UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Twist1 in Cellular Mechanosensing

A dissertation in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Spencer C. Wei

Committee in charge:

Professor Jing Yang, Chair
Professor Adam Engler
Professor Kun-Liang Guan
Professor Stephen Howell
Professor Tony Hunter
Professor Mark Kamps

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The dissertation of Spencer C. Wei is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

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DEDICATION

I would like to dedicate this work to my family, which has made this all possible.

EPIGRAPH

There is frequently more to be learned from the unexpected question of a child than the discourses of men.

John Locke

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

2D – Two dimensional

3D – Three dimensional

AFM – Atomic force microscopy

βAPN – Beta aminoproprionitrile

bHLH – Basic helix-loop-helix

CAF – Cancer associated fibroblast

Cav1 – Caveolin-1

CDH1 – E-cadherin

DNA - Deoxyribonucleic acid

DCIS – Ductal carcinoma *in situ*

ECM – Extracellular matrix

EGF – Epidermal growth factor

EMT – Epithelial mesenchymal transition

FAK – Focal adhesion kinase

FFPE – Formalin fixed paraffin embedded

FSP1 – Fibroblast-specific protein 1

G3BP2 - GTPase Activating Protein (SH3 Domain) Binding Protein 2

HER2 – Human epidermal growth factor receptor 2

LCIS- Lobular carcinoma *in situ*

LOX – Lysyl oxidase

LOXL1 – LOX-like 1

MET – Mesenchymal epithelial transition

MMP-3 – Matrix metalloproteinase-3

MMTV – Mouse mammary tumor virus

mRNA – Messenger ribonucleic acid

MRTF – Myocardin related transcription factor

MSC – Mesenchymal stem cell

NES – Nuclear export signal

NLS – Nuclear localization signal

NTF2 – Nuclear transport factor 2

Pa – Pascal

PA – Polyacrylamide

PDGF – Platelet derived growth factor

PLA – Proximity ligation assay

rBM – Reconstituted basement membrane

RCC – Renal clear cell carcinoma

ROCK – Rho Kinase

RRM – RNA recognition motif

SCS – Saethre-Chotzen syndrome

SHG – Second harmonic generation

siRNA – Small interfering ribonucleic acid

shRNA – Short hairpin ribonucleic acid

SH3 – Src homology 3 domain

TAM – Tumor associated macrophage

TCGA – The Cancer Genome Atlas

TIL – Tumor infiltrating leukocyte

TNBC – Triple-negative breast cancer

TGF-β – Transforming growth factor β

LIST OF FIGURES

LIST OF TABLES

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Chapter 1, in part, will be submitted for publication as an article titled “Between a Tumor and a Hard Place: The Relationship between Tissue Rigidity and EMT”. I was the primary contributor along with my coauthor Jing Yang.

Chapter 2, in part, has been submitted for publication as an article titled "Matrix stiffness drives Epithelial-Mesenchymal Transition via a Twist1-G3BP2 mechanotransduction pathway". I was the primary contributor along with my coauthors Jeff H. Tsai, Laurent Fattet, Yurong Guo, Albert Chen, Robert Sah, Susan Taylor, Adam Engler, and Jing Yang.

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VITA

2008 Bachelor of Arts, University of California, Berkeley

2014 Doctor of Philosophy, University of California, San Diego

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

The Role of Twist1 in Cellular Mechanosensing

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Mechanical forces are recently recognized as potent regulatory signals of cellular behavior in a wide range of biological contexts, including tumor progression and stem cell differentiation (Calvo et al., 2013; Dupont et al., 2011; Engler et al.,

2006; Jaalouk and Lammerding, 2009; Leight et al., 2012; Levental et al., 2009; Paszek et al., 2005). Matrix stiffness is controlled by deposition and modification of extracellular matrix, especially collagen (Provenzano et al., 2006; Provenzano et al., 2008). In breast tumors, the presence of fibrotic foci, i.e. dense clusters of collagen fibrils, is a marker of increased matrix stiffness and correlates with disease progression and poor survival (Colpaert et al., 2001; Hasebe et al., 2002). This correlation is consistent with the use of manual palpation to detect breast tumors, as the lesions are much harder than the surrounding normal tissue. These observations raise the question of how mechanical inputs from the tumor microenvironment are transduced into transcriptional outputs to drive tumor progression. Herein, I show that the transcription factor Twist1 is an essential mechano-mediator that promotes epithelial-mesenchymal transition (EMT) in response to increasing matrix stiffness. High matrix stiffness promotes nuclear translocation of Twist1 by releasing Twist1 from its cytoplasmic binding partner, G3BP2. Loss of G3BP2 leads to constitutive Twist1 nuclear localization and synergizes with increasing matrix stiffness to induce EMT and invasion. In human breast tumors, increasing matrix stiffness and reduced expression of G3BP2 predict poor survival. These findings reveal a Twist1-G3BP2 mechanotransduction pathway that responds to biomechanical signals from the tumor microenvironment to drive EMT during tumor progression.

Chapter 1

Introduction

1.1 Breast Cancer

Breast cancer is the most prevalent cancer in women in the United States, accounting for approximately 41% of female cancer diagnoses(Siegel et al., 2012a). Cancer mortality in women and men in the United States has declined at a rate of approximately 1% per year recently (1999-2008) however the survival rates of patients with metastatic disease remains low(Siegel et al., 2012b). The 5-year survival rate of patients with distant disease is 23.4% compared to 83.8% and 98.6% for patients with regional and localized breast cancer(Howlader et al., 2011). In 2011 there were over 229,000 new diagnoses of breast cancer and over 39,000 mortalities, the vast majority of which were due to metastatic disease(Siegel et al., 2012b). This highlights both that metastatic breast cancer is a much more deadly disease than localized breast cancer and that we currently lack the treatments to generate efficacious and durable responses in patients.

There are main two types of breast cancer, ductal and lobular, which arise from the mammary ductal epithelial cells or lobular epithelial cells of the milk producing glands, respectively. Each of these types can be identified as either *in situ* or invasive. There are three types of breast carcinoma *in situ*, which include ductal carcinoma *in situ* (DCIS), lobular lobular carcinoma *in situ* (LCIS), and Paget's disease originating in the nipple tissue(Edge, 2010). DCIS is a benign or non-invasive tumor but can give rise to invasive ductal carcinoma (IDC). DCIS is characterized by hyperproliferation of mammary duct cells. These cells however still resemble normal cells and have not

broken through the local basement membrane. Invasive carcinomas on the other hand are highly dyplastic, lose the normal characteristics of the mammary epithelial cells, and invade through the local basement membrane. Invasive carcinomas are divided into 4 stages, increasing in tumor aggressiveness and poor patient outcomes. The components of the staging scoring system are tumor size (T), lymph node positivity (N), and presence of distant metastases (M). The scoring is defined in the TNM system and results in stage definitions for breast cancer, which guides treatment and predicts patient survival(Edge, 2010; Singletary et al., 2002). In Stage I breast cancer, the tumor is localized to the breast and small (less than 2 cm). In Stage II breast cancer the tumor may have spread to regional lymph nodes (up to 3 axillary nodes) and can be up to 5 cm (2 cm if lymph nodes are positive for tumor). In Stage III breast cancer the tumor has spread to regional lymph nodes and is larger than 5 cm (unless there are more than 4 cancer positive lymph nodes) and may have spread to the chest wall and skin of the breast. In Stage IV breast cancer the cancer has spread to distant organs. Outcome correlates with staging, with Stage IV cancers having by far the worst prognosis. The main impetus for this work is to understand the mechanisms by which metastatic tumors arise, with the hope that this knowledge will aid the development of cancer diagnostics and therapeutics.

1.2 Tumor Etiology and Characterization

Where and how cancer arises have been questions asked for millennia. The term ‘cancer’ stems from the initial description of a solid tumor which when viewed spread open, appears with the blood vessels with like the likeness of a crab. Hippocrates and then Galen posited that cancer was a disease caused by an excess of black bile, one of the four *humors*, or bodily fluids. During the 19th century Rudolf

Virchow, among others, led a change in thinking, positing that all cells begat cells; a theory that when extrapolated, meant cancer was a disease of cells. Since then our knowledge of the etiology of cancer grown enormously and the corresponding diagnoses and treatments have transformed. As we have gained more insight into how normal cells work, we have concurrently began to understand how the mechanisms of development and homeostasis go awry during disease. Molecular biology has transformed our understanding of cancer, which as we understand it now, is primarily a genetic disease. Oncogenes and tumor suppressors, were thought to perhaps hold the key to curing cancer. However, as we enter the age of large scale data, we have begun to appreciate how complex cancers and in fact, different they are on an individual basis. The Cancer Genome Atlas (TCGA) project has analyzed 825 breast tumors with matched germ-line tissue, but only identified 3 genes containing somatic mutations in more than 10% of cases(TCGA, 2012). While these three genes, TP53, GATA3, and PIK3CA are potent tumor supressors and oncogenes, this represents a vast minority of breast cancers. Indeed, multi-sampling per patient revealed two tumors from a single patient were more similar than tumors from different patients, indicating great variation between tumor even tumors of the same type(Perou et al., 2000). As such molecular characterization will be critical going forward. Indeed, it has already led to significant advances. Breast cancer can now be classified into four main categories based on the expression of human epidermal growth factors receptor 2 (HER2/ERBB2), estrogen receptor (ER), and progesterone receptor (PR): luminal A, luminal B, HER2 enriched/ER-negative, and basal-like/triple-negative. Patients with luminal A subtyped tumors have the best outcomes, with the lowest risk of recurrence(Voduc et al., 2010). Patients with basal-like/triple-negative tumors, without detectable expression of any of

the three receptors, have the poorest outcomes and constitute approximately 15-20% of all breast cancers(Carey et al., 2006).

Triple negative breast cancers (TNBC) have poorer clinical outcomes and cannot be treated using anti-receptor therapeutics. Most TNBCs are characterized as basal-like, which resemble the basal cells surrounding mammary ducts. Molecular characterization of breast cancers confirms this observation, with TNBC being described as 'basal-like' based on expression profiling(Perou, 2011). Other sub-groups including luminal A and B have comparably favorable outcomes and are responsive to therapeutics that specifically target those receptors. Expression of these receptors and other molecular markers such as the proliferation marker Ki-67 can be used to risk-stratify breast cancer patients. Furthermore, tumor specific therapeutics such as biologics including anti-HER2 monoclonal antibodies, small molecule inhibitors including lapatinib, and hormone antagonists such as tamoxifen can be employed to treat patients. These targeted therapeutics have had a great impact on treatment and have improved clinical outcomes in breast cancer. Despite all we have learned and the great advances since the molecular biology revolution, few cancers are curable and the 5 year survival for metastatic breast cancer remains below 25%(Howlader et al., 2011). However, from a number of examples, it is clear that further understanding of the molecular and cellular events that underlie this disease can give rise to treatments that can cure or turn cancer into a chronic disease.

Recently, molecular and cellular based understanding of cancer has led to the development of effective targeted treatments. These treatments thus far have been mostly aimed at critical processes such as angiogenesis, cell survival, and DNA replication. This approach leverages our knowledge and the postulation that there are

'hallmarks of cancer' which are critical for tumorigenesis(Hanahan and Weinberg, 2000). These hallmarks describe attributes that a cell acquires en route to becoming an invasive cancer cell. It is well recognized that carcinogenesis is a multi-step process that requires a cell to overcome various checkpoints to become transformed. For example, a cell must acquire the ability to sustain unconstrained proliferation, avoid cell-induced apoptosis, recruit an oxygen source via angiogenesis, and acquire invasive and metastatic properties(Hanahan and Weinberg, 2000). Thus, preventing or inhibiting the action of one or more of these hallmarks could be an effective approach to attack a tumor. Because these processes are often driven by cell-intrinsic changes, such as amplification and nucleotide mutation, they can be amenable to inhibition. For example, trastuzumab, a humanized monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), prevents uncontrolled cell proliferation driven by abherrant activation of HER2. This new generation of treatments has been used in conjunction with more traditional treatments including surgery, chemotherapy, and radiation treatments. Further understanding of the effects and interactions of these individual treatments will only improve our ability to combat the disease. Notably, current therapies have had some success in treating local disease in which the tumor has not spread to secondary sites but to a much lesser extent for metastatic disease.

1.3 Breast Cancer Metastasis

Metastatic burden is the cause of mortality in most breast cancer patients. The current prevailing model for metastatic seeding is that tumor cells disseminate from the primary tumor and travel through tissue, lymph vessels, or blood vessels to eventually seed secondary tumors. This process is mediated through dynamic re-activation of a developmental program termed Epithelial-to-Mesenchymal Transition (EMT) and

subsequent cellular reversion via Mesenchymal-to-Epithelial Transition (MET)(Brabletz et al., 2005; Ocana et al., 2012; Thiery et al., 2009; Tsai et al., 2012; Tsai and Yang, 2013; Yang and Weinberg, 2008). Once tumor cells have invaded through the local basement membrane into the surrounding fatty tissue in the breast they are able to migrate from the primary site and seed secondary sites. Tumor cells, once out of the primary site, can intravasate into lymph and blood vessels to later extravasate at distant sites (Figure 1-1). Extravasated tumor cells can then colonize those distant sites to form micrometastases and later macrometastases after they have re-activated their hyperproliferative abilities. Thus, to successfully metastasize, tumor cells must complete a complex multi-step process.

