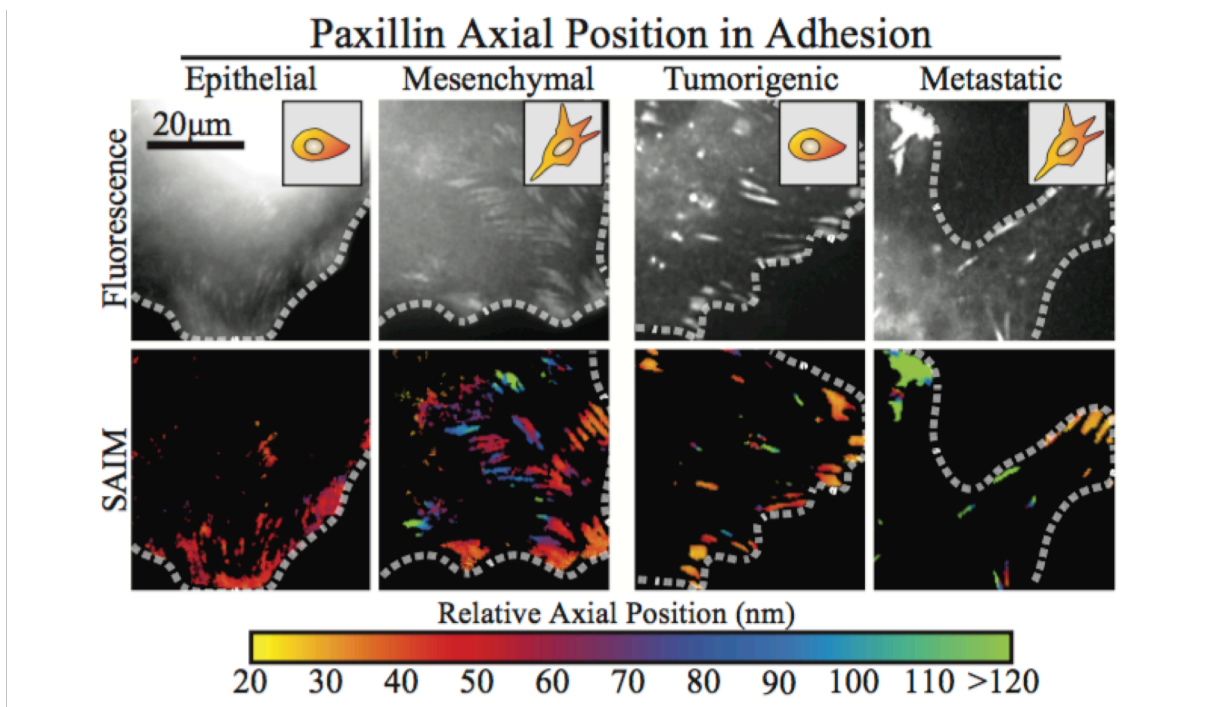


Fig 4.15 Paxillin-GFP axial position in focal adhesions via scanning angle interference (SAIM) super-resolution microscopy. Paxillin position is plotted as relative distance from extracellular matrix. MCF10a +/- TGF β , tumorigenic PYMT and metastatic Met1 cells

When we analyzed paxillin axial nanoscale position in epithelial or tumorigenic cells compared to mesenchymal or metastatic cells, we found a very dramatic and significant change (**Fig 4.15**). Wherein the plasma membrane is **~30nm** from the fibronectin-coverslip interface, we found the N-terminus of paxillin at an average of **~50nm** in epithelial MCF10a and **~65nm** in mesenchymal-like MCF10a (**Fig 4.16A,B**). Intriguingly, when we performed a SAIM screen of 4 other adhesion proteins including FAK, talin, vinculin and zyxin, we observed that all of these constituent adhesion proteins retained their organization between epithelial and mesenchymal like adhesions. Thus the **~15nm** paxillin height change we measured indicates a meaningful difference in the accessible signaling partners present in the higher stratified adhesion layers, as can be seen in an axially scaled model of adhesion architecture proteins^{12,13,218,338}.

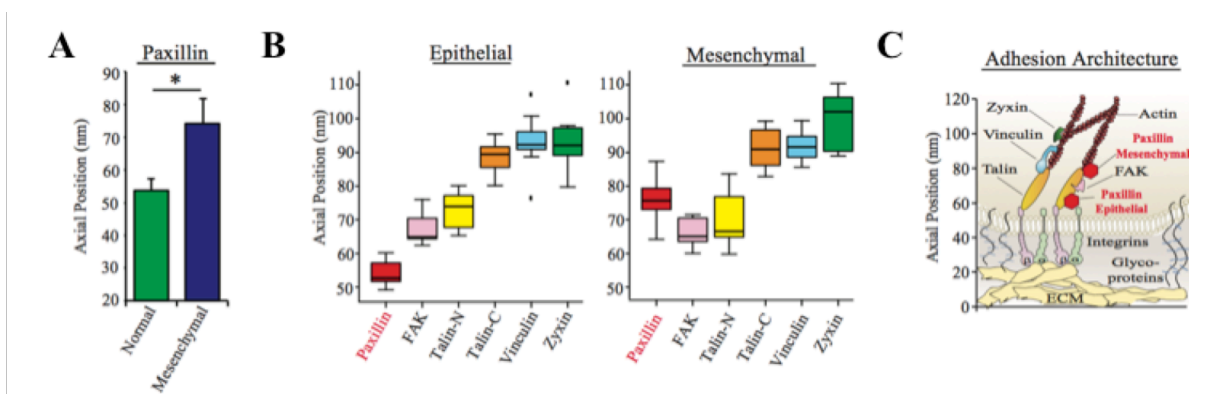


Fig 4.16 **A.** Quantification of paxillin-GFP axial position in MCF10a +/- TGF β . Mesenchymal-like cells have significantly altered paxillin nanoscale position ($p < .05$, $n > 20$ cells). **B.** Super-resolution microscopy screen of various adhesion proteins tagged with GFP in MCF10a +/- TGF β . Paxillin is the only protein to significantly change position, indicating paxillin alone is moving, not the entire adhesion. **C.** Axially scaled model of adhesion architecture proteins in epithelial like and mesenchymal like cells.

4.3.11. Paxillin alternative splicing regulates forces over integrin

Paxillin is a major focal adhesion plaque protein that is responsive to changes in cell contractility and ECM stiffness through phosphorylation of tyrosine residues, and can be alternatively spliced to create paxillin-delta in epithelial cells that do not express tyrosine 31 and 118²³. This provoked us to question if mesenchymal-like cell behavior requires paxillin alpha (full length

including tyrosines 31 and 118) to mechanically stabilize adhesions or drive signaling downstream of adhesion formation. As shown in a protein-scale model of paxillin splicing variants (**Fig 4.17**), paxillin interactions with FAK and Src through tyrosines 31 and 118 can occur via paxillin delta splicing or a synthetic paxillin-F31/118 mutant, which has phenylalanine instead of tyrosine at these residues.

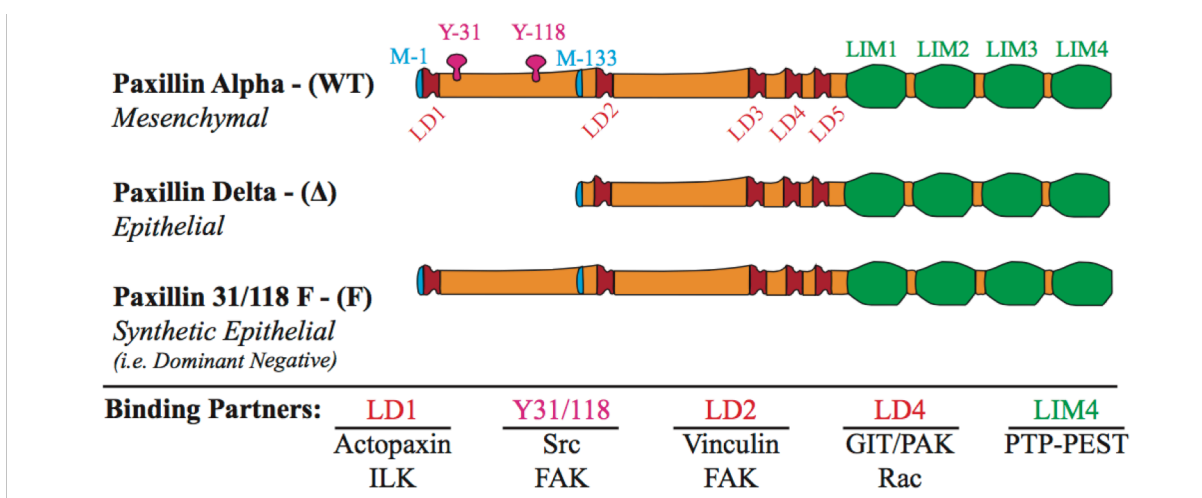


Fig 4.17 Protein-scale model of paxillin splicing variants, including previously described domains and main binding partners.

To test the role of paxillin alternative splicing on force over integrin and external traction force, we implemented the KD2 fibroblast cell line that lacks endogenous paxillin expression and 95% knockdown of Hic5. We then expressed no paxillin, paxillin alpha, paxillin delta or synthetic paxillin 31/118 F and assayed for adhesion formation and force over integrin in the cells (**Fig 4.18**). We found that the average force per integrin significantly increases after EMT.

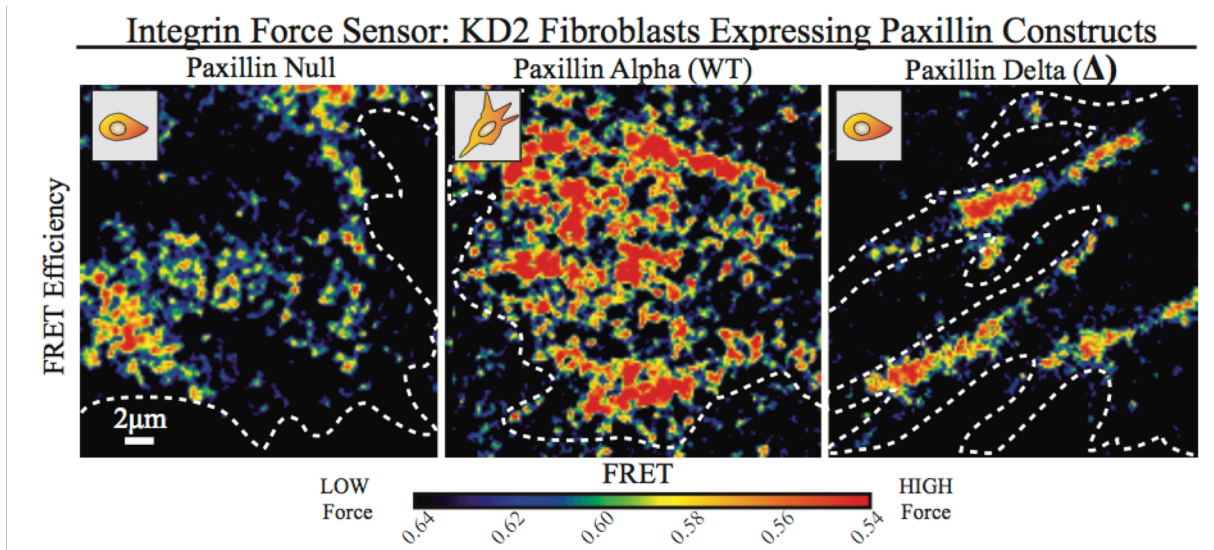


Fig 4.18. Forces over integrins of KD2 fibroblasts expressing paxillin mutants; low FRET values in red indicate high forces per integrin.

4.3.12. Paxillin splice variants alter cell invasion of PYMT, MET1 and MCF10a cell lines.

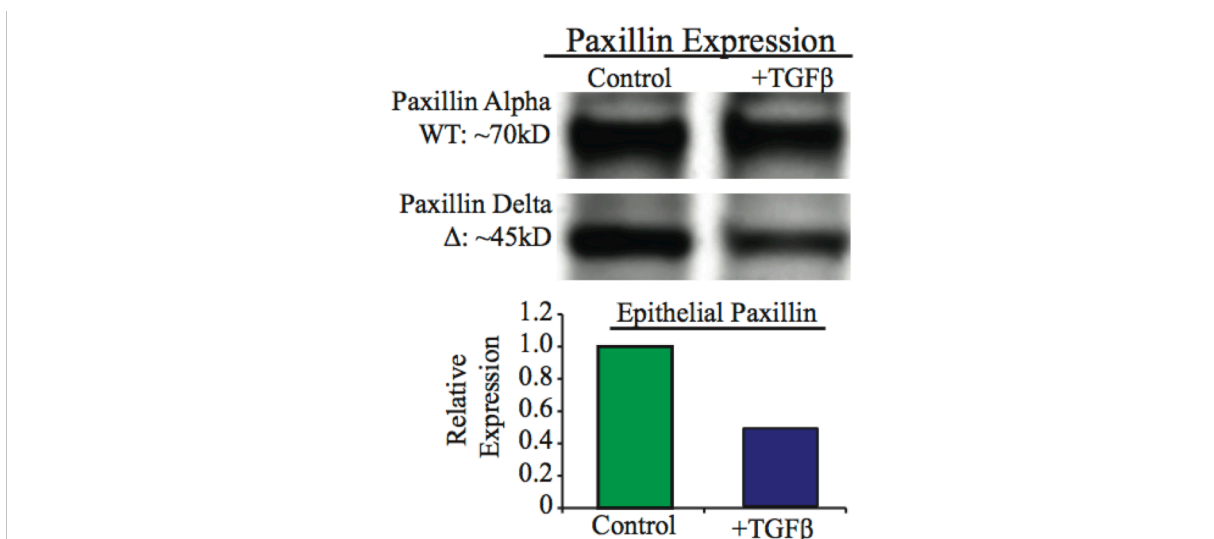


Fig 4.19. Expression of different paxillin isoforms in MCF10a +/- TGF β via Western Blot. Paxillin alpha is ~70kD and paxillin delta is ~45kD. Quantification of the decrease paxillin delta in mesenchymal cells, as normalized to actin expression.

After we confirmed that paxillin splicing could change mechanical forces over integrin in a base fibroblast system, we expressed these different paxillin isoforms in our breast cancer model systems. We first found a decrease in the truncated paxillin delta in mesenchymal MCF10a cells treated with TGF β (**Fig 4.19**), as well as increases in traction force mediated by mesenchymal phenotype alone; similar to our observed differences in PYMT and Met1 cells (**Fig 4.20 A, B**).

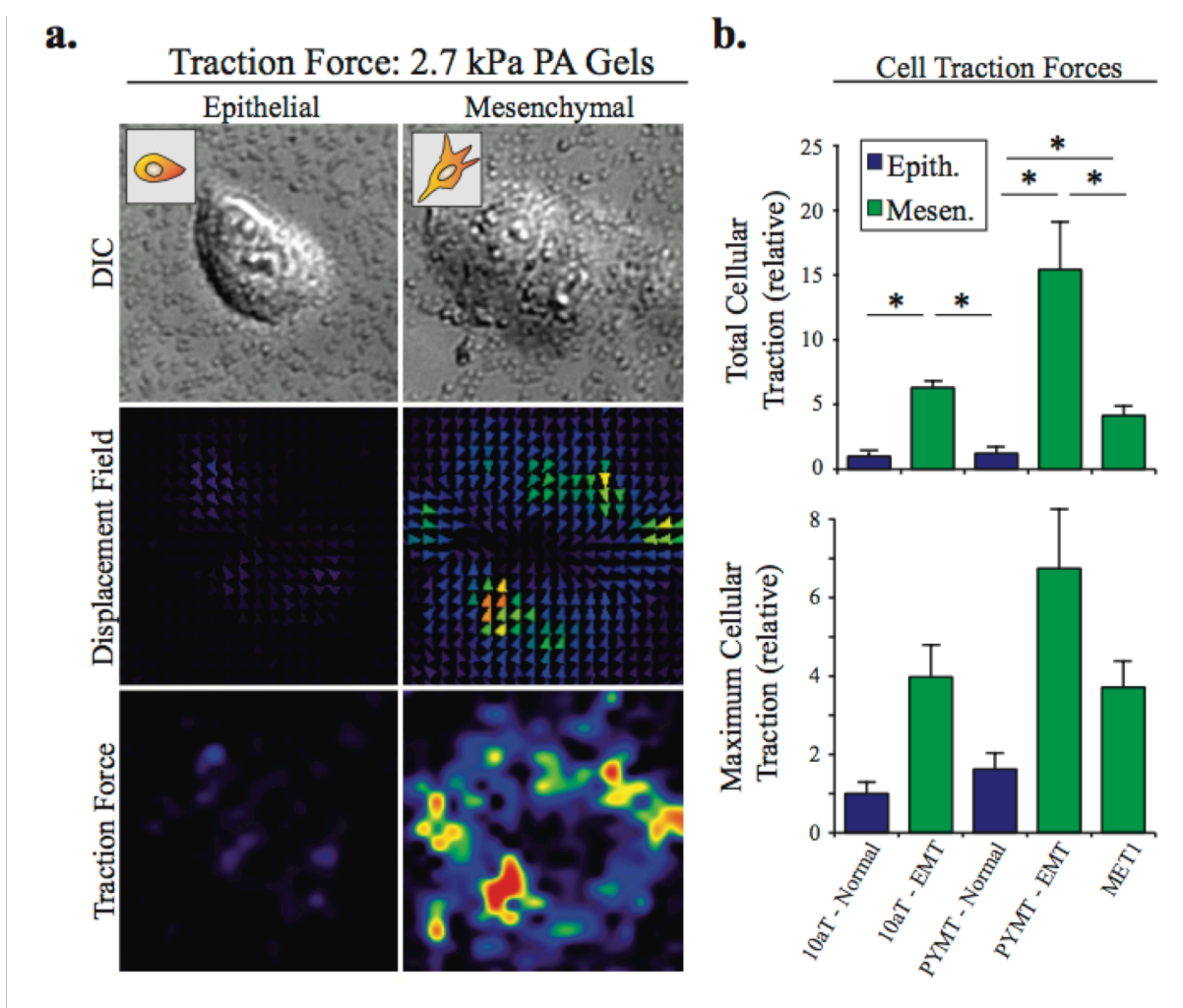


Fig 4.20. A. Left: MCF10a +/- TGF β to induce more mesenchymal-like behavior seeded on 2.7 kPa polyacrylamide (PA) gels conjugated with fibronectin and embedded with fluorescent beads for traction force microscopy. Displacement field is of beads before and after cell lysis, which is used to calculate traction forces. **B.** Quantification of traction force microscopy. At stiffness comparable to tumor ECM (~2.7kPa), metastatic cells versus tumorigenic cells or epithelial cells treated with TGF β to induce mesenchymal behavior are more spread and exert more traction forces on the environment.