Common breast cancer metastasis sites include the lymph node, bone, brain, liver, and lung. It is clear however that signals from the tumor microenvironment can influence this process(Nguyen et al., 2009). It is not currently fully understood how or why different tumors will metastasize to different secondary organs, however. For example, while tumor cells most certainly disseminate from the primary tumor into the lymph and vasculature, it is unclear whether lymph node metastases can also signficiantly contribute to distint site metastases. Moreover, when and how tumor dissemination occurs remain contentious issues. Do tumor cells begin to disseminate early in tumorigenesis or later, after the primary tumor has been fully established? What are the main drivers of a metastatic program; cell intrinsic signals such as genetic mutations or signals from the tumor microenvironment? Despite the recognition that patients most often succumb to metastatic disease rather than the primary tumor, there are no treatments available to effectively treat metastatic disease. A more complete view of tumor metastasis at the physiological, cellular, and molecular level will certainly aid in the treatment and development of new treatments for metastatic disease. The

goal of these treatments will be first to able to stabilize the disease, rendering metastatic cancer a chronic condition and then ultimately be able to eradicate tumor cells.

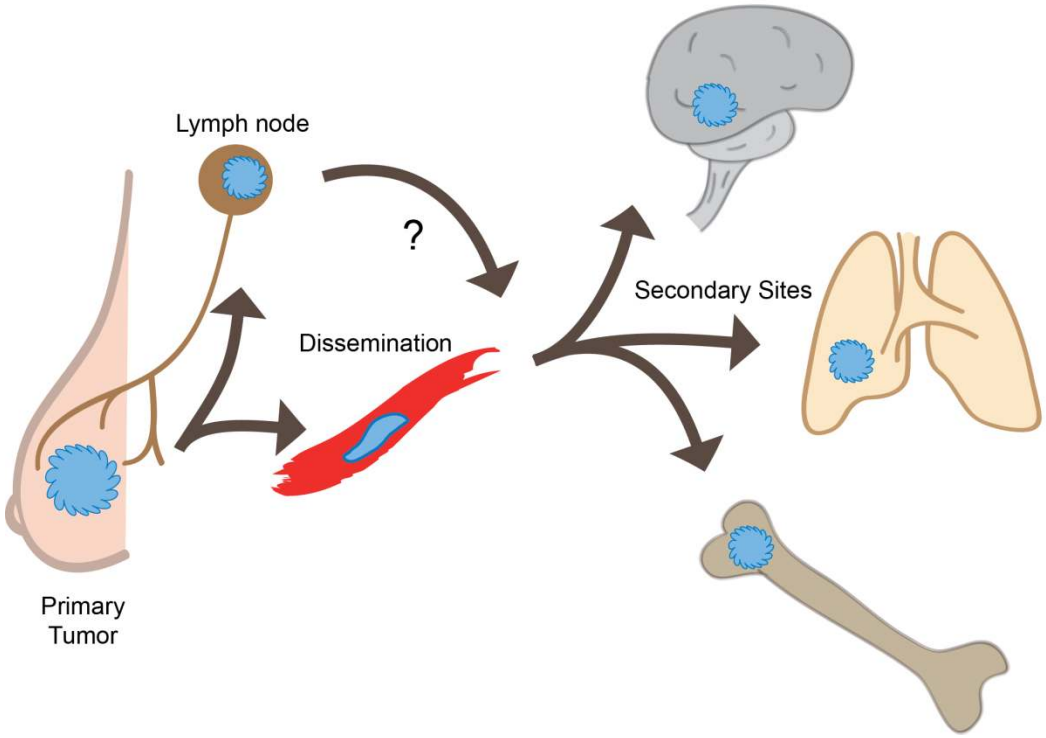

Figure 1-1. Schematic of breast tumor metastasis. Cells disseminate from the primary tumor through vascular and lymph systems. It is currently unclear whether tumor cells from the lymph node metastases also give rise to metastases in distant sites. Common sites of breast cancer metastasis are the brain, lung, liver, and bone.

1.4 The Tumor Microenvironment

How and when metastasis occurs during tumorigenesis has been a difficult question to completely answer, but it is clear now that signals from the microenvironment regulate the metastatic cascade in concert with cell intrinsic changes. As we have learned more about the mechanisms governing this process, the more complex it appears. Even since the relatively recent conception of the discussed 'hallmarks of cancer', it has been become clear that additional inputs, both cell intrinsic and extrinsic,

have significant contributions to tumor progression. Processes such as inflammation, immune surveillance, and cellular energetics have been more recently included as 'emerging hallmarks'(Hanahan and Weinberg, 2011). Input from the tumor microenvironment has been increasingly recognized as extremely influential in directing and facilitating tumor progression. The tumor microenvironment not only provides the context for cellular behavior based on cell intrinsic signals but also can act as a primary contributor. The tumor microenvironment is composed of tumor cells, stromal cells, and extracellular matrix (ECM) – all of which can contribute to tumor progression. The contribution of the extracellular milieu remains relatively unclear, likely due to its heterogeneous nature. In some cases the environmental context can 're-educate' tumor cells. In fact Dolberg and Bissell demonstrated that cues from the extracellular mileau of the avian embryo were sufficient to suppress the robust tumorigenic properties of Rous Sarcoma Virus(Dolberg and Bissell, 1984). This suppression was specific to the microenvironment as dissociation of the same cells from the tissue would release the cells transformed phenotype(Dolberg and Bissell, 1984; Stoker et al., 1990). Biochemical signals from the microenvironment, later identified as TGF-β, were able to overcome suppressive signals from the microenvironment(Sieweke et al., 1990). Many other pro- and anti-tumorigenic microenvironmental signals have since been described. Stromal cells such as cancer associated fibroblasts (CAFs) and tumor associated macrophages (TAMs) have been identified as pro-tumorigenic stromal cells, while other stromal cells such as tumor infiltrating leukocytes (TILs) can be anti-tumorigenic(Hanahan and Weinberg, 2011). For example, CAFs deposit and remodel surrounding ECM in addition to secreting potent cytokines(Orimo et al., 2005). Mammary fibroblasts can influence tumor cell behavior through secretion of soluble factors as well as modify tumor cell responses to changes in the ECM such as ligand

density(Luhr et al., 2012). CAFs can originate from multiple sources, such as stellate cells and adipocytes, which deposit high levels of fibronectin and collagen I(Bochet et al., 2013). The extracellular milieu also contains soluble and latent growth factors such as Transforming Growth Factor beta (TGF-β) which can affect tumorigenesis(Annes et al., 2003). During reorganization, catabolism and anabolism, of the ECM latent molecules stored in the matrix can be released. Interestingly, TGF-β can induce matrix remodeling, suggesting the existence of potential feed-forward mechanisms(Baldwin et al., 2014). Matrix remodeling clearly has a large role during tumor progression, as tumor cells are exposed to and influence processes including inflammation, fibrosis, and angiogenesis. For example, invasive ductal carcinomas with large fibrotic foci (a marker of increased tissue rigidity) and atypical stromal fibroblasts (i.e. CAFs) correlate with significantly poorer outcome(Hasebe et al., 2011). Understanding how these and other extracellular signals regulate tumor cell activity and interact with intrinsic changes in tumor cells such as epigenetic and genetic alterations will be critical for future research and therapeutic development.

1.5 Tissue Rigidity in Breast Tumors

Recently, the mechanical properties of tumors have been shown to have active roles in regulating tumor progression. This makes intuitive sense as breast tumors have been identified by manual palpation since ancient times as tumors appear as hard lumps compared to normal tissue. Indeed, over 95% of primary breast cancers are palpable and perhaps more interesting, in 55% of tumors palpation was the only clinical indication(Mahoney and Csima, 1982). Interestingly, in a long-term recent study on the efficacy of mammograms, 50% of non-palpable tumors were overdiagnosed(Miller et al., 2014). This raises the possibility that tumors of low stiffness do not pose as great of a

risk, although this observation may be due to a number of other variables. This suggests that the changes in the mechanical properties in and around the tumor may be a critical and early event during carcinogenesis. Also lending support to this idea is the fact that increased mammographic breast density is a risk factor for women, with density correlating with variables that include tumor size, lymphatic and vascular invasion(Aiello et al., 2005). However, whether density has an active role in promoting tumor progression remains relatively unclear. There is increasing evidence that high breast density not only influences breast cancer outcomes tangentially by obscuring mammographic screening, but also contributes directly to tumorigenesis(Kolb et al., 2002). Increased mammographic breast density is caused by the relative increase in glandular and fibrous connective tissue compared to the adipose tissue that normally comprises much of the breast.

Breast density and the hardness of the tissue or 'tissue rigidity' are tightly linked as the fibrous tissue has greatly increased tissue stiffness. Indeed, the presence of fibrotic foci, dense clusters of fibroblasts and collagen fibers, within a breast tumor can be used as a metastatic marker(Colpaert et al., 2001). Furthermore, the higher the ratio of foci per tumor, the worse the prognosis was – indicating that the effect of fibrotic foci is additive, supportive of a scalable response. In addition to correlating with poor prognosis, the presence of fibrotic foci correlated significantly with proliferative index, tumor size, and grade. Fibrotic foci also have prospective predictive power in invasive ductal carcinomas, correlating significantly with tumor recurrence and distant metastasis(Hasebe et al., 2002). Because fibrotic foci are composed of dense clusters of collagen fibers and fibroblasts, the presence and organization of these factors can be used as a surrogate readout for increased tissue stiffness. Collagen deposition and fiber formation are a main source of the mechanical rigidity in tumors and can in fact promote

breast cancer cell invasion(Provenzano et al., 2008). Furthermore, dramatic changes in collagen expression and thus organization can be observed during tumor progression(Kauppila et al., 1998). The stiffness, or elasticity, of normal mammary glands is approximately 170 Pa while that of an average breast tumor is above 5000 Pa. How cells sense, respond to, and modulate the dramatic range of tissue stiffnesses within the body remains to be fully understood (Table 1-1). Notably, the elasticity of glass and plastic, common substrates for cell culture, are well outside of the range of physiological substrates, raising the question of whether and how this difference may affect cellular behavior.

Table 1-1. Elastic moduli of relevant tissues and materials. The elastic moduli of various tissue and substrates used in biological applications. Adapted from (Butcher et al., 2009; Engler et al., 2006; Paszek et al., 2005).

Substrate	Approximate Stiffness (Pa)
Blood, fluid	<100
Neuron	100
Mammary Gland	170
Reconsituted Basement Membrane	175
Brain	1,000
Collagen (4.0mg/ml)	1,600
Average Breast Tumor	5,000
Muscle	10,000
Osteoblast	20,000
Collagenous Bone	100,000
Plastic	2.78×10^9
Glass	69×10^9

Indentation atomic force microscopy force mapping of human and mouse breast tumors indicates that the rigidity of invasive tumors is highly heterogenous. Furthermore, cancer tissue is significantly stiffer (1-2 kPa) than the surrounding adipose

tissue (~300 Pa)(Plodinec et al., 2012). Consistent with this observation malignant murine mammary epithelium is significantly stiffer than the corresponding tissue(Lopez et al., 2011). Thus, while tissue stiffness has been implicated as having a critical role in breast cancer progression, whether it plays an active role and what that role is has remained unknown.

Interestingly, DCIS does not usually present with a palpable mass. Most commonly DCIS is diagnosed because of an abnormal mass detected by radiographic mammography(Dershaw et al., 1989). In fact, 70 to 80% of DCIS cases are diagnosed by radiographic evidence alone without the presence of a palpable mass(Fonseca et al., 1997). This raises the possibility that increasing tissue rigidity may be a later event during carcinogenesis which promotes the acquisition of invasive properties. Consistent with this hypothesis dramatic modifications to the ECM can be observed through tumor progression(Burke et al., 2013; Provenzano et al., 2008). In fact, contribution to the tumor microenvironment by stromal cells such as cancer-associated fibroblasts (CAFs) can modulate the invasiveness of tumor cells(Dumont et al., 2013). Thus reorganization of the mammary gland microenvironment is likely carried out coordinately by co-opted resident and recruited stromal cells as well as tumor cells themselves. Feed forward mechanisms likely exist to promote ECM reorganization. CAFs present one such example, as they contribute to the deposition and modification of collagen fibers but themselves are highly responsive to changes in matrix stiffness(Calvo et al., 2013; Dumont et al., 2013).

Fibrosis and increasing tissue rigidity are tightly linked phenomena. Both processes involved dramatic reorganization of the ECM which results in the formation of fibers of ECM components, most notably collagen. In the case of fibrosis, ECM

remodeling results in scarring and large regions of relatively acellular dense ECM. Fibrosis itself can also drive tumor progression and enhance the metastatic potential of tumors. However, this effect may be mediated through lysyl oxidase (LOX) catalysis of collagen crosslinking, suggesting that fibrosis promotes metastasis, at least in part, by stiffening the matrix(Cox et al., 2013). In the context of breast carcinomas, increasing tissue rigidity is largely the result of the formation of organized collagen fibers. Indeed, the presence of fibrotic foci can be used as a surrogate readout for increasing matrix stiffness and the accompanying poor survival outcomes(Colpaert et al., 2001; Hasebe et al., 2002; Van den Eynden et al., 2007). It is difficult to separate these phenomena in the context of human carcinomas *in vivo* however data derived from mouse models and *in vitro* models suggests that increasing matrix stiffness has a potent effect in isolation(Levental et al., 2009; Paszek et al., 2005; Samuel et al., 2011). This suggests that other properties of fibrosis such as inflammation do not constitute the bulk of the effect on tumor progression.

1.6 The Functional Consequences of Changes in Matrix Stiffness

Matrix stiffness has been shown to have potent effects in a variety on contexts ranging from stem cell differentiation to cancer metastasis. Contextual cues from the microenvironment have long been thought to regulate cell behavior. Weiss and Garber demonstrated in 1952 that the physical ‘textures’ of clots could regulate heart fibroblast migration(Weiss and Garber, 1952). Since then many groups have built on this and other pioneering work, and demonstrated a critical role for physical cues from the microenvironment. It was recently demonstrated that matrix stiffness could direct mesenchymal stem cells (MSCs) towards particular lineages, independent of other signals(Engler et al., 2006). Interestingly, MSCs differentiated according to the rigidity of

the host organ. For example, on substrates of low stiffness similar to that of brain, MSCs differentiated towards a neuronal lineage while on a harder substrate similar to that of muscle, MSCs became myogenic. Similar observations have been made in other contexts such as adipocyte hormone secretion, in which matrix compliance and cellular tensile stress affect key biological outputs(Ghosh et al., 2013). Interaction with the basal lamina and ECM regulates mammary acini functions such as β-casein expression(Alcaraz et al., 2008). In the disease context, misregulation of ECM deposition and modification as well as of cellular mechanosensors can have profound consequences. The main cellular mechanosensors for changes in matrix stiffness are integrins. Integrins form heterodimers composed of one α and one β subunit, with the combination defining matrix ligand specificity. Because collagen is a main source of ECM mechanical integrity, β1 integrin, which is essential for collagen recognition, is critical for mechanosensing.

β1 integrin is critical for many biological process and as such complete loss of β1 integrin is embryonic lethal(Fassler and Meyer, 1995). Conditional knockout of β1 integrin have demonstrated a key role during mammary gland development(Faraldo et al., 1998). Deletion of β1 integrin from the basal cells of mammary glands, which are exposed to the ECM, leads to significant defects in mammary gland development and homeostasis(Taddei et al., 2008). Interestingly, deletion of β1 integrin from luminal cells of the mammary gland also leads to dramatic effects on mammary gland development(Li et al., 2005). Indeed, in organotypic cultures of mammary epithelial cells, mechanical cues have been shown to have profound effects on branching morphogenesis(Nelson et al., 2006). Furthermore, loss of β1 integrin dramatically attenuates tumor progression in the MMTV-polyoma middle T driven transgenic mouse model(White et al., 2004). Many of these effects likely stem from β1 integrin's interaction with the basement membrane.

Blockage of β1 integrin by antibodies induces apoptosis in mammary epithelial cells by simulating loss of contact with basement membrane(Boudreau et al., 1995). These and other studies have demonstrated a key role for collagen and mechanical cues in the mammary gland. β1 integrin has critical mechanosensory functions in other organ systems as well, demonstrating this is a conserved and essential process. β1 integrin activation by mechanical cues induces focal adhesion formation and mediates a mechanosensory cellular response(Wang et al., 1993). This effect is dependent on the adhesion function as non-adhesive receptors were unable to transduce mechanical signals. Notably, the unique biology of each organ system defines the mechanism and response to mechanical cues using similar mechanosensory systems. For example, pharmacologic inhibition of mechanosensing has a positive effect in ameliorating experimental pulmonary fibrosis while loss of mechanosensing function via loss of β1 in urothelium renders mice incontinent(Kanasaki et al., 2013; Zhou et al., 2013). Elucidating how mechanical signals are processed by different cell types to produce and regulate distinct biological processes will be critical in our understanding of disease progression and development of therapeutic approaches.

Mechanical cues have critical roles in most biological processes, notably embryogenesis, adult tissue homeostasis, and tumor metastasis. Because of a number of lines of evidence supported a role of mechano-regulation of EMT, as will be discussed in further depth in Chapter 2, it seemed feasible that matrix stiffness could drive tumor metastasis through induction of EMT. As EMT is activated during both development and tumor progression, mechanoregulation could be a conserved regulatory process.

1.7 Epithelial Mesenchymal Transition

EMT is a developmental process that is aberrantly activated during tumor progression. Elizabeth Hay first coined the phrase 'epithelial-mesenchymal transition' and later demonstrated some of the first cellular consequences of this program(Hay, 1995). In both the context of disease and development EMT allows for the acquisition of invasive traits and facilitates cell migration. EMT was first described as a critical process during gastrulation in which cells invaginate from the primitive streak to form the mesoderm from the primary epithelium(Acloque et al., 2009; Hay, 1995; Nieto, 2011; Thiery et al., 2009; Viebahn, 1995; Yang and Weinberg, 2008). This primary EMT event during development is critical for the formation of mesoderm from the primitive streak as well as for neural crest migration. The secondary mesenchyme from the ectoderm, gives rise to a variety of tissues that include the dermis, satellite cells, pancreatic endocrine cells, melanocytes, smooth muscle, and craniofacial tissue (Duband et al., 1995; Thiery et al., 2009; Yang and Weinberg, 2008). EMT is again involved later during development, playing a critical role in the formation of tissues including heart valve and cushion and secondary palate formation(Yang and Weinberg, 2008). This primary EMT event during early development gives rise to mesenchymal tissues. A secondary EMT event occurs during later stages of development and in adult tissues generates fibroblasts and stromal tissues(Zeisberg and Neilson, 2009). In both contexts EMT is primarily characterized as the loss of epithelial attributes including strong cell-cell adhesions and apical basal polarity with a concurrent gain of mesenchymal attributes including fibroblast like morphology and migratory and invasive properties (Figure 1-2).

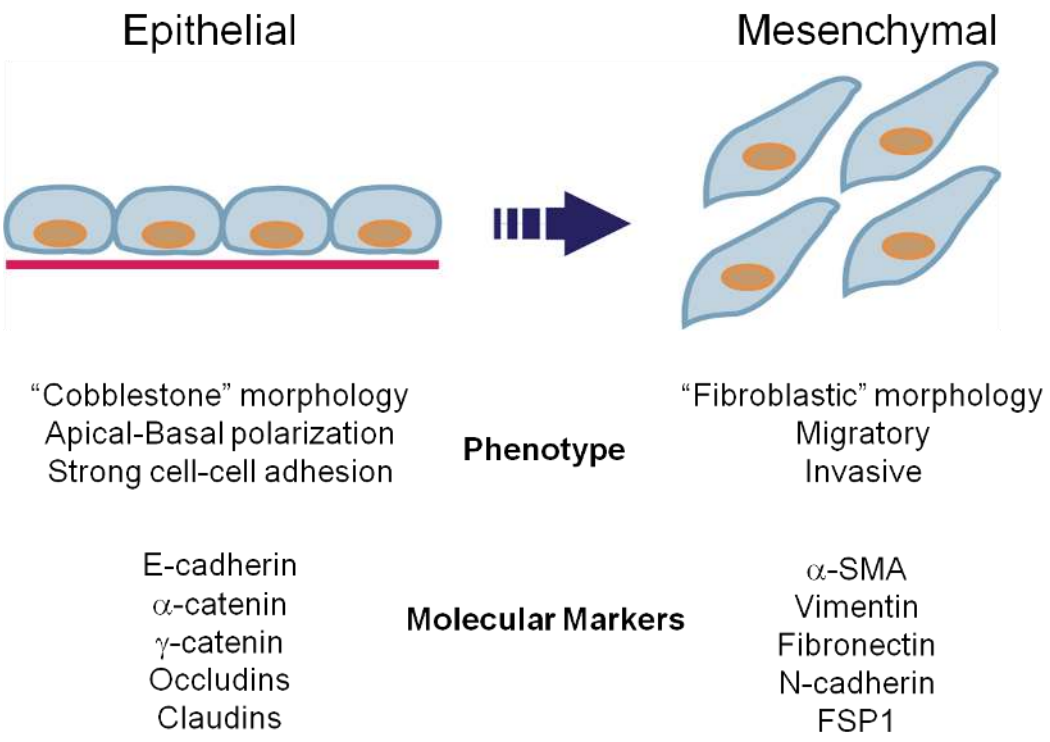

Figure 1-2. Schematic of EMT. Cellular phenotypic, morphological, and behavioral traits are accompanied by molecular changes during EMT including a downregulation of epithelial molecular markers and an upregulation of mesenchymal molecular markers.

Epithelial cells *in vivo* form sheets with strong cell-cell contacts mediated by adherens junctions, tight junctions, and desmosomes as well as gap junctions which mediate intercellular communication. These single cell thick sheets are crucial for barrier formation, secretion, and absorption in tissues such as colon, mammary duct, skin, and bronchial epithelium. These functions rely on strict regulation of junctions as well as polarity, each of which is defined by a variety of molecules. Adherens junctions are composed of transmembrane cadherins and a complex of catenins which binds to the cytoplasmic tail of cadherins and signals to the acin cytoskeleton(Harris and Tepass, 2010). This allows homophilic interaction between cadherins, to tightly connect neighboring cells and regulate cell contractility and shape. Tight junctions are composed

of occludin and claudin proteins and ensure the formation of a water tight barrier, which is critical for the maintenance of ion gradients and the transepithelial electrical resistance(Schneeberger and Lynch, 2004). Gap junctions mainly consist of connexin proteins and allow for the diffusion of small molecules less than 1 kDa, which mediates cell-cell communication(Giepmans, 2004). Apical-basal polarity in epithelial cells is critical to determine the directionality of absorption, secretion, and gradient formation. Polarity is defined by three main complexes constituted of Crumbs, Par, and Scribble proteins among others(Moreno-Bueno et al., 2008). Together these complexes mediate a variety of epithelial functions within an epithelial layer including maintenance of sheet integrity, ion gradient regulation, small molecule diffusion, absorption, and secretion. During EMT, these epithelial structures and functions are lost due in large part to the downregulation of the molecules that define these structures. Thus, epithelial markers include adherens junction proteins such as E-cadherin, α-catenin, β-catenin, γ-catenin, tight junction proteins such as occludins and claudins, gap junction proteins such as connexins, and polarity proteins such as Crumbs, Par, and Scribble. Concomitant with the loss of epithelial characteristics and associated molecular factors is the gain of mesenchymal characteristics and molecules.

Mesenchymal cells are fibroblastic in nature with spindle like morphology and generally increased migratory and invasive capabilities. EMT gives rise to mesenchymal cells such as fibroblasts that constitute the connective tissue surrounding epithelium. These cells contribute to the generation and remodeling of the ECM by secreting proteins such as fibronectin, proteases such as matrix metalloproteases, and growth factors such as EGF(Acloque et al., 2009; Radisky, 2005; Yang and Weinberg, 2008). They also undergo a switch in cadherin expression, downregulating E-cadherin which is replaced with N-cadherin. This cadherin switching allows for the separation of cells from

epithelial sheets and subsequent migration, which occurs during development and then aberrantly during tumor metastasis(Wheelock et al., 2008). Expression of intermediate filaments, mainly vimentin, is also dramatically increased during EMT, which facilitates cell adhesion and migration and perhaps contributes yet to be elucidated processes(Mendez et al., 2010). Thus EMT is associated with an increase in expression of canonical mesenchymal markers including ECM proteins such as fibronectin, fibroblast-specific proteins such as FSP1/S100A4, mesenchymal cadherins such as N-cadherin and cadherin11, and intermediate filaments such as vimentin.

A variety of signals have been shown to induce EMT including growth factors, hypoxia, morphogens, and inflammation(Yang and Weinberg, 2008). Soluble growth factors including Fibroblast growth factor (FGF), Wnt, and Epidermal growth factor (EGF) and TGF-β have been shown to induce EMT(Ciruna and Rossant, 2001; Garcia-Castro et al., 2002; Lu et al., 2003; Oft et al., 1998; Shah et al., 1997; Yang and Weinberg, 2008). Because EMT is a critical process during development and tissue homeostasis, it must be tightly regulated both at the cell and tissue level. Thus it is logical that intercellular signaling through transmembrane molecules such as Notch also regulate EMT. Furthermore, signals from the microenvironment including hypoxia and inflammation can also induce EMT(Timmerman et al., 2004; Zavadil et al., 2004). Hypoxia acts through activation of the transcription factor hypoxia inducible factor -1α (HIF-1α)(Krishnamachary et al., 2006; Yang et al., 2008). Inflammatory signaling through the NF-κB signaling pathway is also able to induce EMT(Lopez-Novoa and Nieto, 2009). Cellular stresses such as oxidative stress also can induce EMT and may be involved as downstream mechanisms in a variety of pro-EMT signaling pathways(Radisky et al., 2005). Interestingly, expression of MMP-3 is sufficient to induce EMT, suggesting that remodeling of the ECM and/or cell surface proteins also

play a significant role in regulating EMT(Lochter et al., 1997a; Lochter et al., 1997b). At a larger scale, MMP-3 promotes mammary carcinogenesis, further supporting its role in tumor progression(Sternlicht et al., 1999). These signals can act in a combinatorial and synergistic fashion to induce EMT(Grande et al., 2002; Higgins et al., 2007; Thiery and Sleeman, 2006). It is clear that regulation of EMT is a complex process that interprets signals from multiple sources. Through identification of permissive and non-permissive signals and deciphering how they interact together, we will be able to more clearly understand regulation of EMT in development as well as disease.

1.8 The Relationship between EMT and Tumor Mechanics

Previous studies have demonstrated that increases in matrix stiffness, or tissue rigidity, could induce a 'malignant phenotype' in mammary epithelial cells. Indeed, it was shown over 20 years ago that EMT could be induced, at least in part, by collagen (a main contributor to matrix stiffness) and that this effect was dependent on β1 integrin(Valles et al., 1996; Zuk et al., 1989). These and other studies support the original hypothesis posulated by Elizabeth Hay, that pathologic changes of the microenvironment could potentially induce EMT and drive the development of invasive carcinomas(Hay, 1995). In fact, Hay and colleagues demonstrated that cell interaction with the ECM could induce EMT(Greenburg and Hay, 1982, 1986). Interestingly, different components of the ECM have widely varying effects on tumors. An intact basement membrane suppressed apoptosis while exposure to other ECM molecules fibronectin and collagen were not sufficient to do so(Boudreau et al., 1995). Despite advances in our knowledge since these pioneering studies, we still do not understand how mechanical signals from the tumor microenvironment lead to transcriptional regulation and subsequent changes in cell behavior. More generally, the mechanisms

by which EMT is regulated during the metastatic cascade remains unclear. While it is generally accepted that EMT underlies the acquisition of migratory and invasive properties by transformed epithelial cells and tumor cells to facilitate their dissemination, how EMT is induced in the primary tumor and subsequently reversed at the secondary site is relatively unknown. Recent reports suggest a dynamic regulation of EMT and MET during tumor metastasis through precise modulation of transcription factors involved in EMT regulation(Ocana et al., 2012; Stankic et al., 2013; Tsai et al., 2012). Further investigation of the complex regulation of EMT by these transcription factors is critical for our understanding of the metastatic cascade.