Unlike KD2 fibroblasts MCF10a epithelial cells struggle to form adhesions or spread at disease level tissue stiffness, 2.7 kPa. However, when the MCF10a were treated with TGF β to induce mesenchymal-like behavior, we observed that paxillin F31/118 significantly impedes cell spreading and traction force generation (**Fig 4.21 A,B**). From these experiments we conclude that paxillin alternative splicing products cannot only regulate forces over integrin but external cell traction forces on the ECM.

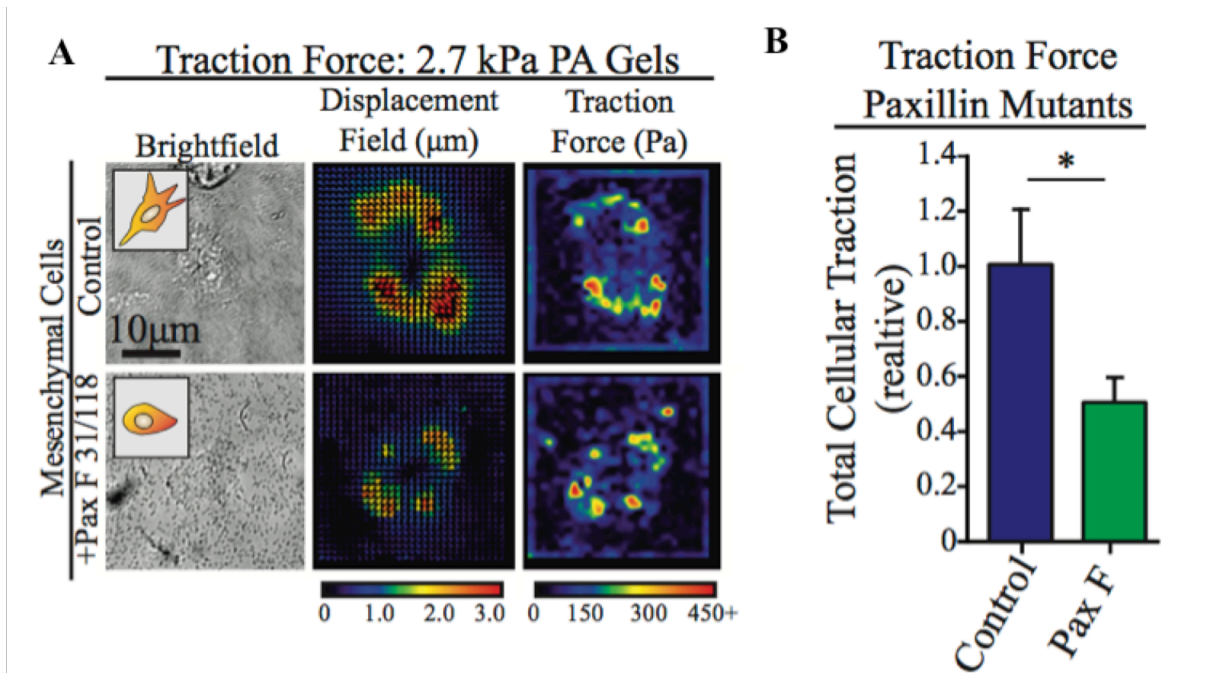


Fig 4.21. A. Traction forces of mesenchymal MCF10a + TGF β expressing control or Paxillin F 31/118 (synthetic epithelial) on 2.7 kPa polyacrylamide (PA) gels conjugated with fibronectin. Displacement field is of beads before and after cell lysis. At stiffness comparable to tumor ECM ($\sim 2.7\text{kPa}$), Paxillin F 31/118 impedes cell spreading and traction force generation. **B.** Quantification of total cellular traction. Paxillin F 31/118 significantly decreases total cellular traction force ($p < 0.05$, $n > 10$ cells).

4.3.13 Mesenchymal paxillin alpha regulates cellular adhesion and mechanosensing

We next explored the relationship between paxillin splicing, cell adhesion formation, and mesenchymal-like cell invasion. We predicted that mesenchymal paxillin-alpha compared to the epithelial paxillin-delta could drive elevated mechanosensing leading to cell adhesion and invasion in tumor-like microenvironments. As we had previously determined that paxillin splicing directly influenced cell behavior on disease like tissue stiffness, we tested if paxillin-WT or tyrosine deficient paxillin-F31/118 overexpression could override a cell's default response to different concentrations of the tumor ECM ligand fibronectin ³⁴².

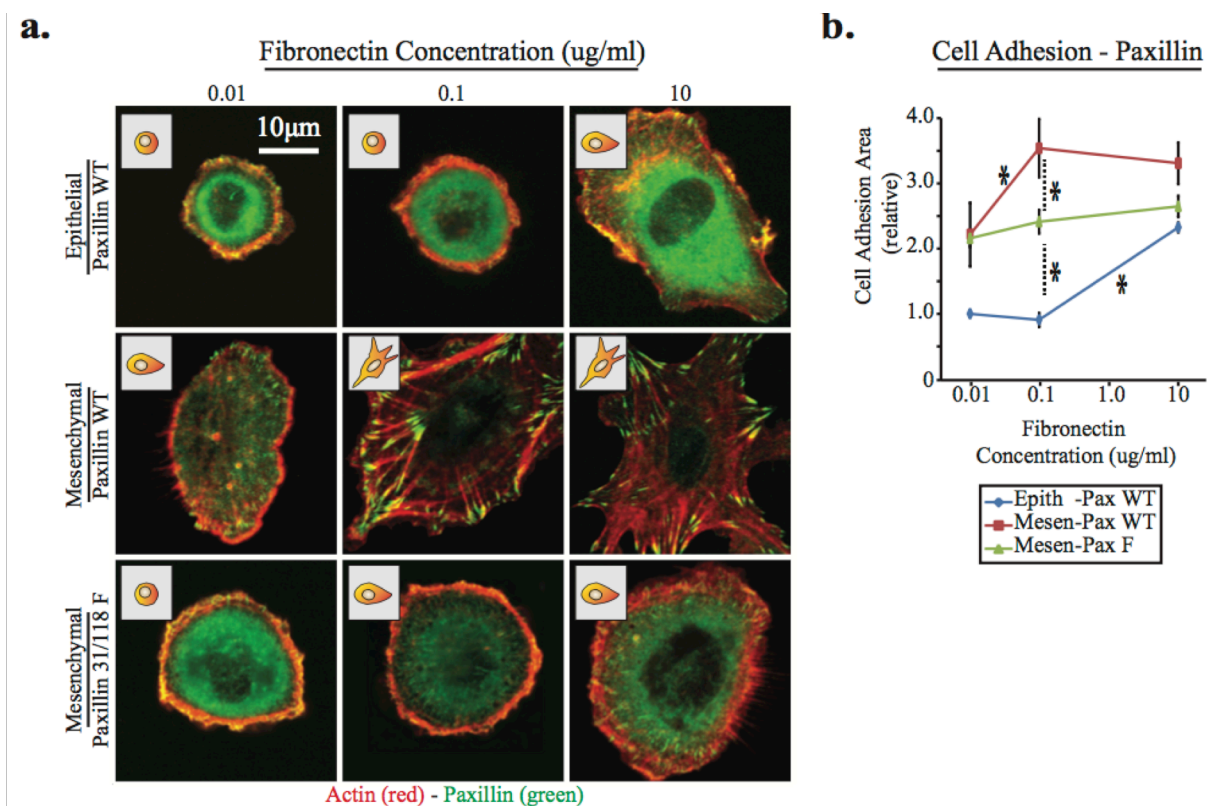


Fig 4.22. **A:** Cellular mechanosensing assay cover slips conjugated with 0.01, 0.1 and 10ug/ml fibronectin and imaged for adhesions (GFP-paxillin-WT or F31/118) and fibrillar actin (phalloidin). MCF10a cells expressing mesenchymal paxillin WT or epithelial paxillin F31/118, +/- TGF β to induce mesenchymal-like behavior. **B.** Quantification of cell adhesion area in fibronectin mechanosensing assay. Mesenchymal-like cells expressing paxillin-WT spread and form adhesions at medium and high ligand concentration significantly more than epithelial-like cells or mesenchymal cells expressing paxillin F ($p < 0.05$, $N > 20$ cells).

Epithelial MCF10a cells expressing paxillin-WT-GFP or paxillin-F31/118-GFP were treated with TGF β to induce mesenchymal-like behavior and seeded on cover slips conjugated with 0.01 (low), 0.1 (medium) or 10 μ g/ml (high) fibronectin; and stained with phalloidin to visualize filamentous actin (**Fig 4.22A**). We observed that mesenchymal-like cells expressing paxillin-WT were significantly more mechanosensitive to fibronectin concentration, as they spread and form adhesions at medium and high ligand concentration; while epithelial-like cells or mesenchymal cells expressing paxillin F were unable to fully spread and adhere (**Fig 4.22B**). To confirm that this mechanosensing phenomenon was not restricted to MCF10a, this experiment was performed with both epithelial 4T1 and mesenchymal-like 4T1 cells treated with TGF β , as well as metastatic Met1 cells treated with TGF β (**4.23 A**). Similar to the epithelial versus mesenchymal-like MCF10a, we found that Met1 or TGF β treated 4T1 cells expressing paxillin-WT spread and form more adhesions at medium and high ligand concentration than non TGF β treated cells, or mesenchymal cells expressing tyrosine deficient paxillin-F31/118 (**4.23B**).

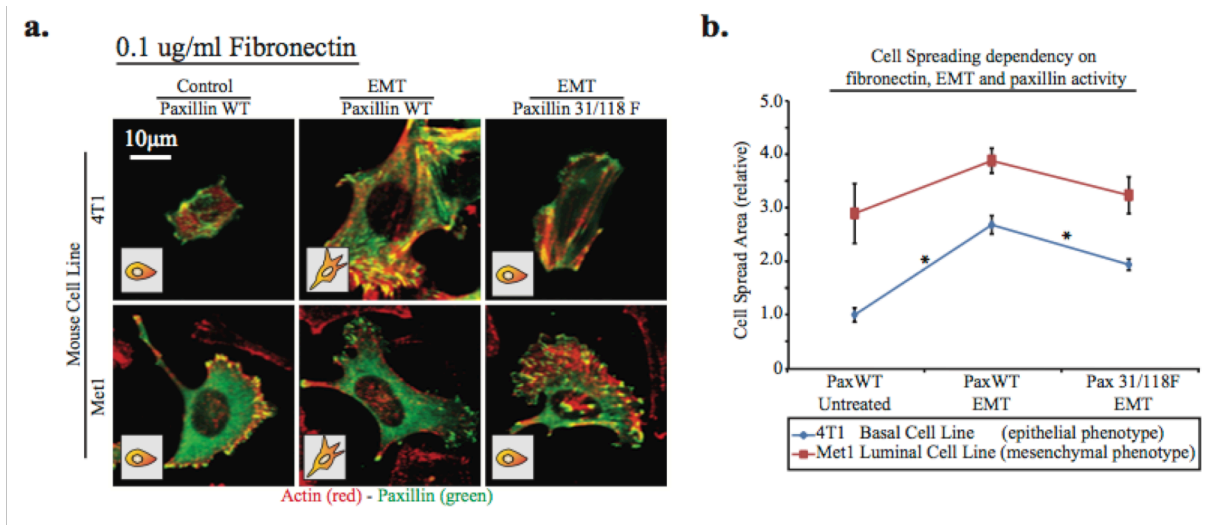


Fig 4.23. A. Cellular mechanosensing assay on cover slips conjugated with 0.1 ug/ml fibronectin and imaged for adhesions (GFP-paxillin-WT or F) and fibrillar actin (phalloidin). Met1 and 4T1 cells expressing mesenchymal paxillin WT or epithelial paxillin F, +/- TGF β to induce more mesenchymal-like behavior. **B.** Quantification of cell adhesion area in fibronectin mechanosensing assay. Met1 or 4T1 TGF β treated cells expressing paxillin-WT spread and form adhesions at medium and high ligand concentration significantly more than non TGF β treated cells or TGF β treated cells expressing paxillin F ($p < 0.05$, $N > 20$ cells).

4.3.14 Mesenchymal paxillin alpha regulates cellular invasion in metastatic cells

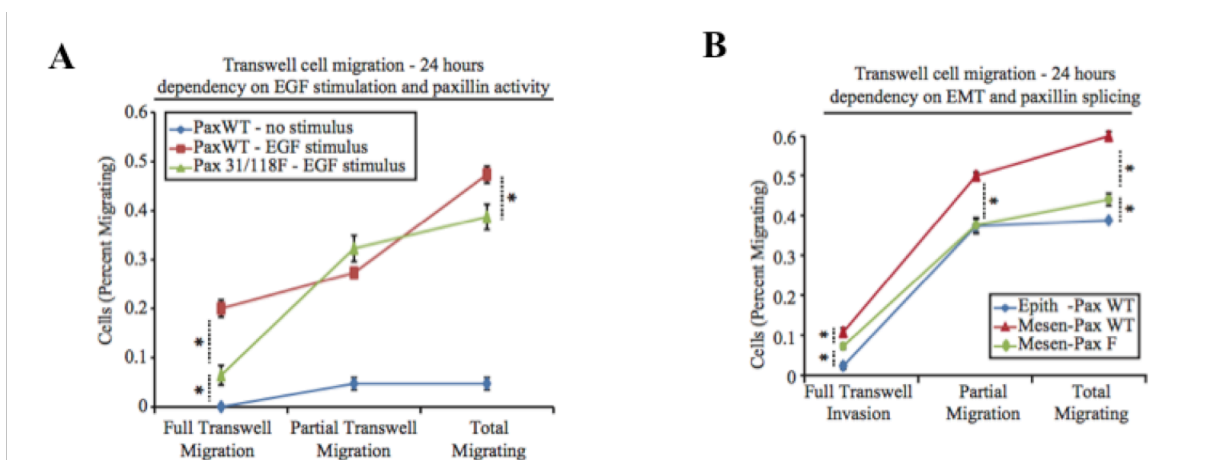


Fig 4.24. A. Transwell cell migration assay of MCF10a cells expressing mesenchymal paxillin WT or epithelial paxillin F, +/- TGF β to induce mesenchymal-like behavior, evaluated via confocal microscopy of cell position in transwell insert. Mesenchymal like MCF10a expressing paxillin-WT demonstrate significantly increased partial migration into transwell insert and complete migration to bottom of transwell ($p < 0.05$, $n > 20$ cells). **B.** Transwell cell migration assay of MCF10a cells expressing mesenchymal paxillin WT or epithelial paxillin F, +/- EGF (10ng/ml) to induce cell migration, evaluated via confocal microscopy of cell position in transwell insert. MCF10a expressing paxillin-WT demonstrate significantly increased complete migration into transwell insert compared to paxillin-F expressing cells and non-EGF stimulated cells ($p < 0.05$, $n > 20$ cells).

To explore the role of paxillin splicing and enhanced cellular mechanosensing in cancer associated environments, we implemented a 3D *in vitro* transwell assay that simulated epithelial wound repair and cell invasion through basal lamina layers ³¹⁹. MCF10a cells +/- TGF β expressing mesenchymal paxillin-WT or tyrosine deficient paxillin-F31/118 were seeded in transwell inserts and exposed with an EGF chemokine gradient to induce cell migration. We found that MCF10a overexpressing paxillin-WT have significantly increased complete migration into transwell insert compared to paxillin-F31/118 expressing cells and non-EGF stimulated cells (Fig 4.24A). Moreover, mesenchymal-like MCF10a have significantly increased partial migration into transwell insert and complete migration to bottom of transwell than epithelial MCF10a (Fig 4.24B).

To evaluate if paxillin splicing could effect survival and growth of metastatic cells in soft, lung-like ECM, we used an *in vitro* soft agar assay ³²⁰. We observed that Met1 cells expressing paxillin-WT had significantly increased colony size (Fig 4.25A), while 4T1 cells expressing paxillin-F31/118 had significantly decreased size (Fig 4.25B).

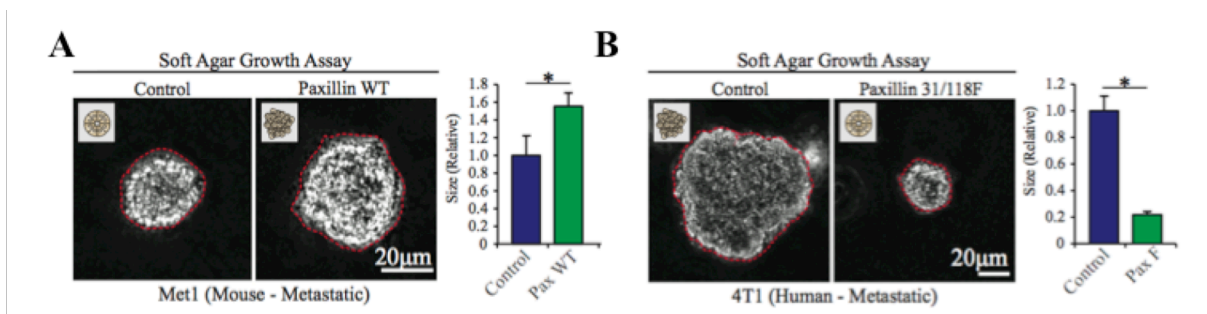


Fig 4.25. **A.** 21 day soft agar growth assay of metastatic Met1 cells, originally seeded as single cells expressing control vector or mesenchymal paxillin-WT. Quantification of cross-sectional-area demonstrates a significant increase in size in the paxillin-WT overexpressing cells ($p < 0.05$, $n > 30$ clusters). **B.** 14 day soft agar growth assay of 4T1 cells, originally seeded as single cells expressing control vector or epithelial paxillin-F. Quantification of cross-sectional-area demonstrates a significant decrease in size in the paxillin-F overexpressing cells ($p < 0.05$, $n > 30$ clusters).