1.9 Transcriptional Regulation of EMT

EMT is regulated by a network of transcription factors in both the developmental and cancer settings that includes Twist1, Twist2, Snail1, Snail2, Zeb1, Zeb2/SIP1, FOXC1, and FOXC2(De Craene et al., 2005; Fang et al., 2011; Ikenouchi et al., 2003; LaBonne and Bronner-Fraser, 2000; Mani et al., 2007; Nieto et al., 1994; Yang et al., 2004). These factors operate in a number of feedback and feed-forward signaling loops to form a complex signaling network(Sandmann et al., 2007; Thiery and Sleeman, 2006). These transcription factors act through a multitude of signaling pathways to downregulate epithelial genes, most importantly, the epithelial gatekeeper gene, E-cadherin. Loss of E-cadherin facilitates the transition from benign to invasive tumors and has a critical role in maintaining epithelial identity(Frixen et al., 1991; Perl et al., 1998). Early studies which have been since validated numerous times demonstrated an inverse correlation between E-cadherin expression and tumor invasiveness(Schipper et al., 1991). Some of the transcription factors such as Snail1, Snail2, Zeb1, and Zeb2 directly bind to the E-cadherin promoter to repress its expression(Batlle et al., 2000b;

Cano et al., 2000b; Comijn et al., 2001; Eger et al., 2005; Grooteclaes and Frisch, 2000; Hajra et al., 2002a; Vandewalle et al., 2005). Others, including Twist1 activate secondary transcription factors and other targets to induce the EMT program. Twist1 can signal through Snail2 to down-regulate E-cadherin for example(Casas et al., 2011). In the context of *Xenopus* development Snail can regulate other EMT factors including Twist and Slug(Aybar et al., 2003). This highlights the dynamic nature of regulation of EMT transcription factors depending on cellular context as well as the complex interactions between them. Elucidation of the intracellular and extracellular cues that modulate these interactions and regulatory mechanisms will be critical to our understanding of EMT in development and disease. Of the EMT-inducing transcription factors, Twist1 has been shown to be responsive to mechanical cues during *Drosophila* development and thus we hypothesized that Twist1 could link transcriptional regulation and EMT with increasing matrix stiffness during tumor progression(Desprat et al., 2008). This hypothesis will be elaborated upon and tested in Chapter 2.

1.10 The Transcription Factor Twist1

Twist1 is a potent inducer of EMT during both development and cancer(Yang et al., 2004). Twist1 is upregulated and correlates with poor survival in many cancers including breast, oral, glioblastoma mulitiforme, prostate, glioma, endometrial, bladder, gastric carcinoma, (da Silva et al., 2013; Elias et al., 2005; Kwok et al., 2005; Kyo et al., 2006; Mikheeva et al., 2010; Riaz et al., 2012; Ru et al., 2011; Zhang et al., 2007). Twist was first identified as a critical factor for dorsal-ventral patterning and gastrulation during *Drosophila* development(Leptin and Grunewald, 1990; Thisse et al., 1987; Thisse et al., 1988). The critical role Twist plays in mesoderm specification during gastrulation and neural crest migration is conserved in vertebrates(Chen and Behringer, 1995; Gitelman,

1997; Hopwood et al., 1989). Twist expression is restricted to the invaginating cells during gastrulation(Leptin and Grunewald, 1990). Twist induces EMT during gastrulation to facilitate cell migration, acting in concert with other factors. Twist defines the ventral furrow and the migratory cell population in concert with snail but is opposed by huckebein(Reuter and Leptin, 1994). Further evidence demonstrates that Snail and Twist act together to induce mesodermal differentiation, and that Twist induces Snail expression, forming the beginning of a transcriptional network(Ip et al., 1992). Twist is also involved during neural crest migration and the generation of the secondary mesenchyme, processes in which EMT plays a critical role(Chen and Behringer, 1995). Twist1 also has essential roles in the differentiation of multiple cells lineages including myogenic and osteogenic lineages(Bate et al., 1991; Hebrok et al., 1994; Lee et al., 1999). The potency of Twist induced changes in cellular behavior and identity have been observed in multiple contexts. It has even been shown that ectopic expression of Twist is sufficient to induce the development of a rudimentary neural crest in a non-vertebrate chordate(Abitua et al., 2012). This suggests a pivotal role for Twist homologs during evolution, as its activity as a mesenchymal determinant likely was repurposed to drive vertebrate development via emergence of the neural crest. Twist1 is also involved in later, more specific developmental events such as pericardial cushion development and differentiation decisions within the osteoblastic lineages(Bialek et al., 2004; Chakraborty et al., 2010; Firulli and Conway, 2008). Interestingly, despite how critical Twist1 is during mammalian development, depletion of Twist1 after birth is not lethal. In fact, loss of Twist1 in a conditional knockout mouse did not result in significant changes in weight, growth, or other characteristics – with the only detectable phenotype in the hair stem cell compartment(Xu et al., 2013). Twist1 is likely turned off following

development as its potency to induce EMT must be tightly regulated to prevent aberrant cellular migration and invasion.

Consisent with its essential role during development homozygous Twist1 mutations are embryonic lethal. In humans haploinsufficiency of Twist1 leads to Saethre-Chotzen Syndrome (SCS), which is characterized by craniofacial and limb abnormalities(el Ghouzzi et al., 1997; Howard et al., 1997). Heterozygous loss of Twist1 leads to a similar craniosynostosis in mice, supporting a conserved role for Twist1(Carver et al., 2002). Mutations observed in SCS patients ablate Twist1 activity through multiple methods including degradation, mislocalization, and inability to bind to DNA – consistent with its activity as a transcription factor(El Ghouzzi et al., 2000; El Ghouzzi et al., 2001). In fact, mutations that impaired Twist1 nuclear localization were located in the helix dimerization domain that allows for heterodimerization with E12. Furthermore, modulation of the ability of Twist1 to heterodimerize with other factors including Hand2 may drive SCS(Firulli et al., 2005). Indeed, there are additional examples of the regulation of Twist1 localization via heterodimerization, such as its interaction with TCF4(Singh and Gramolini, 2009).

Twist1 is a class II basic helix loop helix (bHLH) transcription factor. bHLH transcription factors are categorized into seven classes(Massari and Murre, 2000). Most pertinent to this work are Class I, Class II, and Class V bhlh transcription factors. Class I transcription factors are defined as ubiquitously expressed, so called E-proteins. This class includes E2-2, E12, and E47 among other transcription factors. Class II transcription factors have tissue specific expression and include factors such as MyoD and Twist. Together, Class I and Class II transcription factors can homodimerize and heterodimerize to regulate transcription in a sequence and tissue specific manner.

These interactions are critical during development for spatio-temporal and lineage defined gene expression. Sequence specific binding to DNA is mediated through the basic domain. These heterodimers bind to E-box sequences, defined as CANNTG(Murre et al., 1989b). This DNA binding activity and specificity is conferred by the basic domain, and was first observed in the bHLH transcription factor MyoD, but then proved to be a conserved function within the transcription factor family(Davis et al., 1990). Dimerization is mediated through helix-helix interaction, mainly through interaction of conserved hydrophobic residues(Murre et al., 1989a). Further DNA binding specificity is defined by the unique homo and heterodimers that are formed between Class I and Class II transcription factors. Consistent with this idea, mutations in the helix domain of Twist1 results in changes in DNA binding affinities(Firulli et al., 2007). This only makes sense in the context of dimerization partner choice, as the basic domain of Twist1 mediates its DNA binding activity. The activity of bHLH transcription factors, such as Twist1, is largely controlled through specificity derived from the particular dimer that is formed(Castanon et al., 2001). bHLH transcription factor heterodimerization allows Class II factors, which generally lack large transactivation domains, to interact and recruit Class I factors, which have transactivation domains(Massari and Murre, 2000). Murre and colleagues first postulated this upon identifying the interaction between Class I and II factors and their specific binding to E-boxes(Murre et al., 1989b).

A further layer of regulation is added by Class V bHLH transcription factors which lack the basic domain responsible for DNA binding but have the HLH domain sufficient for dimerization(Massari and Murre, 2000). Id proteins mainly constitute this class, the first of which was Id(Benezra et al., 1990). Because Class V factors lack the basic domain which is necessary for DNA binding but are able to form heterodimers, they

effectively inactivate bound Class I and II bHLH factors(Benezra et al., 1990). Thus, these dimerization partners form a pool of inhibitory molecules for Class I and II bHLH transcription factors. As a result, relative expression levels of Class V factors can regulate activity of Class II factors in a stochastic manner. As expression of Class V factors increases heterodimerization between Class I and II factors is titrated to low levels, thus decreasing transcriptional activation. Id proteins have distinct specificity for different E-proteins as well as class II factors, and furthermore have defined tissue specific expression patterns(Jen et al., 1997; Riechmann et al., 1994). Consistent with the role of this class to inhibit bHLH transcriptional activation, Id and Twist1 share similar expression patterns during murine development(Evans and O'Brien, 1993). This mode of regulation is not limited to development, and can mediate dominant negative-like regulation in the disease context. For example, expression of Id4 can directly inhibit the function of Twist1, leading to loss of Twist1-induced invasion in a glioblastoma model(Rahme and Israel, 2014). As will be discussed in depth in Chapter 2, Twist1 can be post-translationally modified through multiple mechanism to regulate its activity. It is clear that multiple mechanisms exist to regulate Twist1 activity, as well as other bHLH transcription factors including expression of positive (Class I factors) and negative (Class V factors) regulators and expression of Twist1 itself.

1.11 Downstream Effects of Twist1

Once active, Twist1 regulates the expression of a multitude of gene targets through a variety of mechanisms. Mammalian Twist1 was originally thought to be a negative regulator of transcription as it inhibits differentiation into multiple lineages, including myogenic and osteogenic lineages(Bialek et al., 2004; Hebrok et al., 1994; Spicer et al., 1996). Inhibition of myogenesis occurs through multiple mechanisms

including direct targeting of the basic domain of other bHLH transcription factors(Hamamori et al., 1997). Consistent with this idea Twist1 may have intrinsic transcriptional repressor activity, albeit relatively modest in potency(Vesuna et al., 2008). This inhibitory activity may also be mediated through dimerization with other Class II bHLH transcription factors, MyoD and MEF2, which are essential myogenic factors. This mode of inhibition mimics inhibition by Class V factors, as Twist1 does not contain a potent transactivation domain. Interestingly, it has also been reported that ectopic expression of Twist1 can induce transactivation of Mef2(Cripps et al., 1998). These seemingly conflicting reports may be due to the differences in Twist homologs. *Drosophila melanogaster* Twist is 490 amino acids while *Homo sapien and mus musculus* Twist1 are 201 and 206 amino acids, respectively. Many of the additional amino acids in *Drosophila* Twist constitute a glycine rich N-terminal domain that may have activity as a transcriptional transactivator. While mammalian Twist1 does not contain a well described transcriptional activation domain, it is now thought to mediate transcriptional activation. This is facilitated through heterodimerization and recruitment of Class I bHLH E-proteins which have potent transcriptional regulatory domains. Twist1 has also been reported to mediate transcriptional activation in concert with E12, via at least in part by using its own C-terminal transcriptional activation domain(Laursen et al., 2007). This result may also be explained however by a loss of functional dimerization as the C-terminal WR domain has also been reported to mediate interaction with transcriptional regulators(Lander et al., 2013). Indeed loss of the HLH domain resulted, as expected in a similar loss of transcriptional regulation(Laursen et al., 2007). This transcriptional activation activity can be observed in direct Twist1 targets including Snail2, Bmi-1, and YB-1(Casas et al., 2011; Shiota et al., 2008; Yang et al., 2010). Furthermore, consistent with its role as a transcriptional activator, Twist null *Drosophila*

embryos displayed downregulation of genes with conserved E-box binding sites(He et al., 2011).

It seems that the effect of Twist1 on transcription is highly variable and dependent on context. Nevertheless, its effects on cellular differentiation and behavior are striking. These effects are mediated through a large number of targets. Remarkably, during *Drosophila* mesoderm specification Twist targets directly targets approximately 25% of all annotated transcription factors, consistent with its critical role in early germ layer specification(Sandmann et al., 2007). Indeed, more in-depth analysis of Twist binding in six species of *Drosophila* revealed highly conserved Twist binding to enhancer regions(He et al., 2011). Twist1 also interacts with other transcriptional co-factors to regulate gene expression such as p300/PCAF(Hamamori et al., 1999). In this case, Twist1 inhibits the histone acetylation activity of p300. These and other activities mediate a genome-wide reorganization of epigenetic marks. The number of bivalent genes, which are poised to be expressed, increases two-fold upon Twist1-induced EMT(Malouf et al., 2013). Furthermore, based on digital restriction enzyme analysis of methylation, Twist1-induced EMT results in genome-wide hypomethylation and dramatic changes in H3K27me3 and H3K4me3, causing significant changes in gene expression, including in EMT-related genes such as CDH1 and PDGFRA(Malouf et al., 2013). Twist1 has also been reported to bind in a non-canonical mechanism to other EMT transcription factors, Snail1 and Snail2 via its C-terminal WR domain(Lander et al., 2013). Our understanding of Twist1-mediated transcriptional regulation is further complicated by the recent report that Twist1 directly interacts with the methyltransferases SET8(Yang et al., 2012a). This interaction facilitates H4K20 monomethylation, which is associated with both transcriptional activation and repression, depending on the context. Thus recruitment of SET8 as well as differential

heterodimerization with Class I, II, or V bHLH transcription factors may begin to explain the complicated nature of Twist1 transcriptional regulation. Together these and other studies indicate that Twist1 employs a variety of mechanisms to regulate gene expression at the genome-scale to induce the EMT program.

1.12 Regulation of Twist1

How Twist1 is regulated has been a source of intense investigation, however, remains a complex and yet not completely resolved issue. Twist1 can be regulated by extracellular cues including inflammation, hypoxia, and as will be discussed in Chapter II, mechanical forces. Some of the molecular events that regulate Twist1 in response to these cues have been elucidated in the contexts of disease as well as development. For example, hypoxia in the tumor microenvironment, via HIF-1α and HIF-2α, can modulate Twist1 induced EMT (Gort et al., 2008b; Yang et al., 2008). Other signals from the tumor microenvironment can also modulate Twist1 expression and activity. The relationship between Twist1 and inflammation is a common theme in both cancer and development. For example, Twist1 can be upregulated by chemokine signals such as IL-6(Sullivan et al., 2009). The NF-κB pathway can regulate Twist1 in a variety of contexts. During *Drosophila* development the NF-κB family member, Dorsal, induces Twist expression(Thisse and Thisse, 1992). This regulation is mediated through direct binding of Dorsal to the Twist promoter(Pan et al., 1991). Furthermore, Dorsal can syngergistically regulate transcriptional activation in combination with Twist(Shirokawa and Courey, 1997). In Th1 effector memory T cells Twist1 is induced downstream of NF-κB(Niesner et al., 2008). Twist is also regulated by Notch signaling during development via indirect repression of Twist, thus acting in opposition of Twist signaling(Tapanes-Castillo and Baylies, 2004). Twist1 expression can also be

modulated epigenetically. Direct binding of the histone methyltransferases MMSET/WHSC1 to the Twist1 locus leading to H3K36me2 can induce Twist1 expression(Ezponda et al., 2013). The Twist1 promoter has also been reported to be methylated in cancers, but it is unclear whether this has a functional consequence as promoter methylation, while enriched in malignant breast tumors, did not correlate with Twist1 mRNA or protein expression levels in human samples(Gort et al., 2008a). Twist1 expression can also be repressed post-transcriptionally by microRNAs including the miR-145, 151, and 337 families as well as microRNAs from the DLK1-DIO3 locus(Haga and Phinney, 2012; Nairismagi et al., 2013). Thus, we have a reasonable molecular understanding of the cell's wide array of mechanism to induce Twist1 and EMT. Despite this understanding we still lack a complete picture of how EMT is regulated during development tumor metastasis, in particular.

1.13 Conclusion

Breast cancer metastasis remains a relatively intractable problem. There are many open-ended questions which require answers for us to potentially solve this problem. First and perhaps most generally, does metastasis occur using the same mechanisms in breast cancer and other tumor types? And even if not, does metastasis occur using the same mechanisms in breast cancers of different patients? Recent molecular studies have indicated that tumors of all types are highly heterogenous, both in terms of within a single tumor and comparatively from tumor to tumor(Gerlinger et al., 2012). However, there appear to be choke points in cellular processes that tumor cells become dependent on. These processes might have alternate molecular mechanisms to accomplish the same cellular task, but understanding which pathways and processes are essential for metastasis will greatly facilitate our development of therapeutic

approaches. Can we identify what the chokepoints are during the metastatic cascade? And if so, will we have the tools to exploit these checkpoints and the diagnose when to utilize them? As we enter the era of molecular and genomic medicine, we will require ever more information that we can use to profile tumors and to subsequently identify and attack their weaknesses. The extent to which genetic information can inform on tumor behavior and breast cancer prognosis and treatment may be dependent on the involvement of the microenvironment. Importantly, we will need to understand how diagnostics that are able to probe mechanical and biochemical properties of the tumor microenvironment can be effectively used to augment standard of care diagnostics and classification regimes. For example, could increases in tumor tissue rigidity have a large effect in stage 3 breast cancers but not in stage 1 or 2? These and many other questions will have to be asked as our understanding and appreciation of the roles of the tumor microenvironment continues to grow. Understanding how various aspects of the microenvironment interacts with tumor cells of different genetic profiles and origins will allow for the effective development and subsequent use of appropriate therapeutics to treat breast cancer metastasis.

1.14 Acknowledgements

This work will be, in part, submitted for publication as an article titled “Between a Tumor and a Hard Place: The Relationship between Tissue Rigidity and EMT”. I was the primary contributor along with my coauthor Jing Yang.

Chapter 2

Matrix stiffness drives Epithelial-Mesenchymal Transition via a Twist1-G3BP2 mechanotransduction pathway

2.1 Introduction

Breast tumors are often detected by manual palpation, as they appear more rigid than their surrounding normal tissue. This increase in tissue rigidity, or matrix stiffness, is not merely a byproduct of tumorigenesis, and has been shown to play a significant role during tumor progression(Calvo et al., 2013; Leight et al., 2012; Levental et al., 2009; Paszek et al., 2005). Indeed, matrix stiffness plays a significant role in other key cellular processes such as differentiation and embryogenesis(Dupont et al., 2011; Engler et al., 2006; Jaalouk and Lammerding, 2009). Matrix stiffness is controlled by deposition and modification of extracellular matrix, especially collagen(Provenzano et al., 2006; Provenzano et al., 2008). Organized collagen fiber alignment, present in fibrotic foci, is a surrogate marker for increasing matrix stiffness in the tumor microenvironment. Furthermore, collagen alignment and fibrotic foci are associated with breast tumor progression(Colpaert et al., 2001; Conklin et al., 2011; Hasebe et al., 2002). How changes in the mechanical properties of extracellular matrix are sensed and then converted into biochemical responses to direct cell behavior remains unknown. Studies have shown that human mammary epithelial cells form normal ductal acini when grown on compliant matrices that recapitulate the stiffness of normal mammary glands. On matrices with increased rigidity similar to breast tumors, however, cells lose apical-basal polarity, form weaker junctions and invade through the basement membrane(Levental et al., 2009; Paszek et al., 2005). These cellular changes in response to increasing stiffness resemble many morphological features associated with EMT, a developmental

program also critical for tumor cell dissemination and metastasis(Thiery et al., 2009; Yang and Weinberg, 2008). During EMT, cells lose their epithelial characteristics, including cell junctions and polarity, and acquire a mesenchymal morphology and the ability to invade. Therefore, we set out to understand how matrix stiffness regulates the EMT molecular program to promote tumor invasion and metastasis.

2.2 Regulation of EMT by Matrix Stiffness

The EMT program is orchestrated through a network of transcription factors, including Twist1/2(Fang et al., 2011; Yang et al., 2004), Snail1/2(Batlle et al., 2000a; Cano et al., 2000a; Hajra et al., 2002b), and Zeb1/2(Comijn et al., 2001; Eger et al., 2005). Among them, Twist1, a key inducer of EMT and tumor metastasis, is induced by mechanical forces during Drosophila larval development(Desprat et al., 2008). So we asked whether mammalian Twist1 plays a key role in promoting EMT and invasion in response to increasing matrix stiffness. We employed a collagen-coated polyacrylamide (PA) hydrogel system with calibrated elastic moduli ranging from the ~150 Pascals (Pa) of normal mammary glands to the ~5700 Pa of breast tumor tissues(Johnson et al., 2007b; Paszek et al., 2005) in a 3D Matrigel overlay culture(Bissell et al., 2002; Debnath et al., 2003; Lee et al., 2007).

We employed this culture system for several reasons. First, in order to probe the cellular mechanosensory response, a system was required in which matrix stiffness be quantitatively modulated independent of other variables such as ECM ligand concentration, growth conditions, and substrate. Second, a system that could recapitulate as closely as possible, the acinar morphogenesis *in vivo* would allow for a more accurate interpretation of the role of matrix stiffness in tumor progression. Third, the system needed to be amenable to genetic, cell, and molecular biology applications.

A 3D overlay culture system on polyacrylamide (PA) hydrogels can satisfy these requirements and have been used previously for similar approaches. Acrylamide gels are fabricated utilizing acrylamide polymerization on top of which ECM molecules can be conjugated to allow for cell attachment. By varying the concentration of the crosslinker molecule bis-acrylamide, the elastic modulus of the PA gel can be quantitatively controlled(Engler et al., 2004). This process can generate hydrogels with elastic modulus ranging from 150 Pa to over 50 kPa, which can effectively recapitulate the range of substrate mechanical properties in the human body. Soft tissues such as brain and mammary gland reside in the range of 100-1000 Pa while harder tissues such as muscle and bone are 10-30kPa and above 30kPa, respectively(Butcher et al., 2009; Paszek et al., 2005). As expected, when investigated independently ligand concentration and substrate rigidity, differential responses can be observed(Engler et al., 2004). Thus, density of ECM ligands such as collagen, and matrix stiffness, while closely linked, are independent variables that can each induce distinct biological responses.

Matrix stiffness can be defined by Young's modulus, also known as the elastic modulus, of the substrate. The elastic modulus, λ, is defined by the ratio of a material's stress and strain. Essentially the elastic modulus describes the tendency of a material to deform when strained (pressure is applied). As an example, if 500 Pa is applied to a hydrogel with an elastic modulus of 1 kPa, the hydrogel will elongate one half the length of its axis. The elastic modulus can be tested directly by atomic force microscopy (AFM)(Engler et al., 2004). The thickness of the gel can also affect its mechanical properties if thin enough (less than 20 microns) (Buxboim et al., 2010). Given that hydrogels absorb water and swell and that AFM measurements of PA gels demonstrate dramatic changes in the elastic modulus, gel thickness effects are likely not an issue for

this hydrogel system. PA gels offer a well controlled cell culture system to probe the effects of changes in matrix stiffness on cell behavior. For example, pioneering work by Pelham and Wang demonstrated using PA gels that matrix stiffness can regulate cell migration and focal adhesion formation and dynamics(Pelham and Wang, 1997). Many other groups have since demonstrated that many processes are regulated by substrate elasticity.

The other main aspect of the culture system we have utilized is the incorporation of 3D culture. There has been a growing appreciation for the significance of 3D growth in trying to recapitulate what occurs *in vivo*. Culture of fibroblasts in 3D versus 2D culture leads to a complete reorganization of the cytoskeleton and cellular adhesions, which in turns induces biological changes(Cukierman et al., 2001). 3D culture dramatically alters the formation and function of focal adhesions(Fraley et al., 2010). Interestingly, 1D cell movement along fiber like substrates more closely mimicked migration in 3D than did 2D, suggesting that the fibers in 3D environments(Doyle et al., 2009). Interestingly, while focal adhesion associated proteins do not form punctate structures as in 2D cultures, they still play important roles in cell migration, suggesting that 2D can inform 3D experiments, but the functions and outcomes of molecular factors and biological processes may be altered(Yamada and Cukierman, 2007). 3D culture also modifies the engagement and activity of specific integrin heterodimers, suggesting that the environment changes specificity for ECM ligands(Cukierman et al., 2001). Fibroblasts also strongly respond to simultaneous exposure to ECM ligands on their ventral and dorsal sides, supporting the notion that simply introducing the 3D dimension has critical influence(Beningo et al., 2004). Thus, 3D culture allows for growth in 3 dimensions, including the z-axis, growth with native ECM ligands and growth factors, and presentation of ECM ligands to the entire cell surface. These advantages over

'normal' tissue culture in 2 dimensions allow for more biologically representative outputs. 3D can be achieved by a variety of methods, but the underlying idea is to surround cells with a matrix of ECM protein ligands. Reconstituted basement membrane (rBM) is perhaps the most well known of these methods. Matrigel is a trade name for rBM. rBM, or Matrigel, is isolated from Engelbreth-Holm-Swarm murine sarcoma, a poorly differentiated chondrosarcoma first develop by Engelbreth-Holm and colleagues that was later characterized by Swarm and colleagues(Orkin et al., 1977). Soon after, characterization of rBM led to the identification of the proteoglycan Laminin as a significant contributor to the basement membrane, the first of many components to be characterized(Timpl et al., 1979). Much work on the part of many laboratories has further characterized the content and nature of the basement membrane. rBM consists of many ECM proteins such as Laminin, collagen, and entactin, growth factors such as EGF, TGF-β, and PDGF, as well as proteases among other components(Kleinman and Martin, 2005). rBM was found to be highly biologically active, which was some of the first evidence that the ECM and a 3D environment could greatly influence cell behavior. Endothelial cells, instead of growing in a 2-dimensional sheet, formed tube-like structures, reminiscent of vessels while melanocytes were induced to produce pigment when grown with rBM(Kleinman et al., 1986; Kubota et al., 1988). Many other cell types have shown to be responsive to culture with rBM and in general, rBM induces cellular differentiation.

For mammary epithelial cells, culture in rBM induced the formation of ductal structures that are functionally active, secreting casein proteins(Li et al., 1987). This observation was validated in multiple contexts. Primary murine mammary gland cultures underwent alveolar morphogenesis when cultured in rBM(Barcellos-Hoff et al., 1989). These differentiated alveolar structures were functionally active, secreting caseins into

the lumen and producing lactoferrin and transferrin. It was then shown, also by Mina Bissell's group, that primary human mammary explants and normal breast epithelial cell lines form polarized spheroids, whereas primary or established human breast carcinoma cells did not establish polarity, deposit endogenous basement membrane, or form spheroids(Petersen et al., 1992). These authors in fact proposed that the molecules responsible for sensing basement membrane might as a class of tumor supressors. It was later shown that these sensors, integrins, are critical for mammary morphogenesis and that blockade of integrin activation could attenuate tumor progression(Faraldo et al., 1998; Li et al., 2005; Weaver et al., 1997). The developmental and tumorigenic processes were more complex, however. For example, the basement membrane serves as a critical component in polarization as cells without contact with the basement membrane undergo apoptosis(Boudreau et al., 1995). This process facilitates the formation of an apical lumen. Many of these regulatory mechanisms cannot be observed in 2D culture, and are only fully functional in 3D growth environments. Thus, it seems appropriate when investigating the role of matrix stiffness, which is in large part derived from the ECM, that a more biologically relevant system that includes the ECM is used.