We then employed a 3D collagen invasion assay to evaluate the ability of epithelial versus mesenchymal like cells to invade into the tumor microenvironment³³⁷. We found that paxillin WT overexpression significantly increased invasion compared to control or paxillin F overexpression as determined by cross-sectional area and single cell mesenchymal-like invasion (Fig 4.26C, D). This indicating that paxillin splicing plays a role in cellular plasticity and mechanosensing in cancer associated ECMs including basal lamina extravasation, stromal invasion and survival in a metastatic niche.

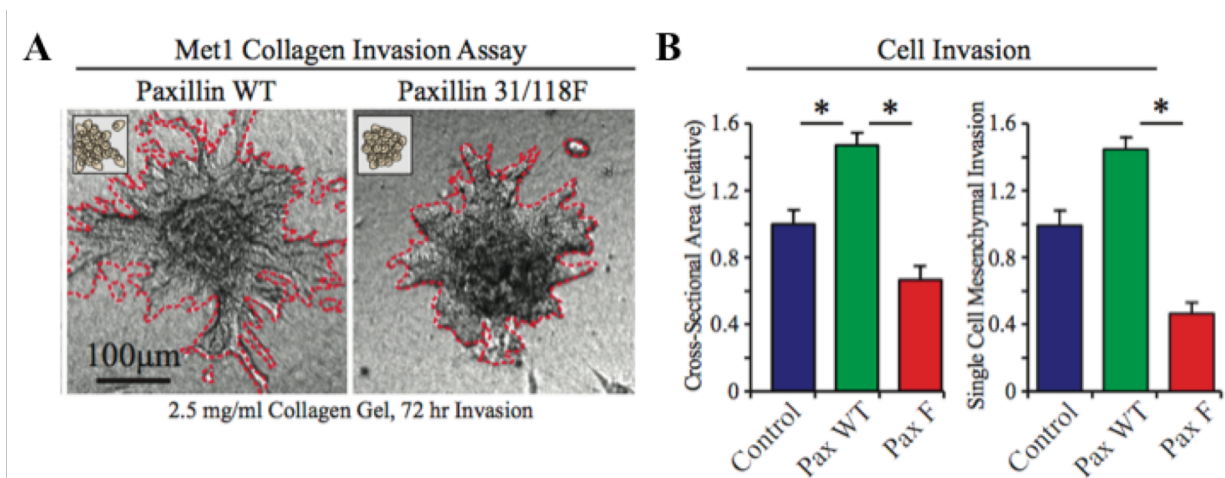


Fig 4.26. **A.** 3D collagen invasion assay of multicellular spheroids composed of metastatic Met1 cells expressing either mesenchymal paxillin-WT or epithelila paxillin 31/118F in 2.5mg/ml stiff 3D collagen gels for 72 hours. **B.** Quantification of invasion. Paxillin WT overexpression significantly increased invasion compared to control or paxillin F overexpression as determined by cross-sectional area and single cell mesenchymal-like invasion ($p < 0.05$, $n > 25$ spheroids).

4.3.15 Paxillin splicing alters cellular mechanotransduction and downstream signal transduction

Mesenchymal-like tumor cell invasion *in vitro* and *in vivo* is thought to require rhoGTPase and FAK activity^{343,344}. Moreover, the tumor microenvironment is known to enhance FAK activity and paxillin positive focal adhesion assembly in culture, and inhibiting either EMT or ECM cross-linking reduces signaling activity and tumor cell invasion^{7,13,305}. We therefore asked whether paxillin splicing increases mesenchymal-like cell invasion by increasing

mechanosensitive integrin reinforcement signaling and mesenchymal gene expression through a FAK/SRC/RhoA/ERK signaling axis.

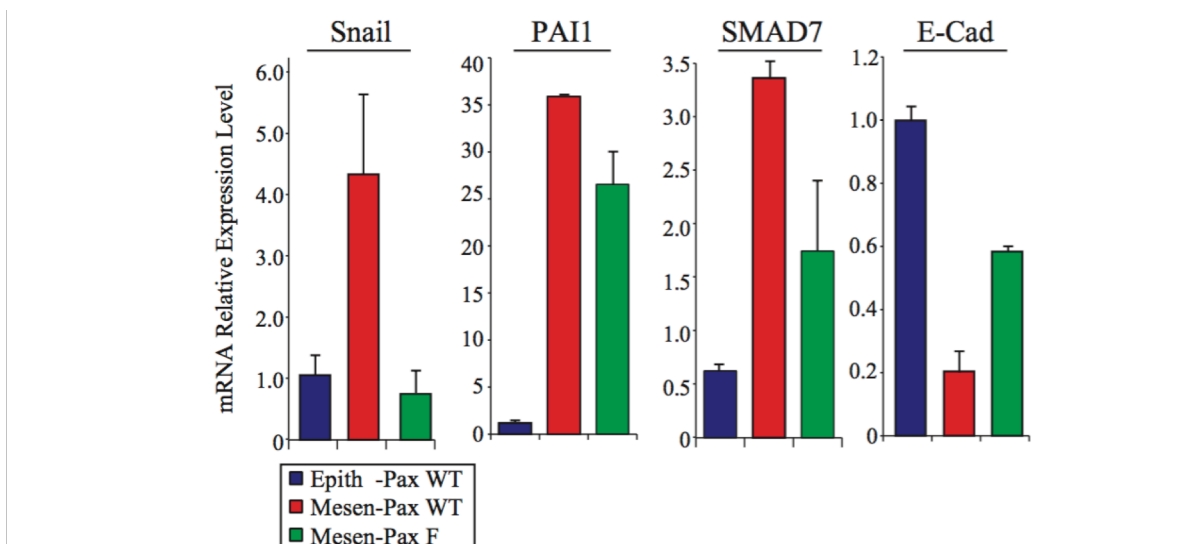


Fig 4.27 Gene expression of mesenchymal associated genes Snail, PAI1, and SMAD7 and epithelial marker E-Cadherin in MCF10a cells expressing mesenchymal paxillin WT or epithelial paxillin F, +/- TGF β to induce mesenchymal-like behavior. Paxillin WT and TGF β increase mesenchymal markers and paxillin F attenuates mesenchymal gene expression.

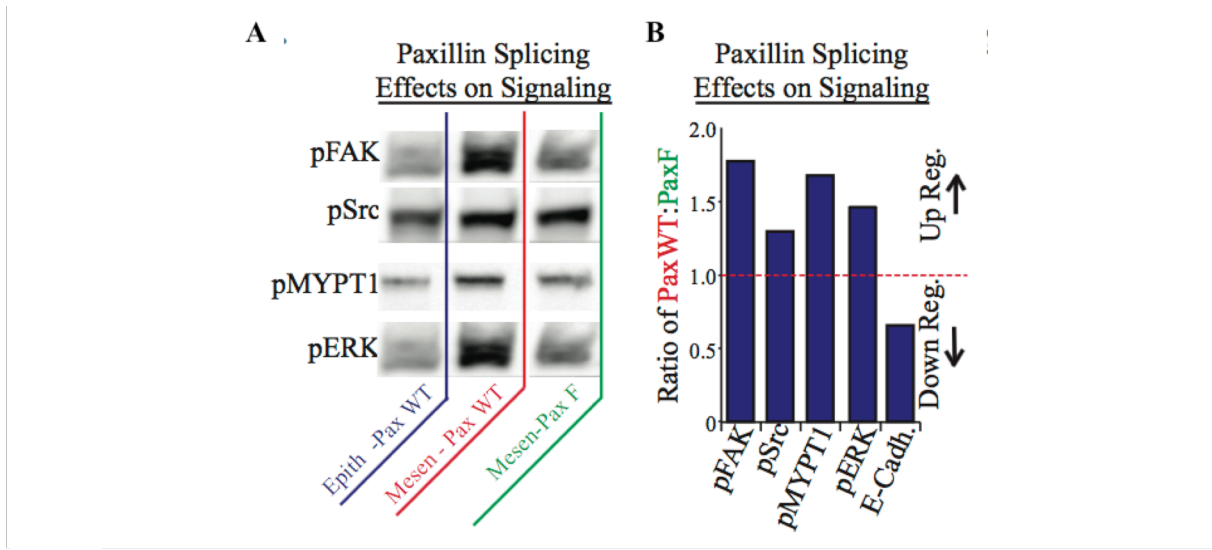


Fig 4.28 A. Paxillin splicing effects on signaling via western blots of phosphorylated proteins in MCF10a cells expressing paxillin variants +/- TGF β . **B.** Quantification of protein phosphorylation in mesenchymal cells expressing paxillin WT versus paxillin F. Paxillin WT expression increases signaling through FAK, Src, MYPT1 and Erk.

We first assayed MCF10a cells +/- TGF β expressing mesenchymal paxillin-WT or tyrosine deficient paxillin-F31/118 for gene expression of mesenchymal associated genes Snail, PAI1, and SMAD7 and epithelial marker E-Cadherin. We observed that mesenchymal MCF10a overexpressing paxillin-WT have increased mesenchymal gene expression and decreased epithelial gene expression compared to paxillin-F31/118 expressing cells and non-TGF β treated cells (**Fig 4.27**). We next assayed for oncogene and adhesion protein signaling via western blotting (**Fig 4.28A**). We found that mesenchymal paxillin-WT expression decreases total cellular levels of E-cadherin, increases signaling through FAK, Src, and Erk, as well as increased RhoA activity as seen through increased phosphorylation of MYPT1 auto-inhibition domains (**Fig 4.28B**). These data confirm that mesenchymal paxillin-WT splicing increases cellular mechanotransduction.

4.3.16 Paxillin mechanical signaling involves a FAK/Src/ROCK/MLCK/ERK protein transduction circuit.

To test the role of Src in cell invasion, we seeded metastatic Met1 multicellular spheroids in collagen gels for 72 hours, and treated with either DMSO, 20nM Dasatinib Src inhibitor (**Fig 4.29 A**), ML-7 myosin light chain kinase (MLCK) inhibitor or Rho Kinase (ROCK) inhibitor Y27632. We found that Src, ROCK, or MLCK inhibition significantly decreased cell invasion as determined by cross-sectional area and single cell mesenchymal-like invasion (**Fig 4.29 B**). These finding further support the presence of a paxillin/Src/RhoA signaling axis in human breast cancer patients.

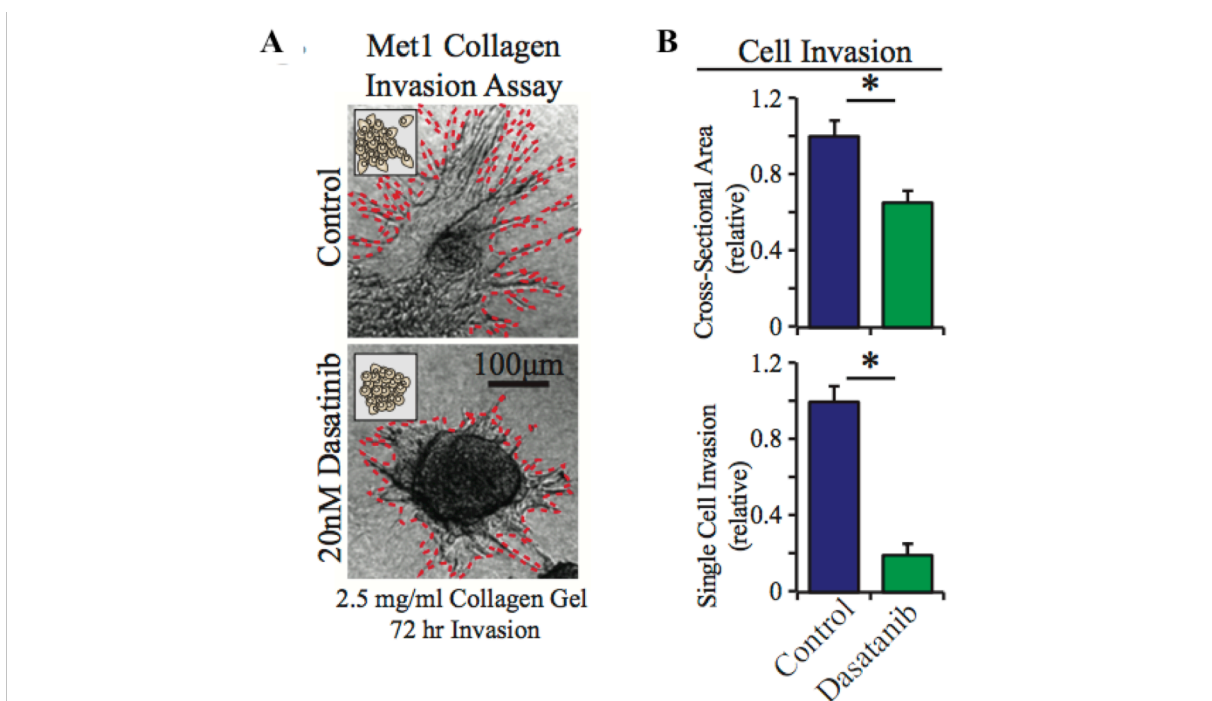


Fig 4.29 A. 3D collagen invasion assay of multicellular spheroids composed of metastatic Met1 cells treated with DMSO or 20nM Dasatinib Src/Abl inhibitor in 2.5mg/ml stiff 3D collagen gels for 72 hours. **B.** Quantification of invasion. Dasatinib addition significantly decreased cell invasion as determined by cross-sectional area and single cell mesenchymal-like invasion ($p < 0.05$, $n > 25$ spheroids).

As paxillin splicing plays a role in RhoA signaling, we tested how paxillin-WT and EMT effects Rac1 splicing and signaling¹⁷⁴. We first tested Rac1 activity via a Raichu-Rac1-GFP-RFP FRET activity sensor³¹³ in MCF10a +/- TGF β to induce mesenchymal-like behavior (**Fig 4.30A**). We found that while there are changes in Rac1 activity localization between epithelial and mesenchymal-like cells, we did not measure a significant difference in Rac1 activity on average across entire cells (**Fig 4.30B**). Furthermore, when we performed quantitative mRNA analysis of Rac1A and Rac1B gene expression, we found that while the mesenchymal-like phenotype induced additional Rac1B expression, paxillin splice variant expression did not affect Rac1A or Rac1B gene expression (**Fig 4.30C**).

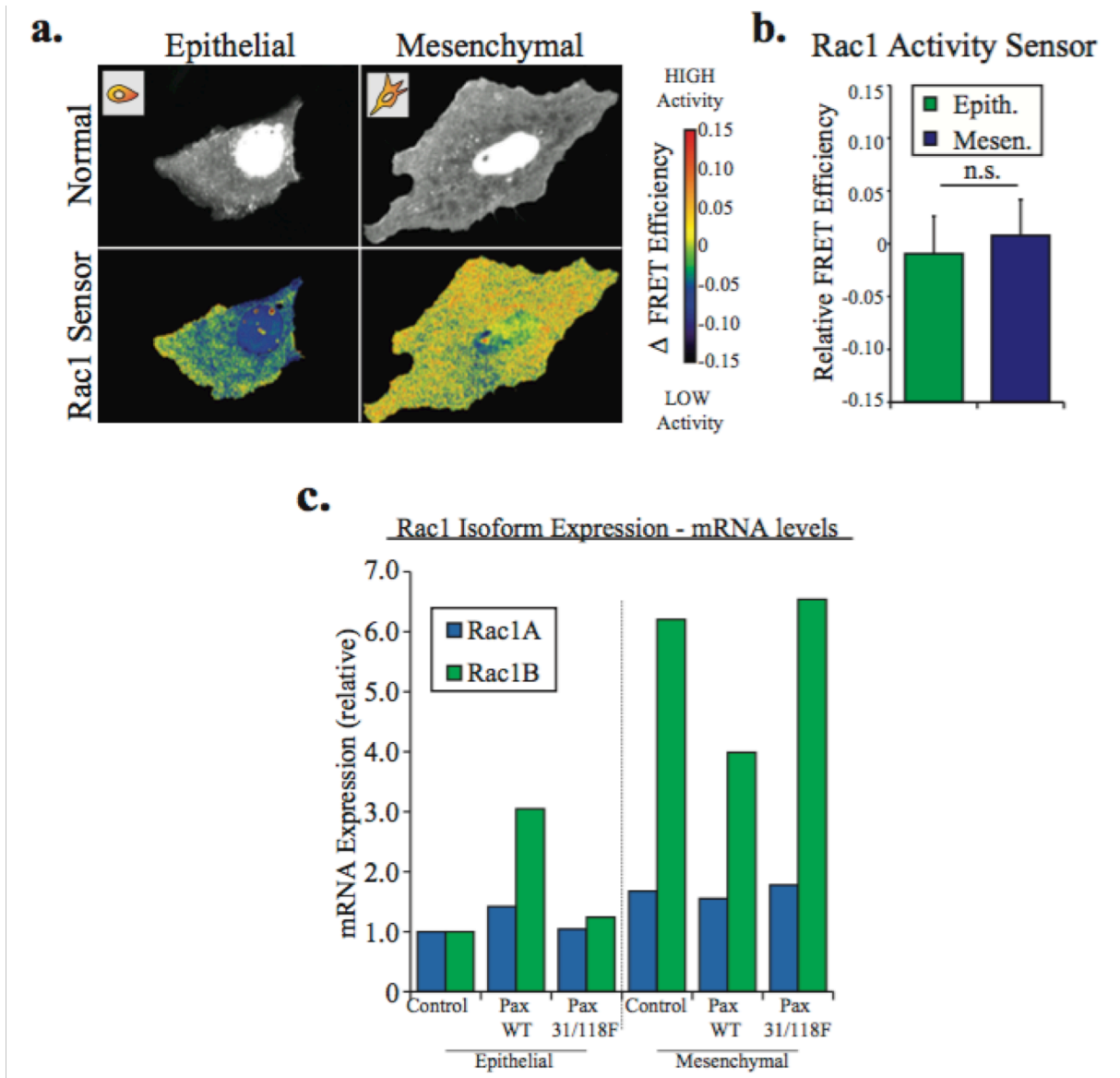


Fig 4.30 **A.** MCF10a cell expressing Raichu-Rac1-GFP-RFP activity sensor, +/- TGF β to induce more mesenchymal-like behavior. Activity of Rac1A as determined by relative FRET efficiency (high FRET indicates high activity) of GFP to RFP via photobleaching FRET method. Between epithelial and mesenchymal cells there appears to be changes in Rac1 activity localization. **B.** Quantification of average Rac1 activity across entire cells reveals no significant changes ($n > 10$). **C.** Gene expression of Rac1A and Rac1B rhoGTPases in MCF10a cells expressing mesenchymal paxillin WT or epithelial paxillin F, +/- TGF β to induce mesenchymal-like behavior. While TGF β increase Rac1B gene expression, there is not significant effects of paxillin splice variant expression on Rac1A or Rac1B gene expression.

4.3.17 Paxillin signaling and expression is a negative prognostic marker in human breast cancer

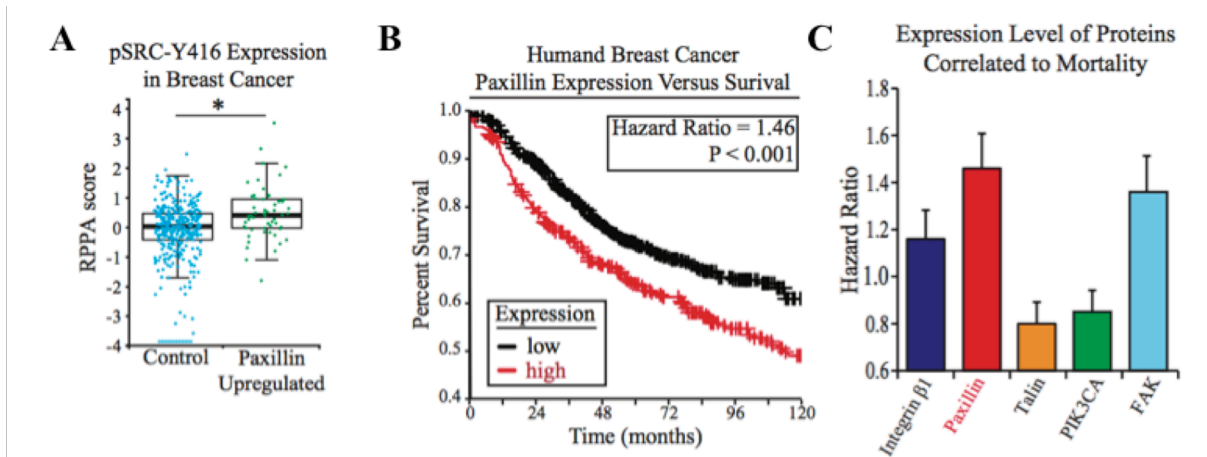


Fig 4.31 **A.** SRC phosphorylation levels via reverse phase protein array (RPPA) in TCGA breast cancer patients separated into control and paxillin upregulated patients. Paxillin upregulation is correlated with a significant increase in Src phosphorylation at tyrosine 416 ($p < 0.05$, $n > 4200$ patients). **B.** Kaplan meier survival curve of invasive breast cancer patients expressing either high or low paxillin. High paxillin level patients have a significantly worse chance of survival ($p < 0.001$, hazard ratio 1.46 at 10 years). **C.** Mortaility hazard ratio for breast cancer patients with high gene expression of several adhesion related proteins. Patients with high FAK as well as Paxillin expression have significantly higher risk of death.

As paxillin signaling and splicing plays a role in mesenchymal like cell invasion, we studied if there was significant association between paxillin and breast cancer progression. We asked if paxillin undergoes mesenchymal splicing in human invasive tumors and cell lines, and if paxillin over-expression significantly increasing patient mortality in human breast cancer. We first evaluated the paxillin-FAK-Src-RhoA-ERK signaling axis in breast cancer patient by conducting a bioinformatics study using The Cancer Genome Atlas (TCGA) data. We evaluated 1104 human breast cancer patients and found that patients with paxillin upregulation had significantly upregulated SRC-416 phosphorylation levels via reverse phase protein array (**Fig 4.31A**). We next employed the TCGA dataset along with the online survival analysis software to assess the role of paxillin on patient mortality³²¹. Invasive breast cancer patients were striated into patients that had cancer detected in the lymph nodes, then separated again into high and low paxillin

expressors. We found that high paxillin level patients have a significantly worse chance of survival; with a p-value <0.001 , and a hazard ratio 1.46 at 10 years (**Fig 4.31B**). When we analyzed the mortality hazard ratio for breast cancer patients with high gene expression of several adhesion related proteins we observed that patients with high FAK as well as Paxillin expression have significantly higher risk of death, but did not find that high expression alone of proteins including $\beta 1$ integrin or talin increased patient mortality hazard ratio (**Fig 4.31C**).

4.3.18 Paxillin splicing is a negative prognostic marker in human breast cancer

Lastly, we analyzed a proteome data set of triple-negative tumors and cell lines for the protein levels of different paxillin splice variants, including epithelial paxillin-delta and mesenchymal paxillin alpha³²². We found that not only is epithelial paxillin-delta significantly more expressed in non-invasive compared to invasive cell lines as in our own previous MCF10a data, but that mesenchymal paxillin-alpha is enriched in triple negative breast cancer tumors (**Fig 4.32A**). Thus, mesenchymal paxillin alpha isoform is required for EMT mediated cell plasticity through a Paxillin-FAK-SRC-RhoA-Erk signaling axis, and paxillin expression levels could be implemented as a prognostic marker for decreased survival (**Fig 4.32B**).

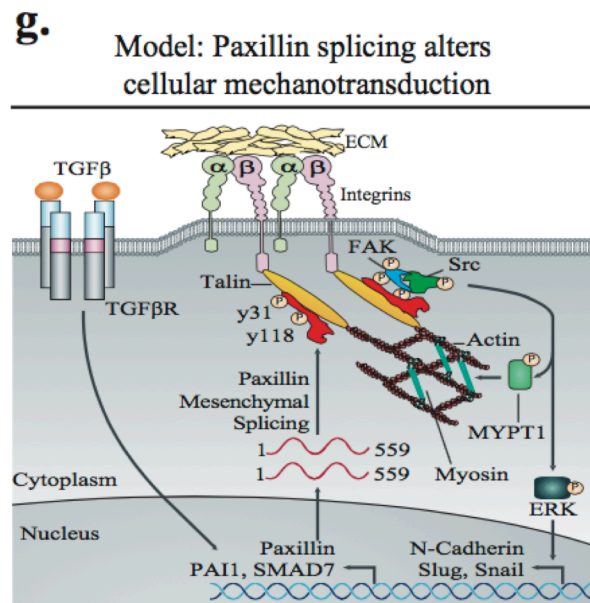
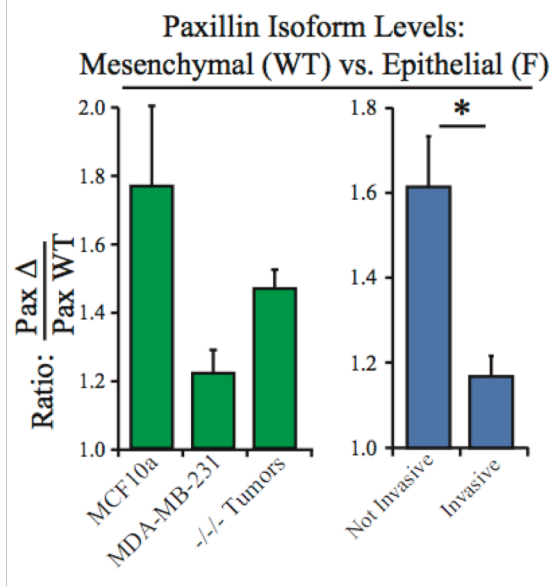


Fig 4.32 A. Paxillin delta (epithelial) versus paxillin alpha (mesenchymal) isoform protein levels in triple-negative tumors and cell lines via mass spectrometry protein analysis. Epithelial like paxillin delta (compared to mesenchymal paxillin WT) is at higher levels in non-invasive MCF10a cells compared to invasive MDA-MB-231 cell lines. In triple negative breast cancer tumors, the ratio of paxillin delta compared to WT, is at a level between MCF01a and MDA-MB-231. Additionally, paxillin delta is significantly more expressed in non-invasive compared to invasive cell lines ($p > .05$, $n > 5$ per condition). **B.** Model of paxillin splicing altering cellular mechanotransduction through Paxillin-FAK-SRC-MYPT1-ERK signaling.

4.4. Discussion

4.4.1. Paxillin, EMT, and Mechanotransduction

The tumor microenvironment and cellular mechanosensitivity are known regulators of malignant progression^{7,8,290}. Using human bioinformatics data, single and multicellular models of tumor invasion with paxillin mutants, super-resolution analysis of focal adhesion architecture via SAIM and 3B microscopy, as well as cellular mechanical measurements via traction and atomic force microscopy we find that epithelial to mesenchymal transition (EMT) induces alternative paxillin splicing that enhances cell plasticity in tumor-like ECM, driving cancer cell invasion. We establish that the mesenchymal paxillin alpha isoform is required for mesenchymal-like cell invasion and cell plasticity, indicated by increased cell spreading, traction and force per integrin on tumor-like ECM. Our data provides detailed molecular mechanism to explain how cells couple increased ECM stiffness to mesenchymal like adhesions and changes in cell signaling events^{13,20}. Our data illustrates how mesenchymal paxillin alpha mediates cell mechanosensation through a Paxillin-FAK-SRC-RhoA axis, facilitating oncogenic signaling through pathways including TGF β and ERK signaling networks^{203,300,303,309}.

4.4.2. Paxillin role as a mechanotransducer and biomarker

Our results emphasize mesenchymal paxillin alpha's role as not only a mechano-switch protein, but as a transducer of a cell signaling circuit through which cells interpret the extrinsic environment to drive altered intracellular signaling^{22,164,299}. Indeed, by demonstrating a critical role for paxillin splicing in mesenchymal-like single cell invasion and survival in invasive breast cancer patients, we provide the first definitive evidence that paxillin-alpha mesenchymal splicing

can be used as a simultaneous prognostic biomarker for cellular mechanotransduction in cancer and malignant disease progression.

4.3. Importance of studying paxillin at the nano, micro and macro scale in 3D

While most available work on cell adhesion and invasion has been performed in 2D systems, our investigations relates and furthers existing cell mechanobiology knowledge to cell behavior in a 3D physiological context at multiple length scales^{13,21,285,337}. We combine studies of nanoscale molecular interactions, multicellular *in vitro* cultures and human bioinformatics analysis to provide a broad view of how cells can plastically employ adhesion molecules to respond differently to normal or disease like ECMs^{13,345,346}. On the computational level, we have improved upon existing motor-clutch cell adhesion simulations^{175,330,331}, by introducing a model that is tunable to cell parameters including the degree of integrin reinforcement, the strength of catch and slip bonds, and the number of motors and clutches. These improvements allow us to use knowledge of adhesion properties and our empirical results to make future predictions of cell behavior on different stiffness matrices; as well as in a cell with variable integrin and myosin composition or a cell with altered integrin reinforcement feedback^{340,341}. Our model allowed us to prospectively study paxillin-mediated integrin reinforcement and myosin contractility, and gave us predictions that integrin positive feedback would increase cell plasticity at disease level tissue stiffness and cause molecular level changes in forces per integrin. Future improvements in the model can now be made to firmly connect molecular protein kinetics to changes in cell behavior including migration and invasion in 2D and 3D environments.

4.4.4. Paxillin nano-architecture

Our experimental data at the nanoscale to cellular level establish mesenchymal paxillin-alpha as a critical molecule in mechanosensation and mechanotransduction. Intriguingly, we observed that while most constituent adhesion proteins retained their organization between epithelial-tumorigenic and mesenchymal-metastatic cell populations, the signaling protein paxillin undergoes a pronounced increase in height of ~15 nm. While super-resolution SAIM microscopy does not firmly confirm change in binding partner, this limitation can be overcome with mass spectrometry studies of genetically encoded biotin ligase probes ³⁴⁷. Thus the paxillin height change that we observe most likely plays a role in inducing cell signaling that drives the highly invasive and motile phenotype characteristic of mesenchymal-like cells. This provocatively suggests similar mechanism of nanoscale architecture regulation for other plaque proteins with scaffold functions including mechanical proteins talin and vinculin, or possibly signaling platforms including p130cas or zyxin ^{218,290,338,348}. Moreover, our data verifies that integrin reinforcement through paxillin or mesenchymal transition can increase force across single integrins dictating cell adhesion and spreading as previously postulated ^{170,317,349}.

4.4.5. Paxillin dependent mechano-sensitivity can drive cell invasion

At the multicellular level, our data suggest that cells undergoing mesenchymal-like single cell invasion or leader invading cells employing cell-ECM adhesion, employ paxillin signaling through tyrosines 31 and 118 to transduce signaling directly through FAK and Src, as well as indirectly through a RhoA-ERK signaling network ^{299,302,344}. More broadly, our results confirm the importance of mechanosensitivity in tumor like microenvironments and demonstrate the functional importance of paxillin positive cell adhesions by demonstrating that they can regulate

growth factor signaling and cellular plasticity^{8,20,286}. Indeed, our data showed that neither mesenchymal like cell behavior or overexpression of the paxillin-alpha isoform are sufficient for cell invasion, suggesting that cellular mechanosensation collaborates with key oncogenic pathways to drive malignant transformation. Thus our investigations support previous findings that paxillin downregulation decreases invasion and metastasis in a mouse model of breast cancer lung metastasis, and that paxillin upregulation is correlated with tumor progression and decreased survival in gastric cancer^{350,351}.

4.4.6. Future *in vitro* and *in vivo* studies

As we observed paxillin mesenchymal splicing in human invasive tumors and cell lines, and determined that paxillin over-expression significantly increasing patient mortality in human breast cancer, we conclude based on our and other's data that paxillin is a biomarker for negative outcomes in human breast cancer, and potentially in gastric and colorectal cancer among others^{299,300,350–352}. Future work is now needed to identify the entire circuit of proteins in the cellular mechanotransduction pathway turned on by the mesenchymal paxillin-alpha splice form. Notably, from our studies we have already identified and tested the role of Src-inhibition in tempering 3D mesenchymal-like invasion of metastatic cells, and found that the Src/Abl small molecule inhibitor dasatinib at 10nM can strongly attenuate invasion. This confirms previous finding that dasatinib can be used to treat triple-negative tumors that are often mesenchymal in behavior, and resistant to epidermal growth factor receptor (EGFR) signaling inhibition^{296,353,354}. Moreover future studies could be used to identify novel proteins involved in protein level splicing and alternative translation of paxillin and these could be targeted for therapeutic treatment^{23,355}. Consequently, therapies that target paxillin mesenchymal splicing, altered

cellular mechanosensation, or downstream FAK-Src-RhoA-Erk signaling, could have great utility in the treatment of breast tumors that are mesenchymal in phenotype and not responsive to growth factor receptor inhibition^{354,356}.

4.4.7 Contributions

Matthew Rubashkin and Valerie Weaver conceived and initiated the project. Matthew Rubashkin, Valerie Weaver, Alexander Dunn and Armen Mekhdjian designed experiments. Matthew Rubashkin and Michael Pickup performed the data analysis on the human bioinformatics data. Matthew Rubashkin, Luke Cassereau and Fui boon Kai performed the single and multicellular experiments of tumor invasion. Matthew Rubashkin, Christopher Dufort and Guanqing Ou performed the SAIM super-resolution analysis. Stevan Tan and Armen Mekhdjian performed the 3B super-resolution experiment and analysis. Armen Mekhdjian and Matthew Rubashkin performed the integrin force sensing experiments. Matthew Rubashkin and Matthew Barnes performed the atomic force microscopy experiments. Matthew Rubashkin performed the traction force experiments. Janna Mouw, Michael Pickup and Matthew Rubashkin performed the biochemistry experiments. Armen Mekhdjian performed the BirA proteomics experiments. Michael Davidson, Jonathan Lakins and Fui boon Kai designed and built fluorescent protein constructs. Louis Prah, David Odde and Matthew Rubashkin designed and implemented the *in-silico* motor-clutch model of cell adhesion. Matthew Rubashkin Armen Mekhdjian, and Louis Prah prepared the figures with assistance from Valerie Weaver, Alexander Dunn and David Odde. Matthew Rubashkin and Valerie Weaver wrote the manuscript with editorial assistance from all authors.

4.4.8 Acknowledgements

I graciously thank the team that worked on the paxillin project, Armen Mekhdjian, Louis Prah, , Janna Mouw, Fuiboon Kai, Christopher Dufort, Guanqing Ou, Matthew Barnes, Luke Cassereau, Steven Tan, Michael Pickup, Jonathan Lakins, Michael Davidson, David Odde, Alexander Dunn, and Valerie Weaver. We would like to thank Qingzong Tseng for providing traction force microscopy software and assistance, Alice Chang and Masatoshi Morimatsu for assistance with integrin force sensing experiments, Anne Ridley and Tony Ng for Rac1 Raichu FRET biosensor, as well as Allan Bradley for his hyperactive piggyBac transposase. Additionally, we would like to thank Scott Vande Pol for the paxillin null KD2 fibroblast cell lines, Zhenjie Xu and Zena Werb for the 4T1 cell line, and Harold Moses for the PYMT-Flox3 and Met1 cell lines.