We thus employed a 3D overlay culture system on PA hydrogels which allows for the development of mammary acini at the appropriate stiffness. Cells are plated in a 3D environment consisting of rBM but allowed to attach to PA gels, thus exposing cells to mechanical cues in a 3D environment, independent of changes in other culture variables. Using this culture system, it has been previously shown that increasing matrix stiffness can induce a malignant phenotype in mammary epithelial cells(Paszek et al., 2005). We sought to investigate the molecular mechanism that underlies this cellular phenomenon. We used non-transformed human MCF10A(Soule et al., 1990) and

tumorigenic mouse Eph4Ras(Reichmann et al., 1989) mammary epithelial cells in our studies because unlike normal mammary epithelial cells *in vivo*(Xu et al., 2013), both cell lines already express endogenous Twist1, suggesting that genetic or epigenetic alterations predispose them to tumor progression(Blick et al., 2008; Eckert et al., 2011). However, notably MCF10A cells have defective tight junction formation due to chromosomal alterations. Both cells developed polarized ductal acini surrounded by intact basement membrane on the compliant matrix of 150Pa (Figure 2-1, Figure 2-33). In contrast, at 5700Pa high matrix stiffness, cells presented a partial EMT phenotype, with loss of apical-basal polarity, reduced E-cadherin at adherens junctions, and increased Fibronectin expression (Figure 2-6 and 2-7)(Paszek et al., 2005).

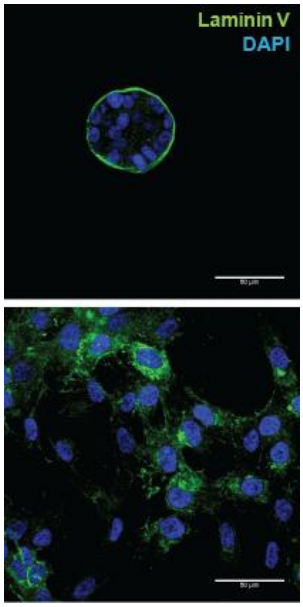

Figure 2-1. Compliant matrices induce acinar basal polarization. Confocal microscopy of MCF10A cells grown in 3D culture on varying matrix rigidities stained for Laminin V (green) and DAPI (blue).

Using this 3D culture system, we tested whether Twist1 is required for induction of EMT and invasion in response to high matrix stiffness. We generated Eph4Ras and

MCF10A cells expressing shRNAs against Twist1 and tested their mechanosensing competence (Figure 2-2, 2-3).

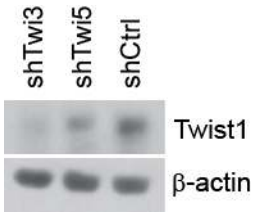

Figure 2-2. Generation of stable Twist1 knockdown cell lines. Cell lysates from Eph4Ras cells expressing control or Twist1 shRNA analyzed by SDS-PAGE and probed for Twist1 and β-actin.

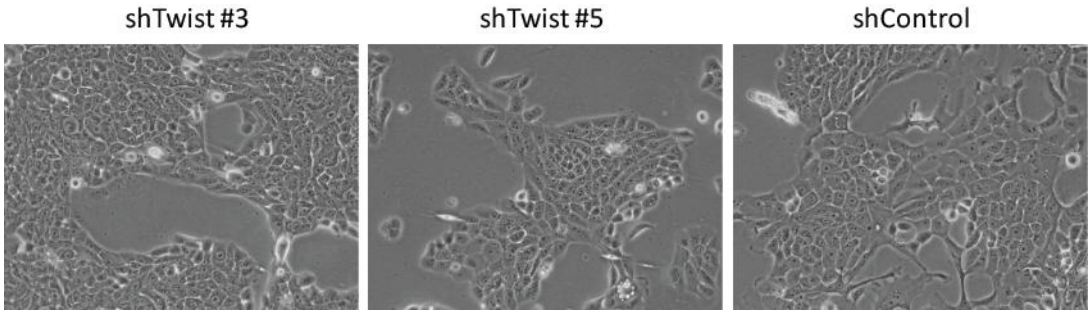

Figure 2-3. Brightfield images of stable Twist1 knockdown lines. Brightfield images of MCF10A cells expressing control and shTwist1 shRNAs.

Significantly, knocking down Twist1 prevented the invasive phenotype at 5700 Pa (Figure 2-4A, 2-4B, and 2-5); instead, these cells formed basally polarized acini with strong junctional E-cadherin on rigid matrix (Figure 2-8A).

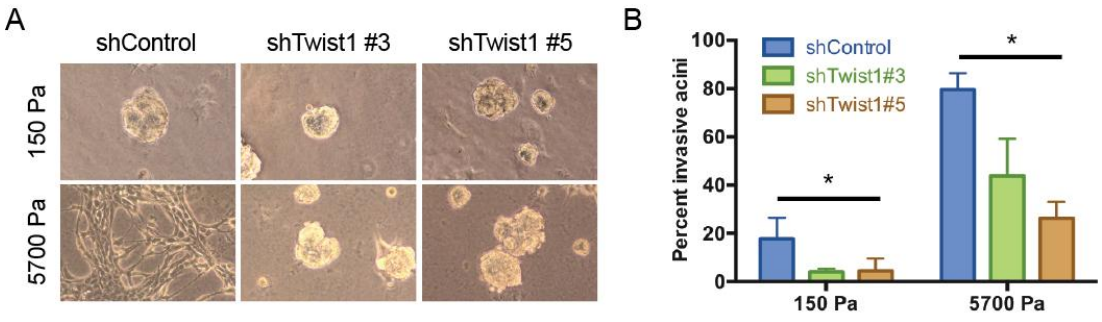

Figure 2-4. Twist1 is required for mechanosensing in Eph4Ras cells. (A) Brightfield images of Eph4ras cells expressing control or Twist1 knockdown shRNAs after 5 days growth in 3D culture on PA hydrogels with indicated rigidities. (B) Quantification of invasive acini in 3D culture described in from 3 independent experiments (*, P<0.001) (right).

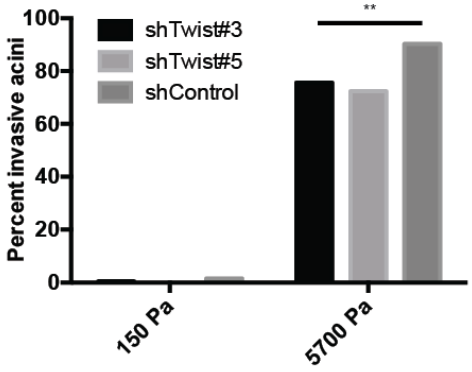

Figure 2-5. Twist1 is required for mechanosensing in MCF10A cells. Quantification of invasive acini of MCF10A shTwist1 cells in 3D culture (**, P<0.001; a representative experiment).

This suggested that Twist1 is critical for mechanosensing and the induction of an invasive phenotype at high matrix stiffness. Since high stiffness alone was not sufficient to induce a complete EMT, we further investigated whether Twist1 is also required for the induction of a full EMT by mechanical signals in concert with the EMT-inducing biochemical signal TGF-β(Xu et al., 2009). TGF-β is a classical EMT inducer during development and tumor progression and is sufficient to induce EMT in Eph4Ras

cells(Oft et al., 1998; Potts and Runyan, 1989). Consistent with published data(Leight et al., 2012), although TGF-β was not sufficient to induce EMT on soft matrix, rigid matrix together with TGF-β triggered a complete EMT, evidenced by both immunostaining and qPCR analysis of EMT markers (Figure 2-6, 2-7, 2-8, 2-9, and 2-10).

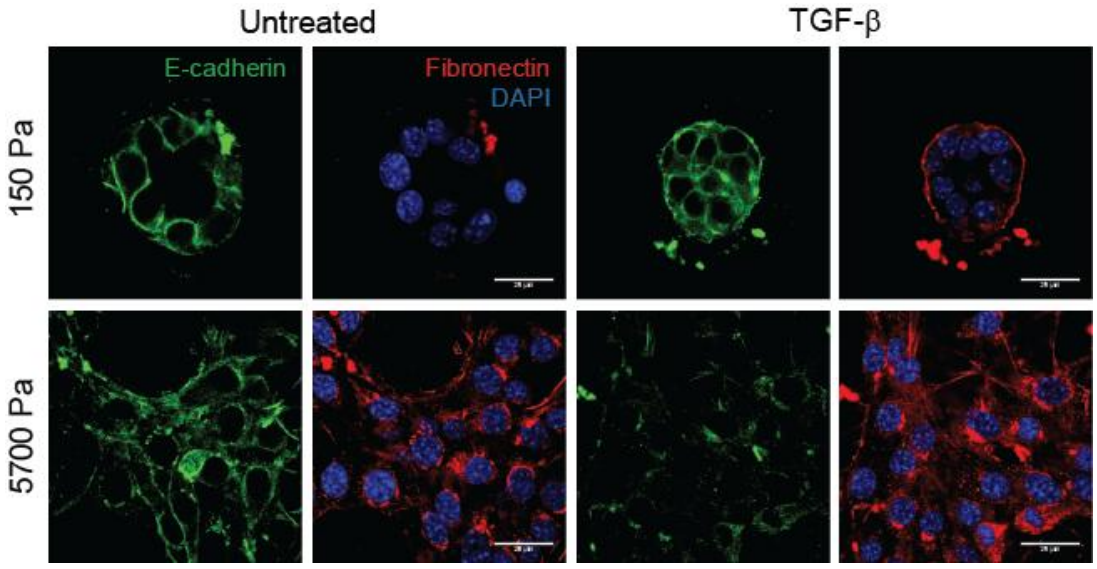

Figure 2-6. High matrix stiffness and TGF-β induce EMT in Eph4Ras cells. Confocal images of Eph4Ras cells grown for 5 days in 3D culture in the absence or presence of 5ng/ml TGF-beta stained for E-cadherin and Fibronectin.

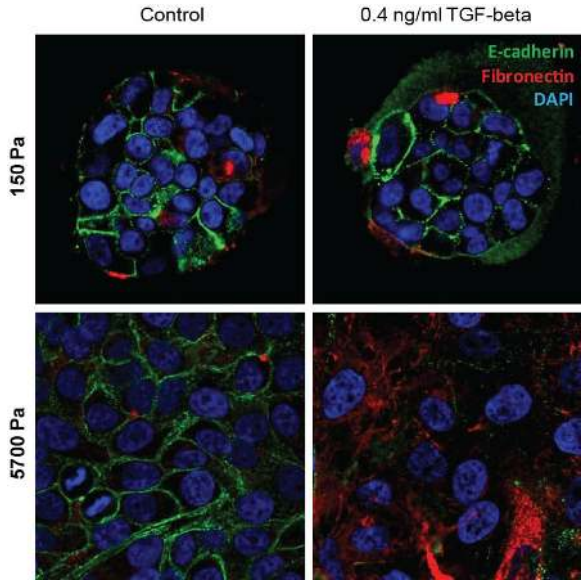

Figure 2-7. High matrix stiffness and TGF-β induce EMT in MCF10A cells. Confocal images of MCF10A cells grown for 7 days in 3D culture on varying matrix rigidities stained for E-cadherin (green), fibronectin (red), and DAPI (blue).

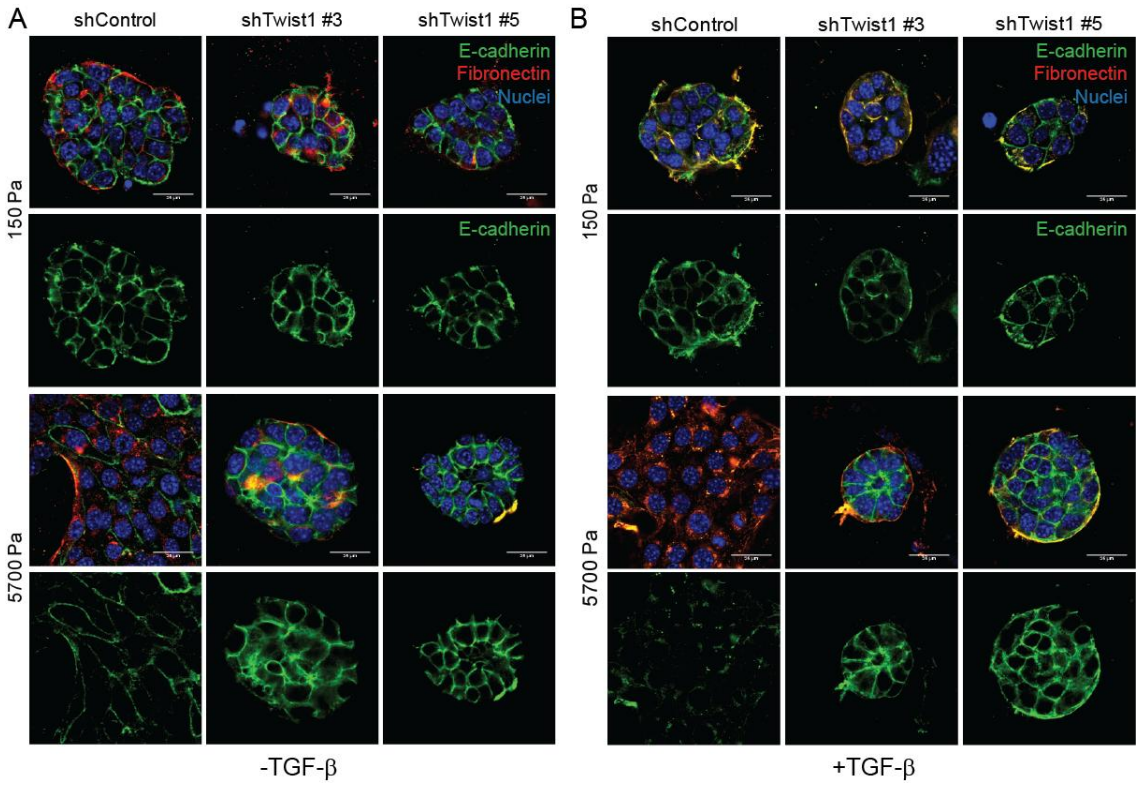

Figure 2-8. Twist1 is required for matrix stiffness and TGF-β induced EMT. Eph4ras cells expressing control or Twist1 shRNAs were cultured in 3D culture with indicated rigidities in the (A) absence or (B) presence of 5ng/ml TGF-β for 8 days and stained with E-cadherin (green), Fibronectin (red), and DAPI (blue).