4.4.9 Funding

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Chapter 5. Future Directions and Conclusion

5.1. Ongoing Projects

5.1.1 Intersection of glycobiology and mechanobiology

The cell glycocalyx, the carbohydrate coating around cells which includes the glycoprotein Mucin family such as Muc1, has been implicated in cell proliferation, migration, invasion and metastasis^{357–361}. An example of the importance of the glycocalyx is the high levels of Muc1 found in breast tumors. Muc1 is found in over 90% of breast cancers and dysregulation of Muc1 cellular localization can lead to tumor progression^{360,362,363}. However, the role of Muc1 as it relates to cell mechanics in cancer is not adequately understood.

In order to understand the biology of Muc1, Matthew Paszek of the Hammer lab in collaboration with the Weaver lab had modeled integrin activation in the context of a variable glycocalyx³⁶⁴. Important to note is that while integrin heterodimers are ~15nm in length, the cell glycocalyx on the outside of an epithelial cell can vary in length from 10 to over 100nm; therefore requiring deformations of the plasma membrane, compressions of the glycocalyx or reorganization of the glycocalyx in order for integrins to engage with their ligand^{330,364,365}. Muc1 is a perfect example of a large glycoprotein in the glycocalyx, consisting of a short intracellular signaling domain, and a very large extracellular domain of repeating O-glycosylation domains. When Muc1 is highly glycosylated, it is predicted that the Muc1 extracellular domain would adopt a rigid rod formation of ~100 to 150nm in length^{364,365}. Therefore this long rigid rod could serve to regulate integrin adhesion formation.

Integrin activation is a two-step process (**Fig 5.1**)^{14,188,364,366}. First, integrins must find and bind to an extracellular matrix ligand. This is a slow process. Second, if enough force is placed over integrins, either through intracellular actin-myosin contractility or through glycocalyx resistance to deformation, integrins

are locked into an activated confirmation that resists detachment from an extracellular ligand. Due to deformation in the actin cytoskeleton, integrins can now more easily locate extracellular ligands, increasing the speed of integrin-ligand interactions, a fast kinetic trap. To Investigate the role of the cancer glycocalyx in cancer cell adhesion and survival we assayed integrin adhesion formation in the prescence of Mucin1 overexpression as well as introduction of synthetic glycopolymers produced by Carolyn Bertozzi's group at UC Berkeley ³⁶⁷. We predicted that longer glycopolymers such as Muc1 would increase cell adhesion by stimulating integrin activation. We found that long (80nm) glycopolymers increase adhesion formation and alter membrane topology of non-adhesion area (**Fig 5.2A, C**). Interestingly, we also found that large glyco-mimetics actually slowed down the intial rate of integrin binding, which we attributed to the increased difficulty of an integrin finding a ligand through a thick glycocalyx mesh (**Fig 5.2B**).

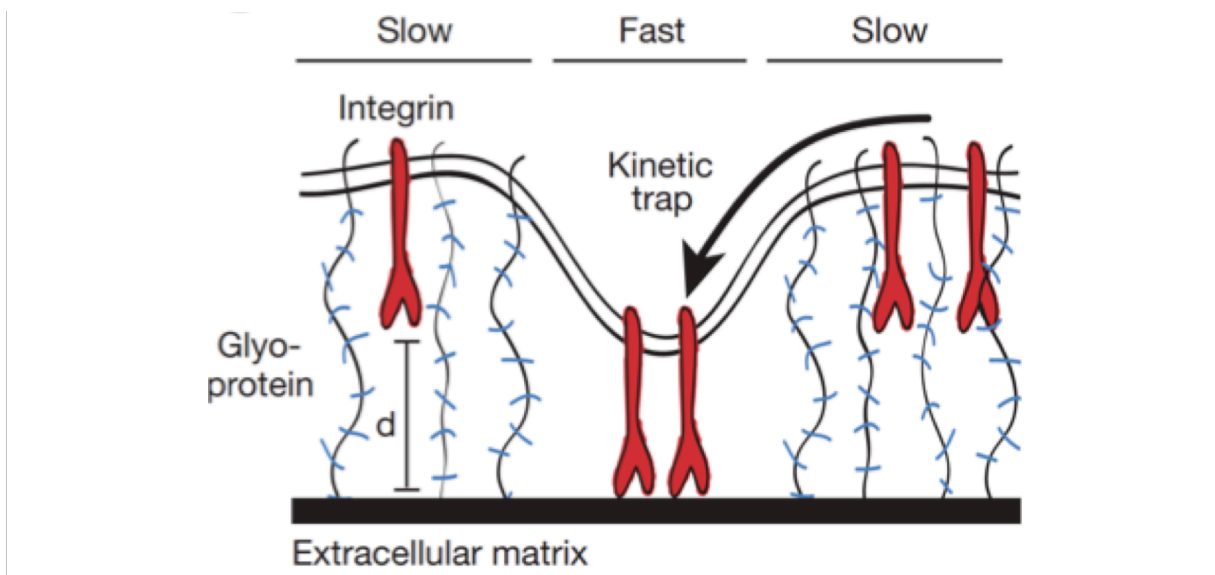


Fig 5.1. The glycocalyx mediates integrin activation. Integrin activation is a two-step process of ligand binding, and force activated stabilization. A large glycocalyx layer can enhance integrin binding through kinetic trapping of integrin molecules and increased force activated stabilization.

Figure prepared by Matthew Paszek

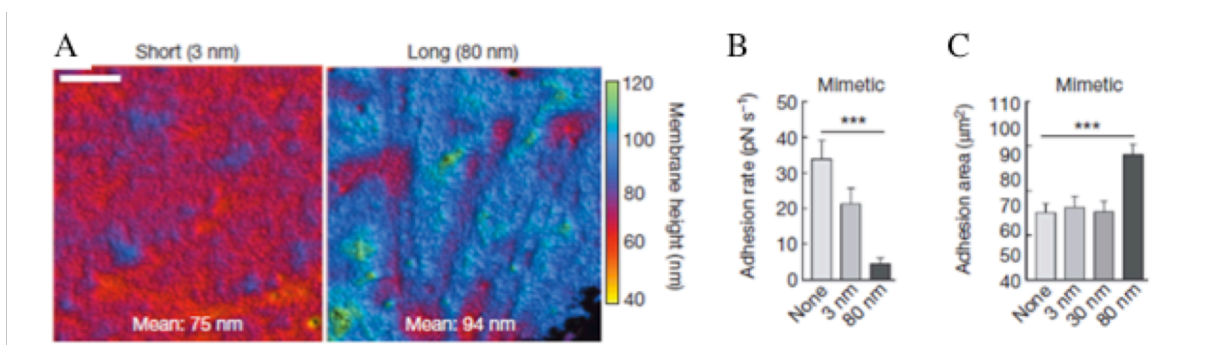


Fig 5.2. Glycocalyx mediated alterations to adhesion architecture, kinetics and organization. **A.** Epithelial MCF10a cells loaded with either short or long synthetic glycopolymers. SAIM images of membrane height demonstrate that long glycopolymers increase the mean membrane height to ~20nm higher above the extracellular matrix. **B.** Atomic force microscopy adhesion rate experiment, wherein a AFM cantilever is used to measure adhesion formation rates of cells loaded with different glycopolymers. Long polymers decrease adhesion rate **C.** Quantification of adhesion area of cells again loaded with different glycopolymers. Long polymers increase adhesion area.

Figure prepared by Matthew Paszek and Matthew Rubashkin

We also found that the cancer glycocalyx mechanically primes integrin-mediated growth and survival, by exerting force on integrin bonds, which in turn drives integrin signaling that increases cell survival in minimally adherent environments. We determined that the glycocalyx exerts force on integrin bonds by measuring the force over individual glycoproteins, and through Newton's first law, conclude that this force would reciprocally push on the plasma membrane loading integrin that would be stretched under tension. My contribution to this project was development of a Muc1 compression sensor and respective FRET and fluorescence-lifetime imaging that was used to measure glycoprotein compression. For the FLIM imaging, I developed the following protocol in collaboration with Holly Aaron at the UC Berkeley Microscopy Imaging Center. Time-domain fluorescence lifetime imaging microscopy (FLIM) was implemented with an inverted Zeiss LSM510 Axiovert 200M microscope with a Plan NeoFLUAR 40x/1.3 DIC oil-immersion objective lens, equipped with a TCSPC controller (SPC-830 card; Becker & Hickl, Berlin, Germany) as described previously³⁶⁸. mTrq2 was excited with 440 nm light generated by frequency doubling of 880 nm pulses from a mode-locked Ti:Sapphire laser (Mai-Tai, Spectra-Physics, 120-150 fs pulse width, 80 MHz repetition rate, and Frequency Doubler and Pulse Selector, Spectra-Physics, Model 3980). The emission light was passed through a NFT 440 beamsplitter, directed to the

fiber-out port of the confocal scan-head, filtered with a 480BP40 filter (Chroma Technology, Rockingham, VT) and detected by a PMC-100 photomultiplier (Becker & Hickl). The pinhole was set to give an optical slice of $< 4.0 \mu\text{m}$. Images of 386×386 pixels were averaged over < 120 seconds. Data analysis to produce an intensity image and a FLIM image was done off-line using the pixel-based fitting software SPCImage (Becker & Hickl), assuming double exponential decay during the first 8.5 ns of the 12.5 ns interval between laser pulses. Images were scaled to 256×256 pixels and no binning was used. Lifetime distributions were calculated for a masked portion of the FLIM image, generated with a triangle algorithm threshold of the photo count intensity image.

These findings were published in Nature in 2014, and will be used in the future to investigate glyocalyx mediated cancer cell invasion and metastasis in several different cancers including breast, pancreas and brain^{14,369}.

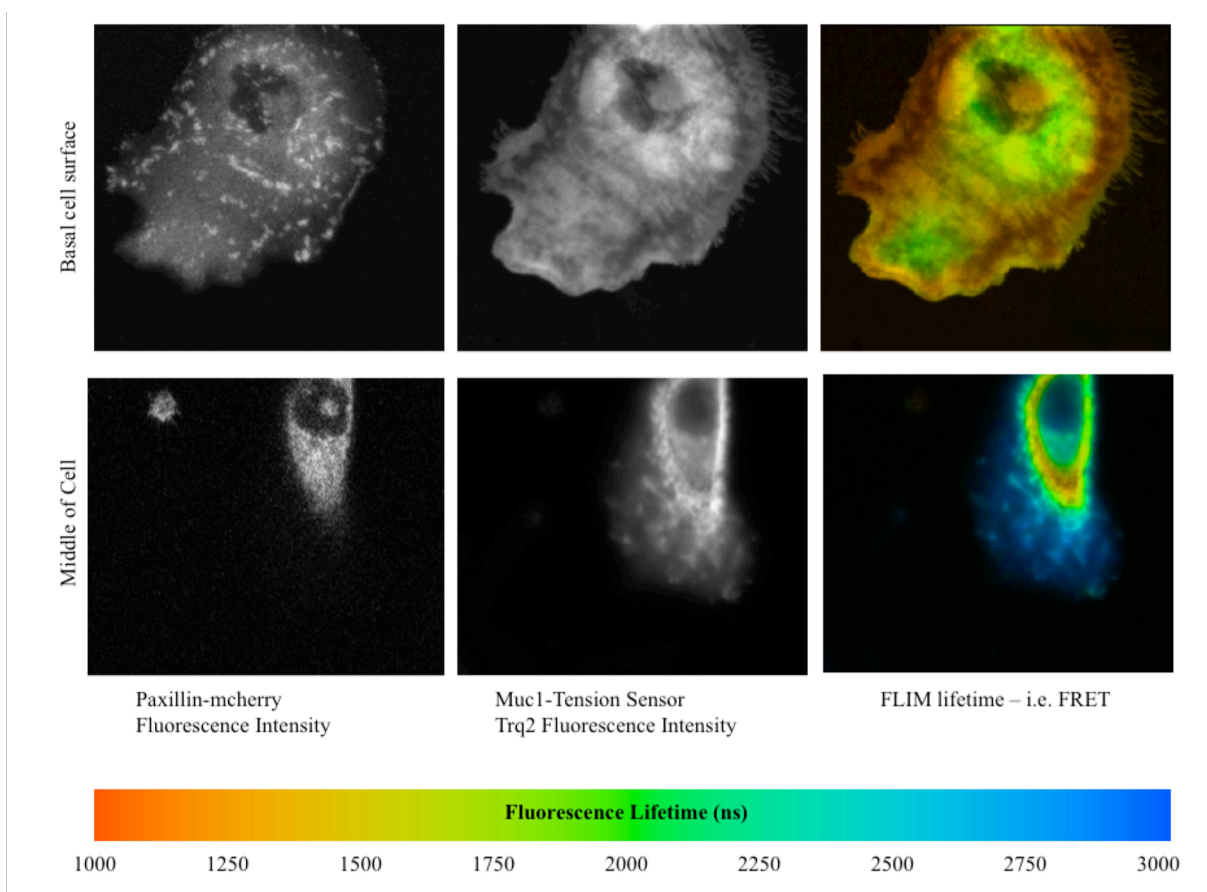


Fig 5.3. Glycocalyx Mucin1 Compression Sensor. The sensor consists of Muc1 with a mTrq2 – entropic-spring – mVenus between the plasma membrane and extracellular domain. Shorter fluorescence lifetime indicates the fluorescent probes are closer together and the molecule is under compression. Shorter lifetimes with higher compression can be found in adhesions (as indicated by paxillin-mCherry). Longer fluorescent lifetimes can be seen in the probe that is localized to the cell cytoplasm or non-ventral (bottom) cell membrane.

Figure prepared by Matthew Rubashkin

5.1.2 Nanoscale architecture of talin

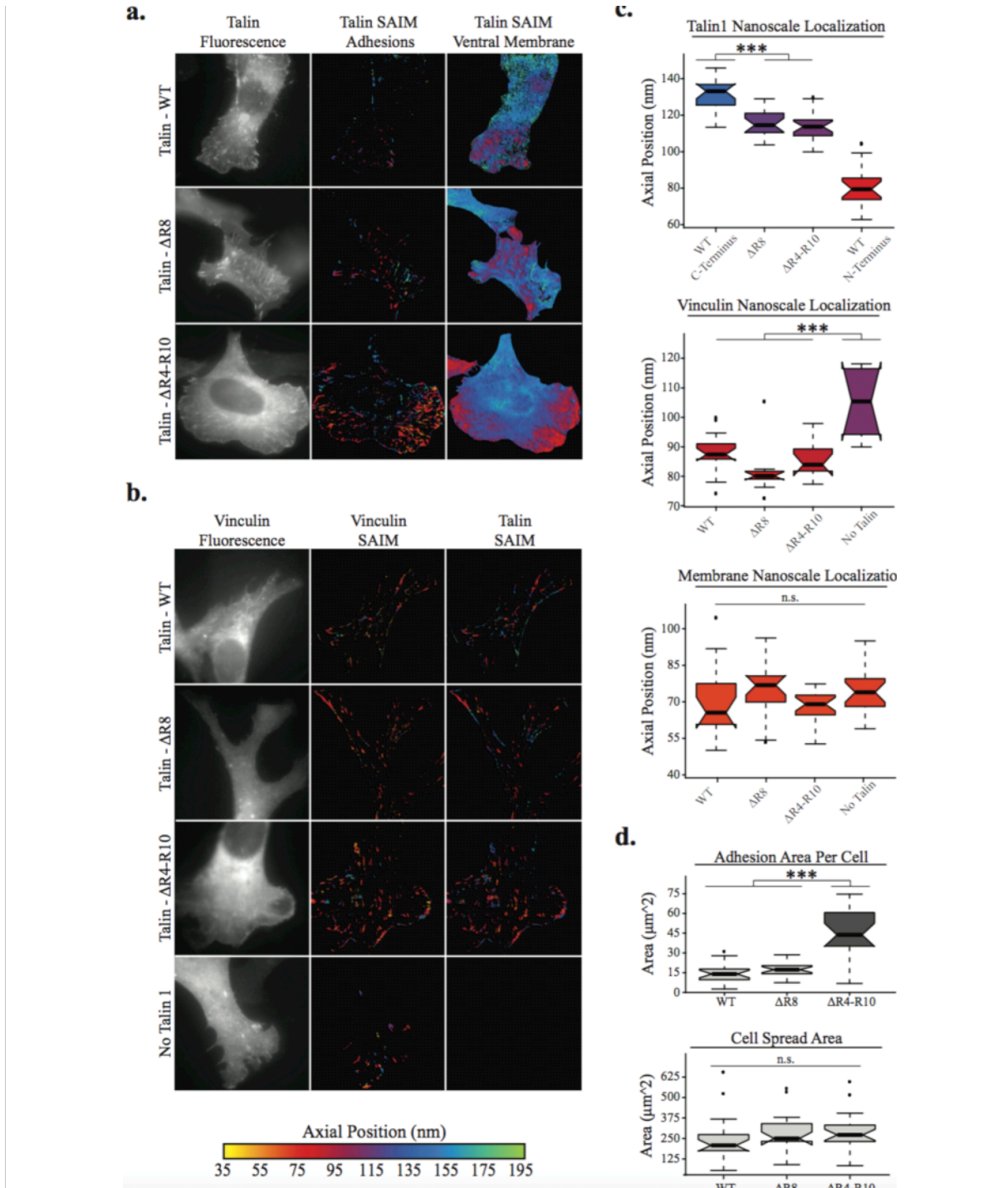


Fig 5.4. Talin adhesion architecture. **A.** MCF10a epithelial cells expressing talin mutants conjugated to GFP and vinculin-mCherry for adhesion visualization. Adhesions are identified via fluorescence microscopy, and SAIM microscopy is implemented to determine talin and vinculin axial position. **B.** Talin $-/-$ fibroblasts expressing talin mutants and vinculin-mCherry, analyzed via SAIM microscopy. **C.** Talin, vinculin and membrane nanoscale position. **D.** Adhesion area per cell as calculated by fluorescence masking of adhesions and whole cell area.

Figure prepared by Matthew Rubashkin

Alongside vinculin (Chapter 3) and paxillin (Chapter 4) signaling in focal adhesions, talin plays a key role in adhesion formation, force engagement and maturation^{177,180}. Moreover, adhesion signaling is influenced by talin engagement and vinculin binding to talin, driving cell adhesion and growth factor receptor signaling^{182,194}. To study talin activity, we studied talin nanoscale architecture in adhesion formation (**Fig 5.4 A,B**). Similar to previous findings using iPALM microscopy, we first found that the talin N and C terminus were separated by ~40nm (chapter 2)^{170,218,251} (**Fig 5.4 C**). Following those results, we implemented talin mutants developed by David Crichtly and Benjamin Goult to test the role of different talin subdomains on talin function and adhesion architecture.

We found that expression of talin1 WT, Δ R8 (talin mutant lacking the R8 domain) or Δ R4-10 (talin mutant lacking the domains R4 through R10)³⁷⁰ is requisite for vinculin localization at ~100nm, a position which corresponds to an 'active' simultaneous engagement of talin and actin (**Fig 5.4 A,B**). Without these talin1 constructs, vinculin is localized to a height of ~120nm, corresponding to an 'inactive' confirmation and weak interaction with actin alone (**Fig 5.4 B**). We also observed that the C-terminus of talin was lowered by ~20nm in Δ R8 and Δ R4-10 mutants, in comparison to talin1 full length. Outside of adhesions, un-engaged talin1 of all varieties was observed at ~150 nm.

In addition we found that expression of Δ R4-10 increased total adhesion area of cells in comparison to talin1 WT or talin1 Δ R8 expression, while I did not measure a significant difference in WT compared to Δ R8. We predict this is because talin1 Δ R8 still has an auto-inhibition domain, not greatly changing its ability to foster adhesion development, converse to Δ R4-10. We did not observe a significant change in average cell spread area between talin1 WT, Δ R8 or Δ R4-10 expression. Additionally we did not observe changes in the position of the plasma membrane at adhesions in cells expressing no talin1, talin WT, Δ R8 or Δ R4-R10. (In the case of Δ R8 and Δ R4-10 I predict that the barbed end of filamentous actin that talin attaches to are at a lower height in the Δ R8 and Δ R4-10 as I saw that the c-terminus was depressed in height by 20 nm). After Y27632 (10um) ROCK inhibition (resulting in ablation of cell contractility),

proteins including zyxin, myosin and paxillin increase in height 10-30nm; while proteins including talin, vinculin and alpha-actinin which have force-activated auto-inhibition domain do not change in position while they are still in the 'active' nanoscale position in adhesions (this experiment is not included in the attached figure).

From these results we conclude that Talin1 is necessary for vinculin binding in the active nanoscale position in adhesions (**Figure 5.4**). When talin1 is shortened from ~70 to ~50nm in length, the plasma membrane does not change position so presumably actin must be lowered in position. If talin1's auto-inhibition domain is deleted, adhesion area per cell is dramatically increased, similar to deleting vinculin's auto-inhibition domain¹³. In the future we look to compare the organization of the actin cytoskeleton with these various talin mutants as well as other measures of adhesion formation, cell spreading and cell motility.

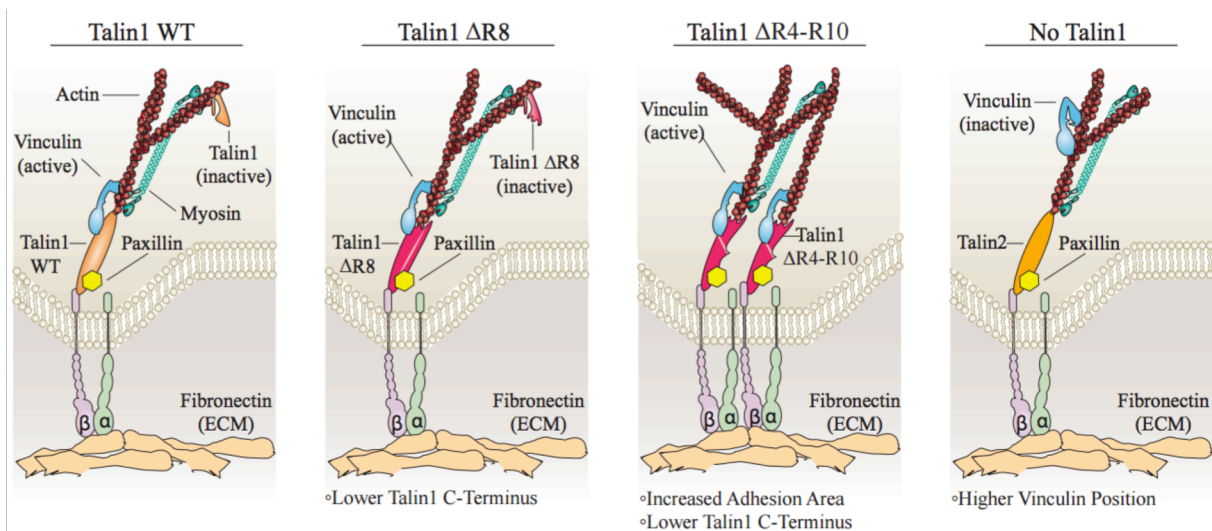


Fig 5.5. Model of adhesion architecture. Talin domains including R4-R10 are critical for vinculin binding and adhesion formation

Figure prepared by Matthew Rubashkin

5.1.3 Mechanobiology of immune synapse formation

Similar to the formation of cell-cell adherens junctions between interacting epithelial cells, immune cells form immunological synapses at the junction of interacting immune cells. At many cell-cell junctions including immunological synapses, receptor-ligand engagement requires nanometer structural alignment^{371–373}. Moreover, the differing size of extracellular domains of signaling proteins including the T-Cell Receptor (TCR) which interacts with major histocompatibility complex class II (MHC II) receptors on other cells is predicted to require molecular-scale changes in membrane topography at cell junctions (**Fig 5.6**). In contrast to the ~15nm size TCR-MHC complex, mechanical junctions at a synapse are predicted to be ~30nm in size from LFA-ICAM interactions, and other transmembrane proteins with large extracellular domains such as the TCR phosphatase CD45 are predicted to be larger than 30nm. The size of these large molecules may control protein activation, nano-domain assembly and receptor signaling³⁷¹. Moreover, nanometer resolution electron microscopy analysis of fixed cells demonstrate that the plasma membrane can vary across a synapse and that these topography changes could correspond to receptor clustering³⁷⁴. Previous studies have implicated released TCR enriched microvesicles at the synapse in topography changes seen in electron microscopy studies³⁷⁵. However, the dynamic interactions of t-cell signaling and nanoscale changes in membrane topology is not well understood, and findings in this area could have impact in the fields of immunology, cancer therapy and immunity.

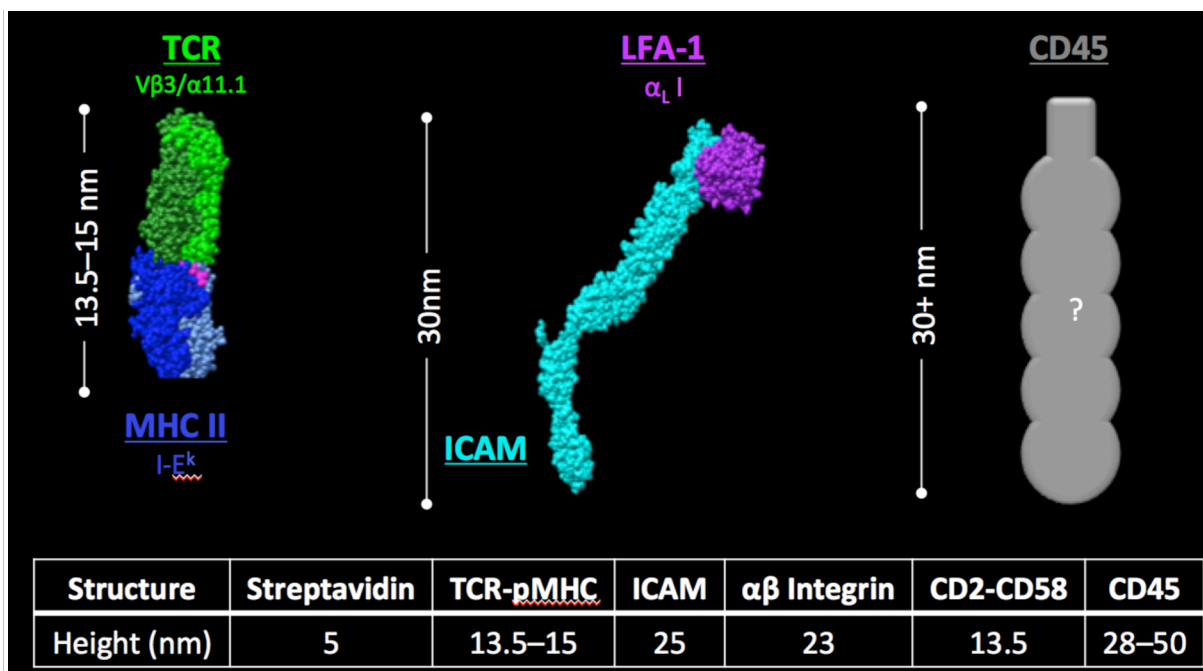


Fig 5.6. Nanoscale structure of proteins in the immune synapse. The TCR-MHCII complex represent the signaling complex between a T-cell expressing TCR and opposing interacting cell such as a dendritic cell. Other dendritic cell proteins include ICAM which can form a mechanical complex with LFA-1. The large phosphatase CD45 is expressed by T-cells and has been implicated in controlling TCR signaling^{422,423}.

Figure prepared by Peter Beemiller

In order to determine the role of membrane topology in immune synapse function and signaling, we partnered with Peter Beemiller and Matthew Krummell to perform SAIM nanoscale measurements of T-cells plated on supported-lipid bilayers that recapitulated a synthetic dendritic cell through presentation of peptide-MHC and ICAM^{371,376}. Through this collaboration and the use of time-resolved imaging in conjunction with SAIM (**Fig 5.7 A,B**), we determined that immune cells including T-cells employ sub-micron scale, rapidly-evolving, ‘nanopodial’ protrusions to interact and scan opposing surfaces (**Fig 5.8 A,B**).

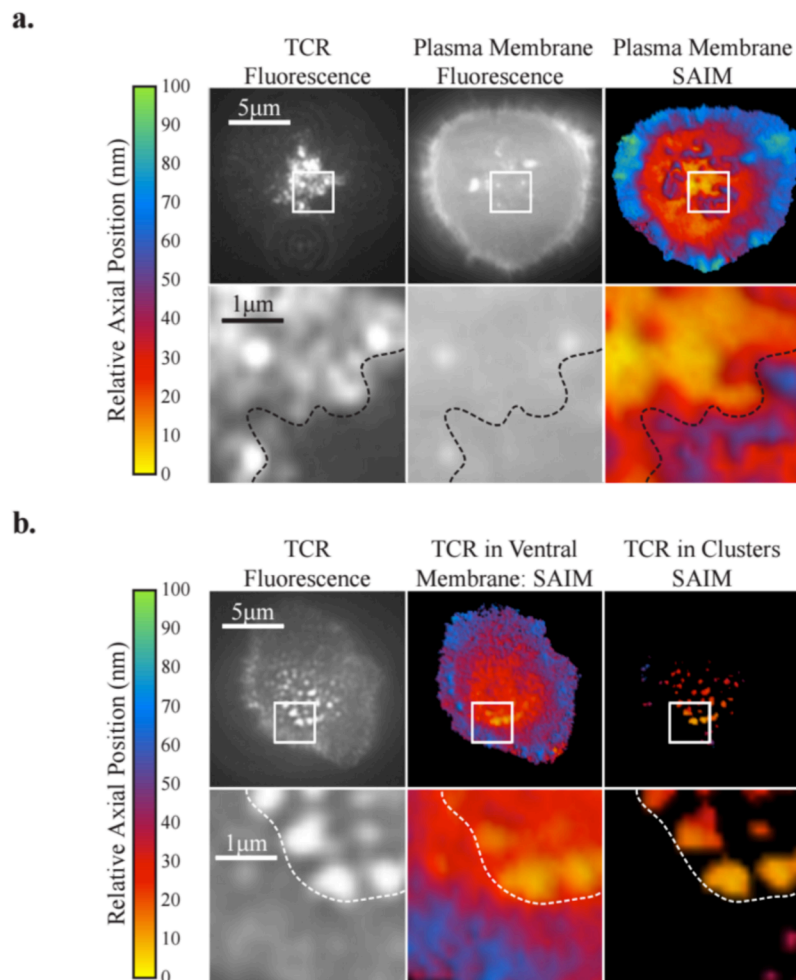


Fig 5.7. TCR and membrane nanoscale topography in primary T-Cells 10 minutes after seeding on supported-lipid-bilayer membranes. **A.** Mature immune synapse with large central supramolecular activation cluster region as indicated by TCR fluorescence (top: whole cell view; bottom: zoom in view). **B.** Immature immune synapse with both central and peripheral supramolecular activation cluster regions. SAIM reveals that the TCRs reside in an area of negative relative axial position (closer to the extracellular supported lipid bilayer membrane); these regions are referred to as nanopods.

Figure prepared by Matthew Rubashkin

Nanopods were formed independent of ligand present on the synthetic dendritic cell (supported lipid bilayer surface), and were formed by many cell types. The function of these nanopods were to bring the TCR into binding range of their ligands; similar to areas of negative membrane curvature that integrin based focal adhesions reside in epithelial cells^{13,14}. In between these nanopods were membrane areas that allowed larger cell-cell mechanical adhesion clusters to form that consist of LFA-ICAM pairs (**Fig 5.8 A, B, C**). Importantly, these nanopods were stabilized when TCRs assembled into clusters, and that TCR

microcluster movement and fusion occurred primarily within nanopods. While binding of peptide-MHC stabilized the nanopods, internal cytoplasmic TCR signaling was not required. That said, actin turnover was necessary for the formation and movement of these nanopods. Therefore nanopod formation represents a powerful mechanical process, which can precede receptor ligand recognition at the immune synapse, but has the power to drive TCR or other small transmembrane proteins to cluster and activate.

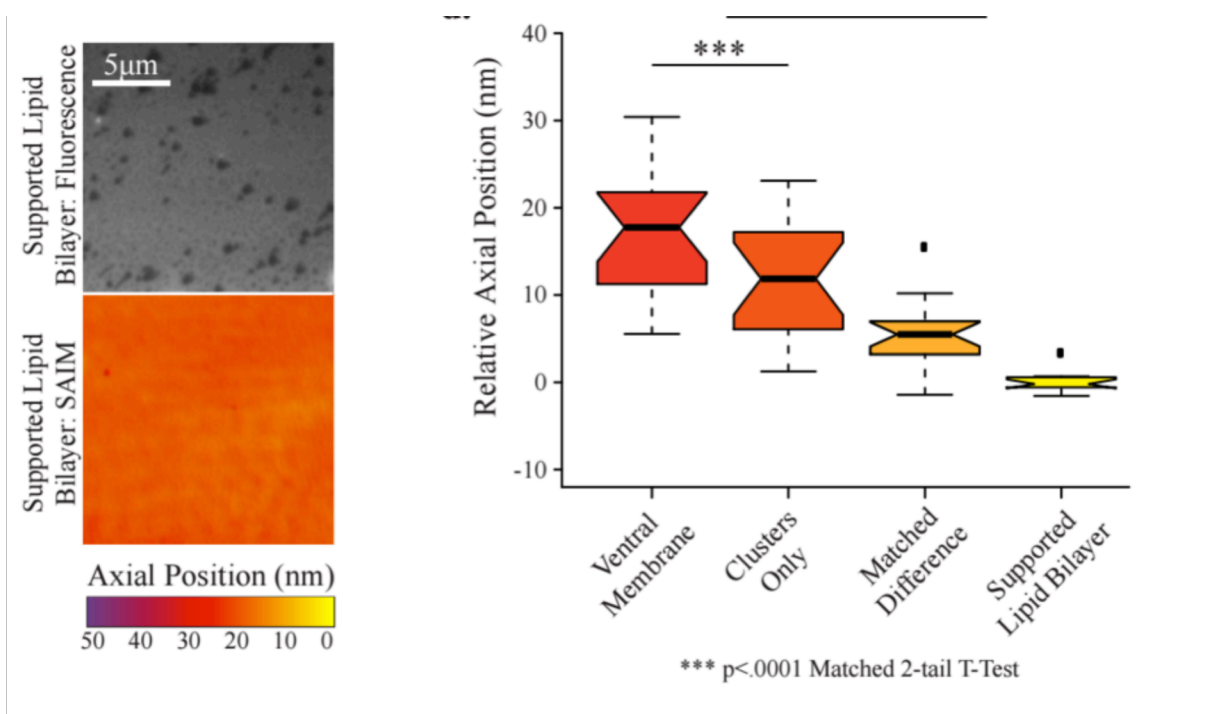


Fig 5.7. SAIM topography measurements of supported lipid bilayer and comparison of the relative axial position of various membrane features. **A.** Supported lipid bilayer on glass substrate seeded with lipophilic and fluorescent DiO dye. SAIM measurements indicate a very flat and uniform surface; while the fluorescence images reveal defects, these are less than 50% different in fluorescent intensity. **B.** Quantification of the relative axial position of the average ventral membrane of immune cells, TCR clusters only, the matched difference between them in individual cells, and a comparison to the supported lipid bilayer. The ventral membrane on average is ~5 nm different than cluster average position, and this is statistically significant.

Figure prepared by Matthew Rubashkin

My role in this project was determining immune synapse nanoscale architecture, specifically if TCR clusters corresponded to protruding nanopods in the plasma membrane, and that LFA-ICAM pairs resided in synapse clefts that receded from the extracellular supported lipid bilayer surface. From these studies of immune cell plasma membrane topography during antigen recognition and cell activation, we conclude that nanopods are general cell structures that are stabilized by receptor signaling, and that nanopods are generated by actin polymerization and stabilized by TCR binding. Future work will involve other cell types and the use of methods include lattice light sheet microscopy to probe immune synapse formation between different cell types in real time with <15nm 3D resolution.