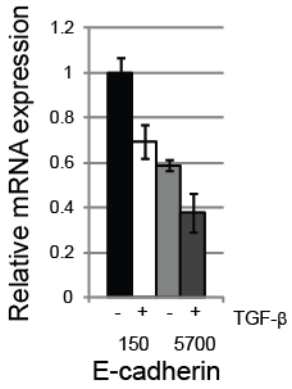

Figure 2-9. Epithelial markers are regulated by matrix stiffness and TGF-β. qPCR analysis of E-cadherin mRNA expression in MCF10A cells in 3D culture on PA hydrogels.

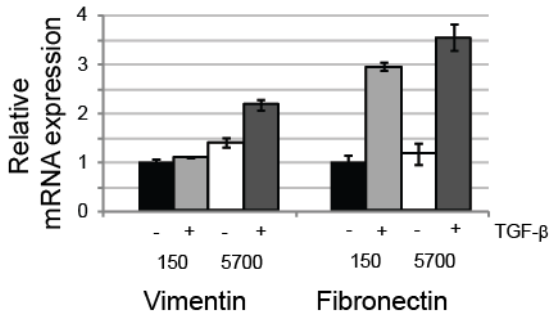

Figure 2-10. Mesenchymal markers are regulated by matrix stiffness and TGF-β. qPCR analysis of the mRNA expression of mesenchymal markers, Fibronectin and Vimentin, in MCF10A cells in 3D culture on PA hydrogels.

This observation is quite interesting as it suggests that mechanical signals from the tumor microenvironment can act as permissive or non-permissive cues. The activity of soluble growth factors such as TGF-β can thus be regulated by integrin activation or perhaps downstream mechanosensory signals. Interestingly, TGF-β can induce Twist1 expression(Eckert et al., 2011). It is also a strong possibility that Twist1-mediated EMT induces TGF-β expression; however this relationship has not been fully investigated. How these pathways interact, in opposition, synergistically, or in parallel was unknown. It was of interest then, whether treatment with TGF-β could overcome loss of Twist1, and allow for the induction of EMT at high matrix stiffness. To test this hypothesis Twist1 knockdown cells were grown on PA hydrogels in 3D culture in the presence and absence of TGF-β. Knockdown of Twist1 completely blocked induction of EMT by TGF-β at high matrix stiffness and rescued acinar development (Figure 2-8). This suggests that TGF-β and mechanosensing pathways at least act together and perhaps synergistically to induce a complete EMT at high matrix stiffness. These data are even more striking when taking into consideration that Eph4Ras cells form a feed-forward autocrine loop when treated with TGF-β(Oft et al., 1998). Together, these data indicate an essential role for Twist1 in mediating matrix stiffness-induced EMT and invasion.

2.3 Mechanism of Mechano-Regulation of Twist1

We next aimed to understand how Twist1 is regulated by matrix stiffness to mediate the invasive response. Given the critical role of Twist1 in cellular mechanosensing, the mechanism by which Twist1 is regulated in response to changes in matrix stiffness is of interest. While there has been significant effort to determine how Twist1 expression is regulated, our understanding of how Twist1 activity itself is regulated remains relatively limited. Mechano-regulation of Twist1 could occur at several levels including modulation of Twist1 mRNA or protein expression, dimerization, and subcellular localization. We first tested whether matrix stiffness affects Twist1 mRNA or protein expression levels. Unlike *Drosophila* Twist whose expression is induced by mechanical forces(Desprat et al., 2008), we did not detect significant increases of either Twist1 mRNA or protein expression levels in human and mouse mammary cells grown under different matrix rigidities (Figure 2-11 and 2-12A)(Desprat et al., 2008). This differential regulation may stem from the natures of the mechanical cues, with compression being very acute and matrix stiffness being a relatively long time scale cue. Thus these signals may employ entirely different molecular mechanisms. Nevertheless, the relationship between Twist and mechanical cues appears to be a conserved feature. To further confirm a functional role for Twist1 in mechanosensing we assayed the expression of other EMT transcription factors in the presence and absence of Twist1 as EMT-transcription factors often regulate each other's expression. We did not observe increases in Snail1/2 and Zeb1/2 mRNA. However, mRNA expression of Snail2, Zeb1, and Zeb2 was Twist1-dependent (Figure 2-11), suggesting that Twist1 functions upstream of these EMT transcription factors during matrix stiffness-induced EMT. The question still begged, however, how matrix stiffness regulates Twist1.

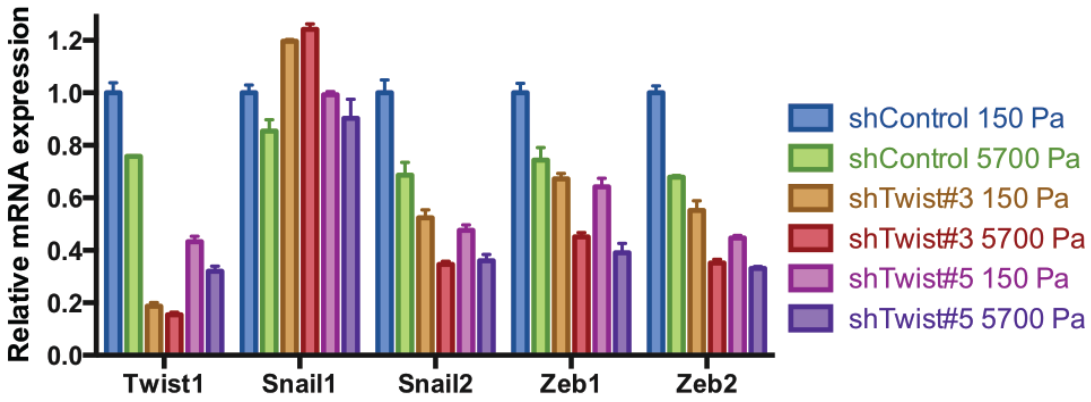

Figure 2-11. Twist1 governs expression of EMT transcription factors. qPCR analysis of Twist1, Snail1, Snail2, Zeb1, and Zeb2 mRNA expression in Eph4Ras cells expressing control or Twist1 shRNAs cultured under indicated matrix rigidities.

2.4 Matrix Stiffness Regulates Twist1 Nuclear Localization

Regulation of the subcellular localization of Twist1 could be a potent molecular mechanism to modulate its activity. Twist1, as a transcription factor is only active when localized in a nucleus. Twist1 can be regulated via its subcellular localization, however it is nearly always reported to be nuclear. This is due to its two functional nuclear localization sequences (NLS)(Singh and Gramolini, 2009). Interestingly, Twist1 does not have a reported nuclear export sequence (NES), suggesting nuclear import-export equilibrium may not be an effective mode of regulation. Sequence analysis identifies a degenerate NES sequence (143LSKIQTLKL151), although functional validation of this sequence remains to be done. The location of this sequence may be of importance as it is lies in the helix-loop-helix domain responsible for dimerization. Thus, if functional this NES might only be available for interaction with nuclear export machinery in Twist1 monomers, providing a unique mechanism for ensuring only dimerized Twist1 complexes bind to the genome. Consistent with this idea, dimerization with TCF4, another bHLH transcription factor, is sufficient to rescue nuclear localization of NLS-

deficient Twist1(Singh and Gramolini, 2009). Taking into consideration that phosphorylation of Twist1 by PKA at T125 and S127 can affect dimerization partner choice, this mechanism provides an intriguing possilibity for Twist1 regulation(Firulli et al., 2005). Indeed, phospho-regulation of Twist1 has been postulated to regulate differentiation(Firulli and Conway, 2008). Twist1 is also regulated via nuclear translocalization in an integrin-dependent manner(Alexander et al., 2006). Inhibition of Rho-associated kinase and β1 integrin, as well as, actin destabilization attenuated Twist1 nuclear localization and downstream target induction in PC-3 prostate carcinoma cells. This study utilized fibronectin coated substrate, which activates β1 integrin clustering, and canonical integrin mediated mechanosensing. However, this study compares gene activity in cells cultured in suspension versus those in an adherent environment and it remains unclear whether this effect is due to adherence or more specifically to integrin activation. Based on the discussed published works, there appears to be sufficient machinery and evidence that modulation of Twist1 subcellular localization could be a potent mechano-regulatory mechanism. Thus, we examined Twist1 subcellular localization in response to increasing matrix stiffness.

Surprisingly, Twist1 was largely cytoplasmic on the compliant matrix of 150Pa and translocated into the nucleus on the rigid matrix of 5700Pa (Figure 2-12B). This observation aligns with our finding that cells undergo EMT at high matrix stiffness as Twist1 regulates transcription to induce EMT. At low matrix stiffness conditions, similar to the normal mammary gland, in which mammary epithelial identity is maintained, Twist1 is effectively inactivated by localization to the cytoplasm. This mechanism for regulation is a corollary to that which occurs during development, in which increasing mechanical stress (compression in the developmental setting) induces Twist1 activity via its expression(Desprat et al., 2008). Furthermore, because this mechanism is not

transcription-dependent, it suggests that Twist1 is both critical for and upstream in the signaling pathway involved in matrix stiffness induced EMT. This is supported by our observation that mRNA expression of the EMT-transcription factors Snail2, Zeb1, and Zeb2 is dependent on Twist1.

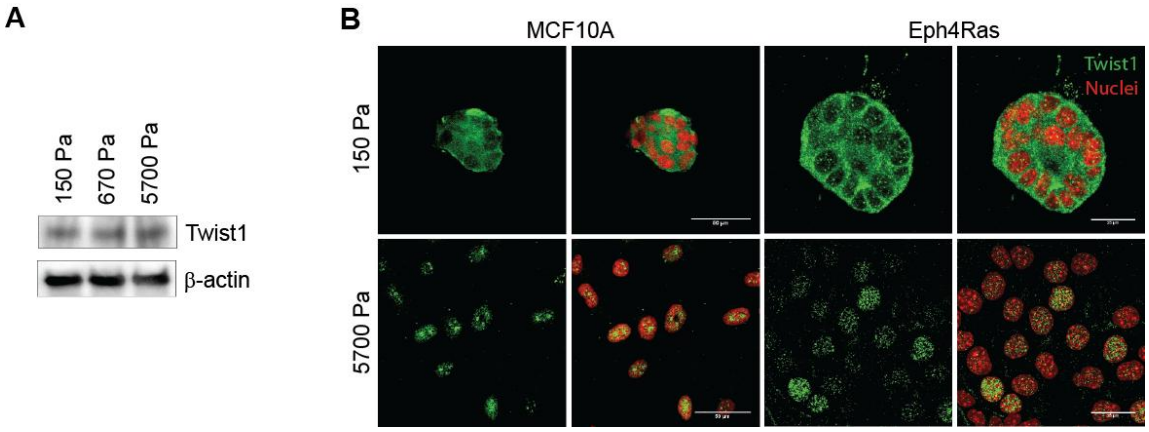

Figure 2-12. Twist1 localization is regulated by matrix stiffness. (A) Cell lysates from MCF10A cells grown in 3D culture on PA hydrogels with indicated rigidities were analyzed by SDS-PAGE and probed for Twist1 and β-actin. (B) Eph4ras, MCF10A were cultured in 3D culture with indicated rigidities for 5 days and stained for Twist1 (green) and nuclei (red).

To test whether this mode of regulation is specific to cells of low invasive capacity we assayed Twist1 localization in a metastatic breast cancer cell line, Bt-549. High stiffness-induced nuclear translocation of Twist1 was observed in Bt-549 cells in addition to human MCF10A and mouse Eph4Ras cells (Figure 2-12B and 2-13), suggesting nuclear translocation of Twist1 is a conserved response to increasing matrix stiffness.

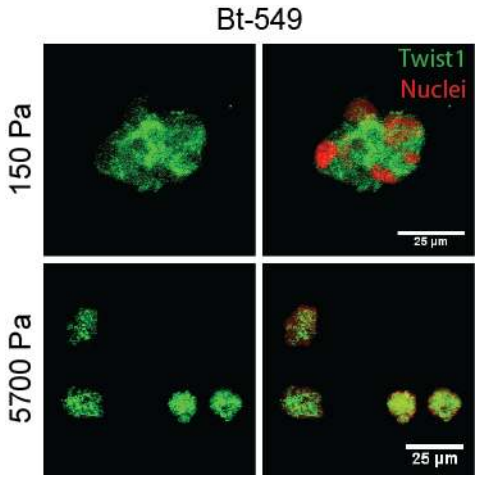

Figure 2-13. Matrix stiffness regulates Twist1 localization in metastatic breast cancer cells. Bt-549 cells were cultured in 3D culture with indicated rigidities for 5 days and stained for Twist1 (green) and nuclei (red).