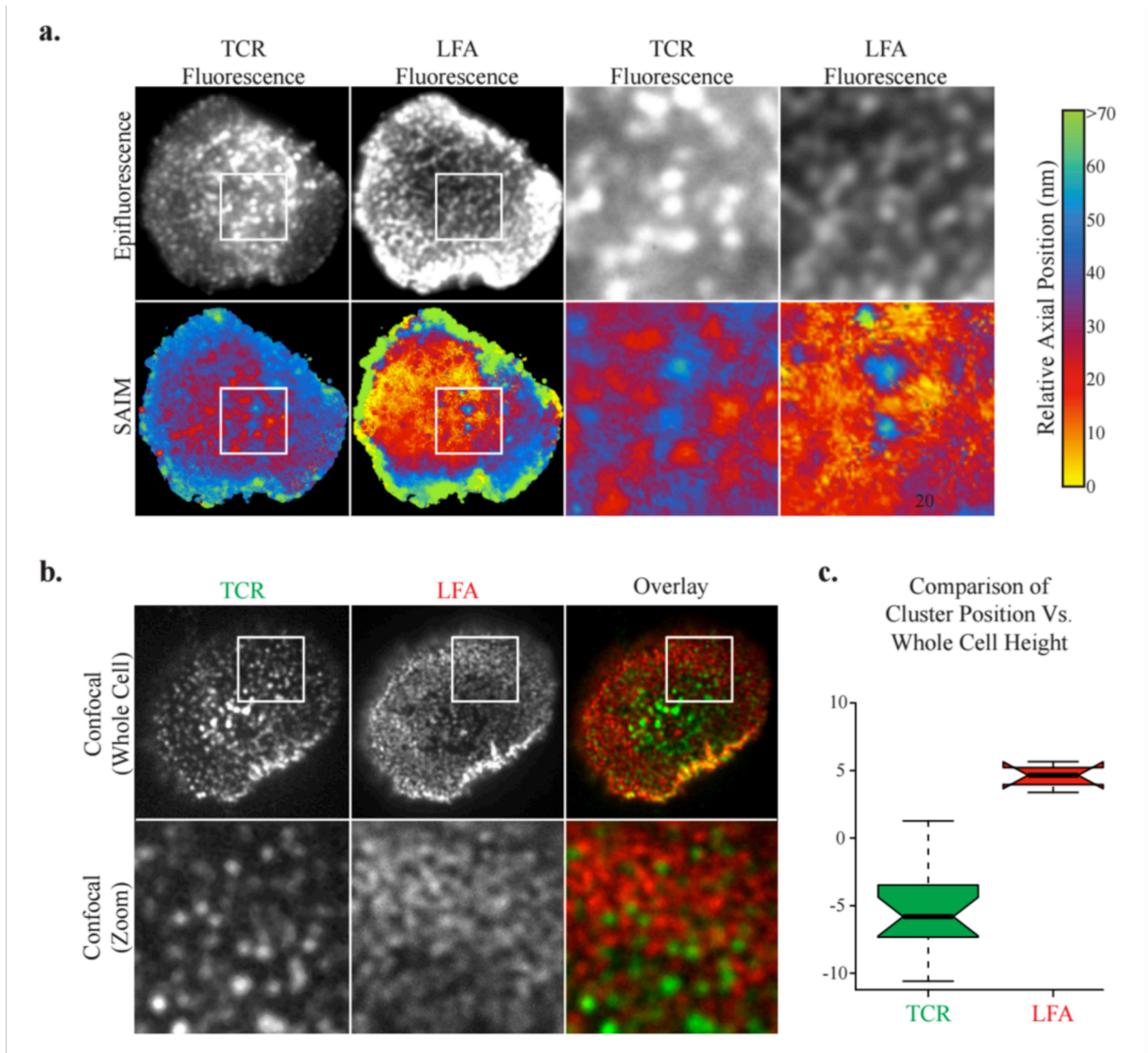


Fig 5.9. SAIM topography measurements of TCR-MHC as well as LFA-ICAM clusters and comparison of the relative axial position. **A.** Maturing immune synapse with peripheral and central supramolecular activation cluster region as indicated by TCR fluorescence (top: whole cell view; bottom: zoom in view). SAIM images when juxtaposed to molecular position of TCR and LFA on the T-cell surface reveal that the LFA resides at a higher average position. **B.** Confocal images of TCR and LFA to further illustrate the microscale exclusion of TCR and LFA clusters in the immune synapse. **C.** Quantification of the relative axial position of TCR and LFA clusters in the ventral membrane. TCR clusters are ~15 nm different in height than LFA clusters, similar to their predicted molecular difference.

Figure prepared by Matthew Rubashkin

5.1.4 Neutrophil motility and membrane nanoscale topography

The mechanical properties of the cell plasma membrane affect many cell processes including adhesion, motility and immune surveillance^{377–379}. The plasma membrane is known to influence intracellular events from cytoskeleton remodeling, to endocytosis to cell signaling³⁷⁷. Many signaling molecules responsible for cell motility can be found in the plasma membrane, including particular cytokine responsive members of the JAK/STAT pathway or G protein-coupled receptors (GPCRs). Human neutrophils express GPCRs that are responsive to external stimuli, and these GPCRs are responsible for translating external chemical stimuli to signaling pathways responsible for cell motility and regulating membrane tension³⁸⁰. This includes hydrolysis of the membrane phosphoinositide PIP(2) by phospholipase C (PLC) into secondary messengers³⁸¹. In particular lipid phosphoinositides are known to regulate cell membrane curvature and tension, and feed forward into the PI3K-Akt-mTOR signaling pathway^{262,382,383}.

In collaboration with Alba Diz-Munoz and Orion Weiner we investigated how changes in plasma membrane tension regulate plasma membrane architecture, cell shape, and motility in human neutrophils. We used human neutrophils as a broad model of amoeboid cell motility important to immune surveillance in the body. The Orion lab first confirmed that the mTOR signaling pathway members Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) and phospholipase D-2 (PLD2) activate Akt signaling in conjunction with the mechanistic target of rapamycin (mTOR2) signaling complex³⁸⁴. Interestingly, knockdown of either RICTOR or PLD2 increased membrane tension and increased leading edge number and size in human neutrophils. From this data, we hypothesized that decreased mTOR/Akt signaling through RICTOR-knockdown would increase membrane tension, decreasing membrane folds that allow for effective adhesion formation, which in turn limits cell migration and immune surveillance.

To analyze the role of plasma membrane nanoscale topography in neutrophil activation, we assayed human neutrophils on fibronectin surfaces (**Fig 5.9 A,B**). Cells were differentiated for 3 days then plated

on the wafer-fibronectin surface for 10 minutes before PFA + glutaraldehyde fixation. We first assayed inactive and stimulated cells with FMLP, labelled with CAAX-mcherry and Hem1-GFP (WAVE complex)^{385,386}. We then analyzed activated cells with either Rictor, PLD2 or nonsense shRNA; wherein both Rictor and PLD2 knockdowns had increased membrane tension.

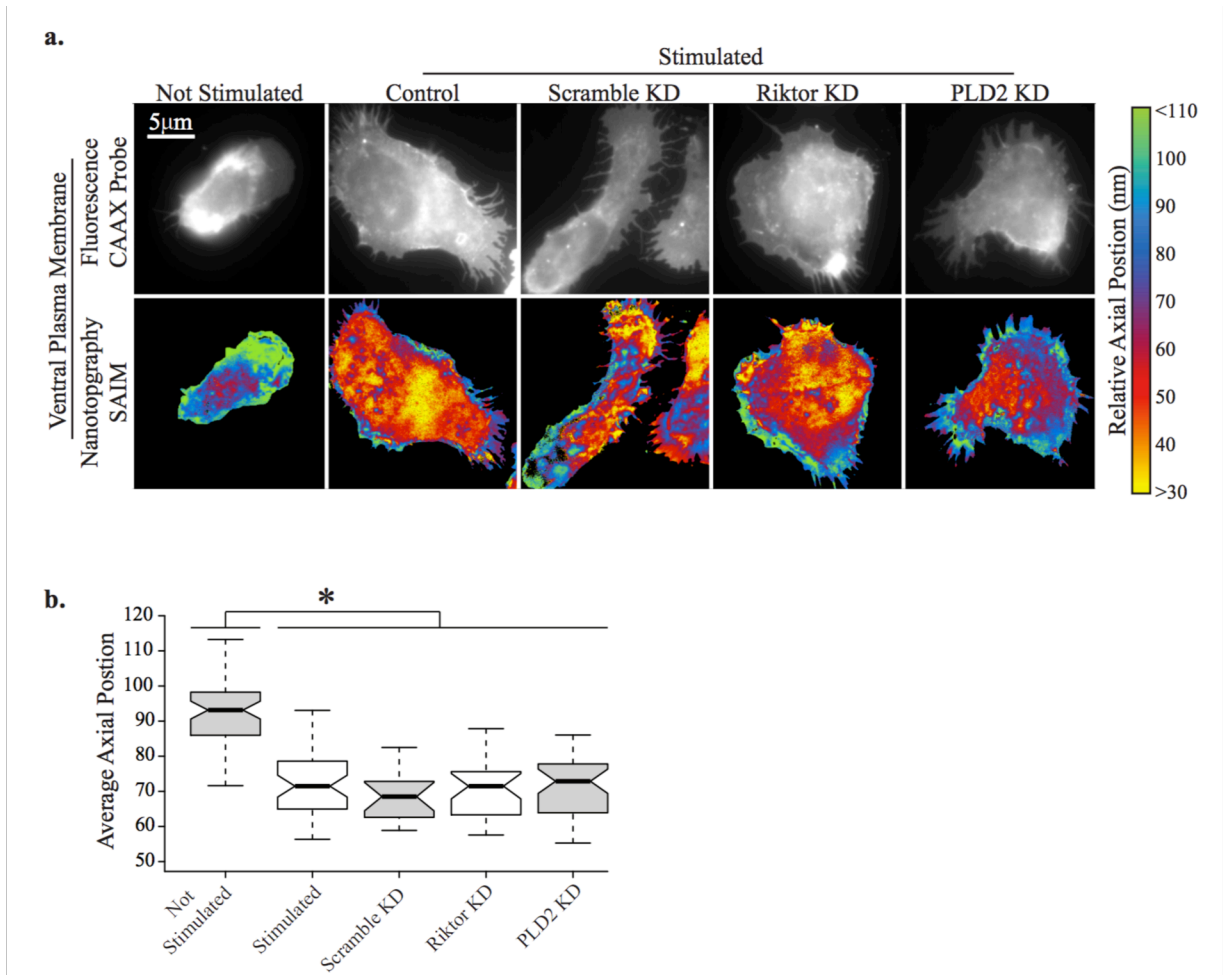


Fig 5.10. SAIM topography measurements of human neutrophils expressing a fluorescent membrane probe plated on fibronectin coated silicon wafers and stimulated with FMLP. **A.** Fluorescent and SAIM images of neutrophils +/- stimulation and with various knockdowns of mTOR signaling pathway members including RICTOR and PLD2. After increases in cellular tension through RICTOR or PLD2 knockdown, cell spreading is increased, including formation of multiple lamellar protrusion. **B.** Quantification of the relative axial position of the neutrophil plasma membrane. Cell activation decreases average axial position, due to the formation of additional areas of negative membrane curvature (protrusion towards the cell surface). However, knockdown of mTOR pathway proteins does not change average axial position, only micro-scale cell morphology.

Figure prepared by Matthew Rubashkin

From the Hem1-GFP probe we observed that inactivated cells extended protrusions, but did not spread on the fibronectin wafers (**Fig 5.9 A**). Activated cells extended protrusions, spread, and exhibited a polarized morphology. Both Rictor and PLD2 KD cells which had increased membrane tension through inactivation of mTOR signaling had increased lamellar and lamellapodial protrusions, enhanced spreading as compared to activated cells, and had defective polarization. This loss of polarity leads to decreased cell motility and persistence of the knockdown cells.

Similar to our previous observations in epithelial and mesenchymal cell types, protrusive membrane areas are 'higher' in axial position, i.e. further away from the ECM than areas of the plasma membrane under the nucleus, in integrin adhesions or non-protrusive membrane areas in epithelial cells. We observed a significant ($P < .01$) difference in unstimulated ventral membrane height in comparison to the stimulated samples (including control, scramble KD, PLD2 KD and Rictor KD) (**Fig 5.9 B**). This membrane on average is ~20nm higher in the unstimulated sample, a large, and potentially biologically relevant variation in membrane axial position. Wherein the high heights correspond to membrane disengaged from the substrate. It is important to note that we were not able to detect changes in average axial position in the stimulated control versus the knockdown samples. Instead, the differences manifested in changed micro-scale in plane membrane organization, appearing to stem from altered neutrophil polarization.

In the future, we will conduct several experiments to analyze the role of other cell surface molecules in regulating membrane tension and transducing information encoded in the plasma membrane to cell migration and other behaviors. This includes staining for an integrin receptor (MAC-1: α M- β 2) implicated in neutrophil attachment to fibronectin ³⁸⁷. This would allow us to clearly correspond depressed (i.e. lowered) membrane heights to cell-ECM adhesion regions. Additionally, we plan to investigate different neutrophil migration phenotypes by dialing back fibronectin concentration to encourage a more mesenchymal motility phenotype. By analyzing various motility phenotypes, we could

better understand how nanoscale membrane topology and membrane tension affects neutrophil migration phenotype.

5.2. Future Directions

5.2.1 Scanning Angle Interference Microscopy for 3D imaging and faster acquisition

Scanning angle interference microscopy (SAIM) has several limitations. These include spatial limitations of a 120um working distance, and a <1um depth of field. This limits imaging to ~2 cells directly above the surface of a glass cover slip and for that cell, only a single 1um slice can be analyzed^{12,251}. Another limitation of SAIM includes long exposure times, of ~20 seconds for 5nm resolution. Lastly, SAIM can only be used for super-resolution imaging in the z-axis of imaging, directly out from the objective; and lacks X-Y super-resolution of other techniques including iPALM²¹³.

In order to address the current shortcomings of the SAIM technique, I have been collaborating with Kate Carbone and Nico Stuurman of Ron Vale's laboratory to implement SAIM in uManager, an open source microscopy platform. The beta version of this package is now available in Java, and will hopefully be included in future releases of uManager and potentially Fiji and ImageJ; the open source image editing software from the NIH. Additionally, the Paszek lab at Cornell University is currently developing a 2nd generation SAIM. This includes the implementation of non step-motor driven mirrors to increase the angle scanning speed, higher power lasers to decrease exposure time, and deconvolution mathematical techniques to allow reconstruction of multiple planes of exposure. Another potential improvement in the technology could include hybridizing SAIM with photo-activation-localization-microscopy techniques through the use of blinking fluorophores and greater X-Y fitting to allow for super-resolution in 3D. In the future, we hope to have 2nd generation SAIM from the Paszek lab integrated as an open source microscopy suite using uManger and the resources of the Weaver and Vale laboratories.

5.2.2 Investigating paxillin and vinculin signaling in different tissue systems and diseases

Alterations in the vinculin-PI3K-Akt and paxillin-FAK-Src-Erk signaling pathways is a negative prognostic signature in invasive human breast cancer¹³ (Chapter 4). While paxillin and vinculin have been implicated in other cancer types, diseases and development, the role of focal-adhesion dependent mechano-signaling is still unclear^{243,267,388–390}. Therefore future work into this area could result in new diagnostic signatures similar to those found with vinculin in invasive breast cancer (Chapter 3) and paxillin in mesenchymal tumor cells and cancers (Chapter 4).

There are several viable approaches for investigating adhesion dependent mechano-signaling. These include the use of vinculin or paxillin null cell types (which primarily includes fibroblasts), and CRISPR-Cas9 gene edited cells or tissues of interest to knockout vinculin, paxillin or their binding partners. For study of vascularization in development or disease, 3D tissue culture experiments can be conducted with shRNA to pathway components. Lastly, alternative model systems such as drosophila could be genetically modified to further study the role of paxillin and vinculin dependent mechano-signaling in organogenesis.

5.2.3 Develop drugs that can target mechanobiology signaling with minimal toxicity

In chapters 3 and 4 I identified several components of vinculin and paxillin dependent signaling axes. Unfortunately these identified components included FAK, Akt and Integrin, clinical targets with currently limited efficacy^{10,31,391,392}. In general, a failed cancer drug in human trials can be due to both redundant protein signaling pathways that allow rapid tumor adaption, and that the drugs cannot be given at high enough levels to kill the tumors due to toxic side effects of the drug. These toxic side effects are often due to expression of the drug target on normal, non-cancerous cells. Therefore in order to avoid toxic side effects of on-target binding of cancer therapeutics to non-cancerous cells, new mechanobiology signaling proteins must be identified that are not commonly expressed in non-transformed tissue. The difficulty in this task lies in the diversity of adhesion proteins, which currently number over 200^{253,393}, as well as

developing drugs that can penetrate the cell plasma membrane. Alternative mechano-signaling targets could also include transmembrane or extracellular proteins involved in mechanotransduction including glycoproteins and galectins, glycocalyx cross-linkers ^{14,394–396}.

5.2.4 Identify patients that would benefit most from mechanobiology interventions

Cancer is a disease characterized by varied cellular changes. These include specific hallmarks including self-sufficiency in growth signals, insensitivity to tumor suppressive signals, avoiding apoptosis, limitless replicative potential, sustained angiogenesis, evasion of anti-tumor immune function, altered metabolism, unstable DNA, inflammation, as well as altered cell-matrix interactions that lead to cell invasion and metastasis ^{25,33,397}. The questions remains how best to identify which patients would benefit the most from targeting of signaling pathways that facilitate cell invasion and metastasis, in the context of malignant progression.

In order to identify patients that would benefit from mechanobiology interventions, several resources could be employed. This includes The Cancer Genome Atlas, which is a repository for gene, protein and clinical information for thousands of cancer patients; which can be employed to investigate different tumor types ^{5,306,321}. Additionally, data repositories including The Human Protein Atlas can be used to compare protein level expression data in cells and tissue of mounted tumor tissues ^{13,398}. The first step in identifying patients with high level of mechano-signaling would be to establish a signature of critical genes involved in this process, preferably a gene set which is necessary to drive the entire mechano-signaling pathway from sensation to transduction to gene regulation. This could include proteins such as specific integrins, paxillin, vinculin and other adhesome proteins, as well as the respective signaling pathway members such as Akt, Src and Erk. After this protein signature was defined at the DNA and protein levels, patients could be stratified by both mechano-signaling and other traditional breast cancer designation such as hormone and Her2 status ^{2,296}. Lastly, extensive testing would have to be performed in

cell lines that aligned or did not align with this signature ³⁹⁹, before testing these drugs in an appropriate mouse model of tumor cell invasion, for instance the mechanically response PYMT model ²²³.

5.2.5. Development of novel tools for evaluating radiation induced extracellular matrix alterations that can induce breast cancer recurrence.

It has been shown that radiotherapy (RT) as part of normal breast cancer treatment can reduce the rate of local recurrence from 35 to 20% and 15 year risk of death by 3.8% ⁴⁰⁰. However, there remains concern over the persistence recurrences of aggressive tumors such as triple-negative breast cancer ⁴⁰¹. The Graves Radiobiology Laboratory at Stanford has recently investigated the role of radiation induced tumor recurrence mediated by circulating tumor cells, CTCs ⁴⁰². CTCs identified in the blood of breast cancer patients are indicative of an unfavorable prognosis for both tumor recurrence and metastasis ⁴⁰³. Hypothesizing that radiation therapy could promote CTC survival and proliferation by providing a supportive environment, the Graves laboratory found that GM-CSF produced by tumor cells in response to radiation recruits migrating tumor cells to irradiated tumors ⁴⁰². Previous work using *in vitro* invasion assays and *in vivo* orthotopic models of breast cancer has shown that radiation can induce 4T1 tumor recurrence in a Granulocyte macrophage colony-stimulating factor (GM-CSF) dependent manner (**Fig 5.10 A**). Therefore, investigations are needed to study how radiation mediates the GM-CSF signaling axis. Specifically, colony stimulating factor 2 receptor (CSF2R α) may be responsible for stimulating GM-CSF responsive circulating tumor cells (CTCs), and as such, future research could entail the use of CSF2R α antibodies to target GM-CSF sensitive CTCs. I would hypothesize that Anti-CSF2R α antibodies would reduce GM-CSF and c-jun/JAK2/STAT3 mediated cell invasion, and would block CTC mediated tumor recurrence after radiotherapy in an orthotopic model of breast cancer.

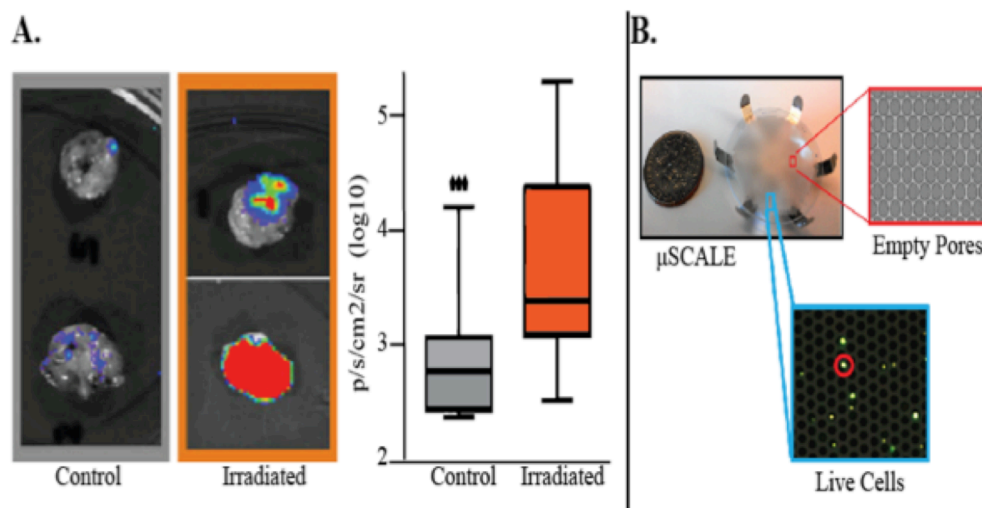


Fig 5.11 A. Radiation can induce tumor recurrence in vivo: Ex vivo bioluminescence images of 20 Gy irradiated 4T1 orthotopic tumors. There is a significant increase in primary tumor reseeded after irradiation⁴⁰²
B. Ultra-high throughput single cell screening platform for drug screening: Micropore array: 4 cm², with 3.3 million 10 μm pores

Figure prepared by Matthew Rubashkin, Edward Graves and Bob Chen

Cell migration is important for many biological processes including immune surveillance and CTC mediated cancer metastasis. In physiological 3D environments, cells navigate diverse environments by integrating mechanical and biochemical signals, including GM-CSF molecular gradients. In Chapter 3, I found that breast cancer cells directly respond to altered extracellular collagen by increasing integrin mediated PI3k/Akt oncogenic signaling, (Rubashkin et al., 2014). A system that allows single cell high throughput screening of novel drug compounds, radiation profiles and chemokine gradients in the context of a 3D cellular invasion assay would be transformative for studying radiation induced extracellular matrix alterations that can induce breast cancer recurrence. Towards addressing this need, the Cochran Laboratory at Stanford has recently developed an ultra-high throughput micropore single cell analysis and screening platform for discovery of protein based drug compounds (**Fig 5.10 B**). This technology is currently being adapted for cell activity screening. A future project could entail implementing a high-throughput functional cancer cell invasion assay for screening of GM-CSF therapeutics post radiotherapy. That said, it remains to be seen if radiation

induced, GM-CSF mediated cell invasion is a dose dependent phenomenon which can be countered by altered radiation dosing, modifications to the extracellular matrix or inhibition of CSF2R α .

5.2.6 Do nanoscale cell mechanics play a role in immune cell activation and cancer-immune targeting?

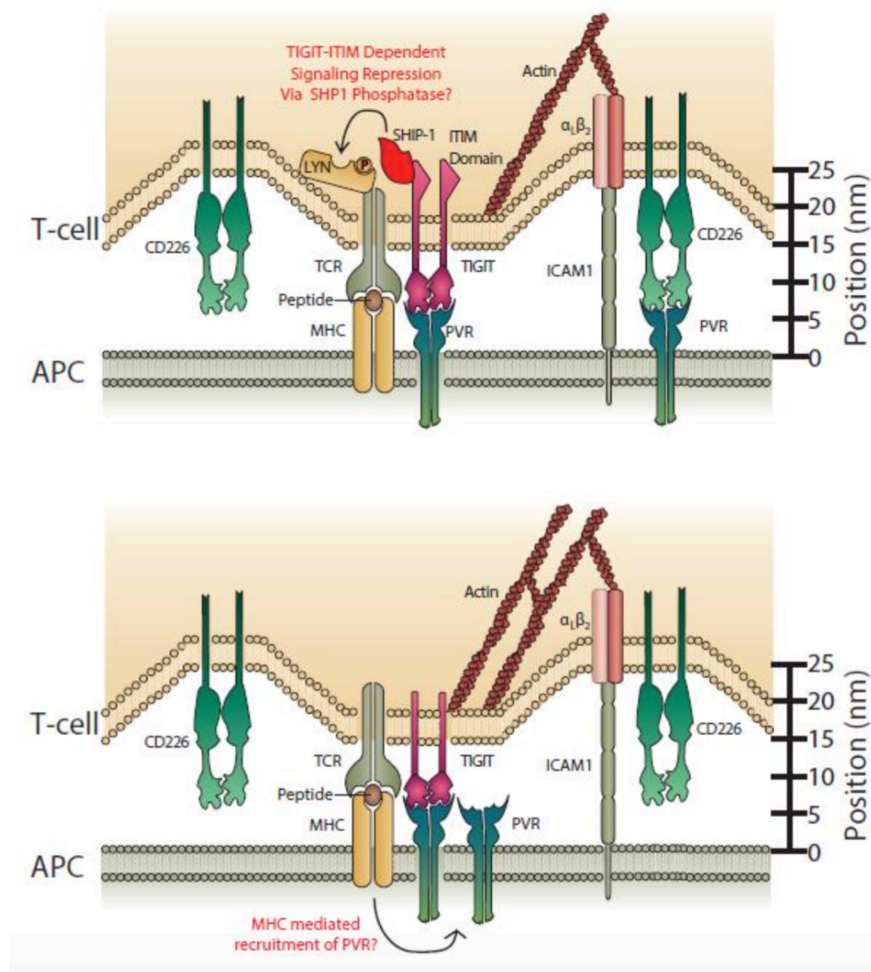


Fig 5.12 Immune synapse between a human T-Cell and antigen presenting dendritic cell. In the immune synapse dendritic cells present a peptide-MHC complex to actively scanning T-cells, for TCR dependent cell activation. PVR is a transmembrane protein on the surface of an antigen presenting cell (APC). TIGIT is a transmembrane protein on T-cells which suppresses signaling by binding PVR, while CD226 is thought to activates TCR signaling on the T-cell surface, also by binding PVR. It is hypothesized that either MHC can recruit PVR on the dendritic cell surface (top panel), or that internal signaling from the TIGIT receptor can reduce activity of the TCR on the T-cell surface (bottom panel).

Figure prepared by Matthew Rubashkin

In the field of cancer immunology, it is unclear how cellular mechanics plays a role in interactions amongst immune cells and transformed cells^{371,404}. In order to study this process, the biology of the immune cell synapse must be better understood. In the immune synapse dendritic or cancer cells, also known as antigen-presenting cells (APCs) present a peptide-MHC complex to actively scanning T-cells, for TCR dependent cell activation. Previous work (Chapter 5.1.3) has implicated plasma membrane nanoscale topography in creating nanopods of receptor clustering and cell signaling. There are several other protein pairs outside of MHC-TCR and LFA-ICAM in the immune synapse. The Poliovirus Receptor (PVR) is a nectin-family member transmembrane protein on the surface of an antigen presenting cell (APC). This includes TIGIT which suppresses signaling and CD226 which activates TCR signaling on the T-cell surface as well as PVR, the binding transmembrane receptor on the APC⁴⁰⁵⁻⁴⁰⁸. It is hypothesized that either MHC can recruit PVR on the APC surface or that internal signaling from the TIGIT receptor can reduce activity of the TCR on the T-cell surface (**Fig 5.11**). This function is similar to the receptor-ligand-decoy triad seen in other receptor-ligand systems including VEGF-RI, VEGFR-II and VEGF on endothelial cells⁴⁰⁹; or the repressor CTLA-4 and activator CD28 on T-cells which can both bind CD80 on the surface of an APC⁴¹⁰.

In order to assay how nanoscale cell mechanics play a role in immune cell activation and cancer-immune targeting, several sets of experiments could be conducted. The first includes employing the lipid bilayer synthetic dendritic cell system to test if plasma membrane nanoscale organization and mechanics dictates PVR, TIGIT, CD226 clustering. This experiment would require plating T-cells expressing fluorescent TIGIT and TCR on synthetic APCs that present peptide-MHC, PVR and ICAM. Readouts would include localization of TIGIT, CD226 and PVR in immune synapse formation. I would predict that PVR would be localized to peptide/MHC-TCR zones and that CD226 is localized to LFA/ICAM zones. Moreover, I would expect that nanopod formation in T-cells or tumor cells leads to size dependent exclusion of CD226 from PVR enriched zone and size dependent inclusion of TIGIT in PVR enriched zone. Increased TIGIT and PVR interactions could then lead to scaffolding of Tyrosine-protein phosphatase non-receptor

type-6 (SHP-1) on the cytoplasmic tail of TIGIT and signaling inhibitory to TCR function (**Fig 5.11**). Other mechanical manipulations that could be performed include alterations in membrane tension through tuning of the mTOR signaling pathway members RICTOR and PLD2 (as seen in Chapter 5.1.4), as well as ablation of specific cytoskeleton components including actin or microtubules.

Another set of future experiments could include investigating the role of PVR dependent epithelial (breast) cancer cell motility in metastasis ^{405,407}. These experiments could include *in vitro* Transwell invasion and 3D Collagen tissue that mimics normal or disease tissue properties; and conducting these assays in the presence of TIGIT or CD226 overexpressing T-Cells. Cancer cell and immune cell motility and homing could then be compared to established modes of motility ⁴¹¹. These assays could also be extended *in vivo* to study PVR dependent metastasis via CRISPR KO of PVR in either orthotopic implantation or tail-vein injection model.

5.3. Thesis Overview

5.3.1 Motivation

Every year in the United States, there are over 200,000 new breast cancer diagnoses, and over 40,000 deaths^{412,413}. Breast cancer is the second most common cause of death for women in the United States, and places a large burden on our health care system. The traditional hallmarks of cancer including sustained proliferative signaling, evasion of growth suppressors, replicative immortality, angiogenesis induction, invasive behavior and apoptosis resistance no longer fully encompass the known distinctive and complementary capabilities that enable tumor growth and metastasis^{24,25}. Importantly, the tumor microenvironment and tensional homeostasis have recently been implicated as critical regulators of epithelial cell tumorigenesis and disease progression in cancer^{7,8,169,288,414,415}. In this thesis I juxtaposed the behavior of breast cancer epithelial cells in 2D and 3D microenvironments and investigated the role of cell spreading, dimensionality and protein diffusion in tumor invasion, progression and metastasis.

5.3.2 Cancer Mechanobiology at the Nanoscale

As researchers' understanding of cellular behavior in 3D progresses, studying signaling in the appropriate context has become essential. From intracellular localization of signaling molecules to molecular gradients at the tissue level, it is apparent that signaling is influenced by a myriad of factors and is a far more complex process than the simple interaction of reactants to form an end product. This recognition has spurred the invention of new technologies to facilitate investigation of signaling in 3D contexts. At the cellular level, novel superresolution imaging tools, such as Scanning Angle Interference Microscopy (SAIM), detailed in **Chapter 2** allow unprecedented characterization of protein localization and interaction dynamics.

5.3.3 Cancer Mechanobiology in 3D

In order to recapitulate the complex and heterogeneous *in-vivo* environment, bioengineered materials serve as *in vitro* mimics of tissue ECM, helping to elucidate the role of cell-matrix interactions in governing intra- and intercellular signaling. Using novel engineered environments, in **Chapter 3**, the role of 3D cell-ECM interactions and force-activated focal adhesion engagement over vinculin is explored. In the future, microfabrication and 3D printing will permit even greater control and recapitulation of multicellular structures, bringing multiple cell types together in physiologically relevant ways and revealing emergent properties at the tissue level. Results from the application of these tools has demonstrated that cell behavior can change dramatically based on the conditions under which they are studied^{15,16,233,416}. As such, it is no wonder the biomedical field has been faced with unprecedented failure rates in translating hard-earned discoveries into clinical progress. Therefore, it has never been more urgent for both basic science and translational research to be conducted in the appropriate context, as highlighted in many experiments throughout this thesis. With the adoption of new methods, findings from the laboratory will become more transferable to improve human health.

5.3.4 Mechanistic Insight Into Invasive Breast Cancers

In **Chapter 4**, we next asked if invading mesenchymal-like tumor cells could also be engaging a specific signaling axis inside of adhesions; similar to the vinculin engagement and Akt signaling we observed in stiff ECM with high ligand availability in Chapter 3. I employed the 3D environments I investigated through a literature search in Chapter 1, and the microscopy technique SAIM, that was developed in Chapter 2. Using these tools we observed that paxillin had altered nanoscale behavior in cells with mesenchymal behavior that drove a FAK-Src-RhoA-

Erk signaling axis. This signaling in combination with paxillin splicing drove cancer cell invasion of triple negative breast cancer cell lines. Moreover, we found that paxillin overexpression significantly increasing patient mortality in human breast cancer, and conclude based on our and other's data that paxillin is a biomarker for negative outcomes in human breast cancer, and potentially in gastric and colorectal cancer^{299,300,350–352}.

Future work is now needed to identify the entire circuit of proteins in the cellular mechanotransduction pathway turned on by the mesenchymal paxillin-alpha splice form. Moreover future studies could be used to identify novel proteins involved in protein level splicing and alternative translation of paxillin and these could be targeted for therapeutic treatment^{23,355}. Consequently, therapies that target paxillin mesenchymal splicing, altered cellular mechanosensation, or downstream FAK-Src-RhoA-Erk signaling, could have great utility in the treatment of breast tumors that are mesenchymal in phenotype and not responsive to growth factor receptor inhibition^{354,356}.

5.3.5. Ongoing and future projects

In the Weaver laboratory there are several ongoing and future projects that I have contributed to, established or furthered. These include studying how the cell glycocalyx influences cell adhesion, invasion and apoptosis resistance with Matthew Paszek, now of Cornell University, studying the nanoscale architecture of talin with Ben Goult, now of Kent University, characterizing neutrophil motility and membrane nanoscale topography with Alba Diz Munoz of the Weiner laboratory, and investigating the role of mechanobiology in immune synapse formation with Peter Beemiller of the Krummel laboratory.

Future studies that could be based off this work include investigating paxillin and vinculin signaling in different tissue systems and diseases, developing drugs that can target mechanobiology signaling with minimal toxicity, and identifying patients that would benefit most from mechanobiology interventions. Other potential future projects could include developing the next generation of SAIM Microscopy for 3D imaging and faster acquisition, studying if nanoscale cell mechanics play a role in immune cell activation and cancer-immune targeting, or developing novel tools for evaluating radiation induced extracellular matrix alterations that can induce breast cancer recurrence.

5.3.6. Outlook

I look forward optimistically to a new era of cancer treatment, where cancer is no longer a fatal disease, but a treatable chronic disorder. I hope that my research into the role of the ECM in driving cancer progression spurs development of drugs that targets this newly identified oncogenic signaling axis.

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