We next tested whether integrin activation is necessary for Twist1 nuclear localization at high matrix stiffness, since mechanosensing responses to matrix stiffness are mediated in part through clustering and activation of integrins(Friedland et al., 2009; Paszek et al., 2005). β1 integrin responds to mechanical signals and induces focal adhesion formation, serving as a cellular transmembrane mechano-transducer(Wang et al., 1993). Consistent with previously published reports, treatment with a β1-integrin blocking antibody (AIIB2) attenuated the malignant response to the ECM(Weaver et al., 1997). Furthermore, in our hands treatment with a β1-integrin blocking antibody prevented nuclear translocation of Twist1 and blocked EMT and the invasive phenotype induced by high matrix stiffness (Figure 2-14)(Levental et al., 2009; Paszek et al., 2005). This indicates that mechano-regulation of Twist1 is dependent on integrin activation and further supports a critical role of Twist1 in mediating matrix stiffness-induced EMT and invasion.

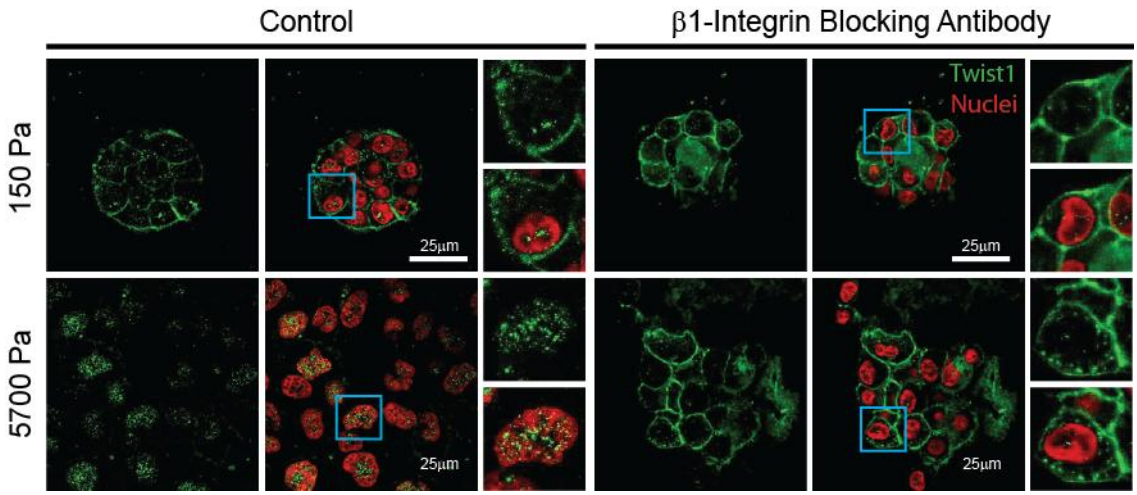

Figure 2-14. Integrin activation is required for Twist1 nuclear translocation. MCF10A cells were cultured in 3D culture with indicated rigidities in the presence of a control IgG or a β1-integrin blocking antibody (AIIB2) for 9 days and stained with Twist1 (green) and DAPI (red).

2.5 The Interplay between Matrix Stiffness and Cell Shape

Matrix stiffness and cell shape changes are closely related. Our observations in 3D culture that increasing matrix stiffness promotes cell spreading concur with previous reports(Dupont et al., 2011; Paszek et al., 2005). This effect is likely mediated through focal adhesions, which are mechanosensitive. Focal adhesions form in response to integrin activation and are acutely sensitive to changes in matrix stiffness. Astonishingly, single focal adhesions are capable of sensing a gradient in the substrate rigidity, which can lead to directed cell movement(Plotnikov et al., 2012). This profound sensitivity to changes in matrix stiffness results in changes in the cytoskeleton and depending on context, regulates cell shape, migration, and behavior. On PA hydrogels at low stiffness (150 Pa), with low levels of integrin activation, the actin cytoskeleton is reorganized to form cortical actin in spheroid cells. At high stiffness cells with high levels of integrin activation and focal adhesion formation, cells spread effectively. In the developmental

context, it is also difficult to distinguish whether effects are directly caused by mechanical cues or the associated changes in cell shape. For example, during germ band extension migrating cells cause acute mechanical compression that induces invagination and regulates cellular differentiation into eventual mesoderm. However, this mechanical compression dramatically changes cell shape(Butler et al., 2009; Leptin and Grunewald, 1990). It is clear that in some circumstances that mechanosensory pathways signal through the cytoskeleton.

Since changes in matrix stiffness also result in changes in cell shape, we set out to distinguish its impact on Twist1 nuclear localization from matrix stiffness. First, we used micropatterning to selectively alter cell shapes without changing underlying matrix rigidity. Eph4Ras cells were seeded on collagen I-coated square micropatterns ranging from 100 μm^2 to 2500 μm^2. The smallest square, with an area of 100 μm^2, prevented any cell spreading while cells on the largest micropattern or completely unpatterned regions were able to spread effectively (Figure 2-15B). Consistently, Twist1 nuclear localization was not affected by changes in cell shape and spreading (Figure 2-15A and 2-15B). This suggests that matrix stiffness directly regulates Twist1 subcellular localization independently of changes in cell shape.

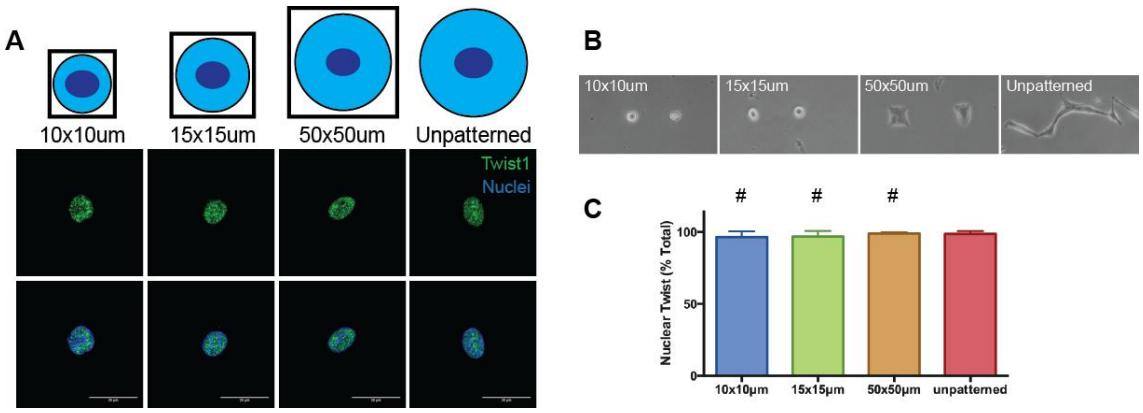

Figure 2-15. Cell shape does not regulate Twist1 nuclear localization. (A) Confocal microscopy images and (B) brightfield images of Eph4ras cells cultured on micropatterned glass coverslips for 6 hours and stained for Twist1 (green) and DAPI (blue). (C) Quantification of the percent of Twist1 localized in the nucleus in Eph4ras cells described in (A) (#, not significant).

This supports the existence of multiple mechanosensing pathways as mechanoregulation of YAP/TAZ, while responsive to matrix stiffness, is dependent on cell shape(Dupont et al., 2011). YAP/TAZ are transcription factors that regulate cell proliferation as part of the Hippo signaling pathway. This is consistent with the role of YAP/TAZ in contact inhibition(Zhao et al., 2007). Another of the few mechanosensitive transcription identified thus far is MRTF. MRTF is mechano-responsive in another distinct mode, as it is responsive to tissue geometry and cell shape(Gomez et al., 2010). Based on our findings and these previously published reports it seems likely that mammalian cells have multiple mechanotransduction pathways, each with distinct sensitivities coupled with distinct biological outputs. Thus, identification and characterization of a novel Twist1-mediated cellular mechanotransduction will be of great interest.

2.6 G3BP2 Mediates Mechanoregulation of Twist1 Localization

To understand how matrix stiffness regulates Twist1 nuclear translocation, we first explored whether Twist1 nuclear import and export rates might be regulated by matrix stiffness. Many transcription factors are regulated through changes in the equilibrium of nuclear import and export. Nuclear import and export is largely governed by importin and exportin proteins. If a protein is regulated in this manner, blockade of nuclear export via inhibition of exportin1 will result in nuclear accumulation due to a shift in equilibrium(Kudo et al., 1999). Treatment of MCF10A with Leptomycin B, a nuclear export inhibitor, did not result in nuclear accumulation of Twist1 on the compliant matrix (Figure 2-16), suggesting that Twist1 is unable to enter the nucleus on compliant matrices, likely due to active cytoplasmic retention and not because of regulation of the nuclear import and export equilibrium. Interestingly, mechanoregulation of YAP subcellular localization was disrupted by LMB treatment, suggesting that YAP nuclear translocation is regulated via the nuclear import and export equilibrium. Furthermore, the differential effect of LMB on Twist1 and YAP suggests that mechanoregulation of Twist1 and YAP may be mediated by distinct mechanisms; an idea that will be explored and elaborated upon later in following sections.

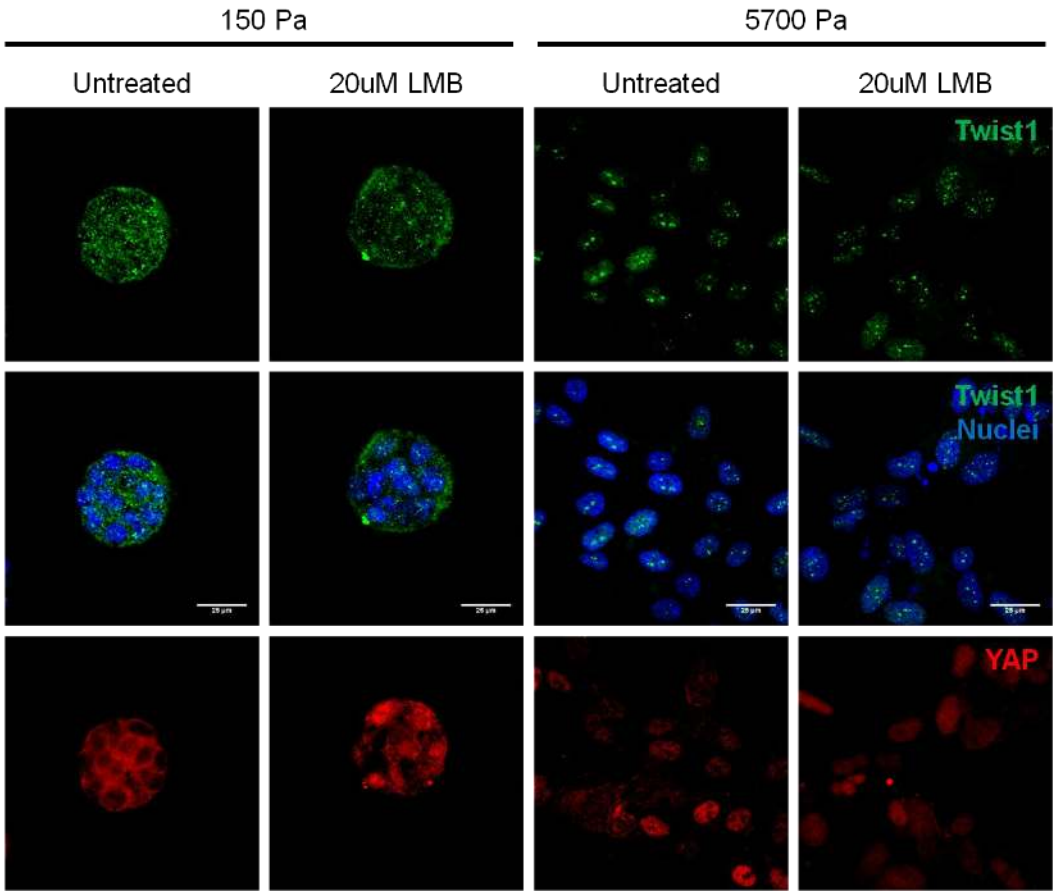

Figure 2-16. Inhibition of nuclear export does not affect Twist1 localization. Confocal microscopy of leptomycin B treated MCF10A cells on PA hydrogels in 3D culture stained for Twist1 (green) and nuclei (blue).

To search for the molecular mechanism of Twist1 cytoplasmic sequestration, we used mass spectrometry analysis to identify Twist1-binding proteins that anchor Twist1 in the cytoplasm (Figure 2-17). Using MCF10A whole cell lysate from cells grown on plastic tissue culture dishes, Twist1 was immunoprecipitated using 5B7 anti-Twist1 hydridoma supernatant immobilized on protein G sepharose beads. Co-immunoprecipitated proteins were analyzed by SDS-PAGE and reversible silver staining. Bands specific to anti-Twist1 immunoprecipitation and not mouse IgG control were isolated and analyzed by mass spectrometry. From the three distinct bands analyzed 8

unique proteins were identified after a stringent subtraction (Table 2-1). The identification of Twist1 further confirmed the specificity of the immunoprecipitation. The identification of KPNA1, a subunit of the importinα, was further validation as Twist1 is shuttled into the nucleus via its canonical NLS sequences by the importin system. Of the novel putative Twist1 binding partners, Ras GTPase-activating protein-binding protein 2 (G3BP2) stood out as a promising candidate to mediate Twist1 cytoplasmic sequestration at low matrix stiffness. The apparent contrast between the conditions of cell growth for this mass spectrometry analysis and Twist1 cytoplasmic interaction, can perhaps be, at least in part, explained by the low protein coverage observed for G3BP2 in the mass spectrometry analysis. Furthermore, Twist1 has a small amount of cytoplasmic detectable when grown in normal glass or plastic dishes. Nevertheless, G3Bp2 presented an attractive mechanism for mechanoregulation of Twist1.

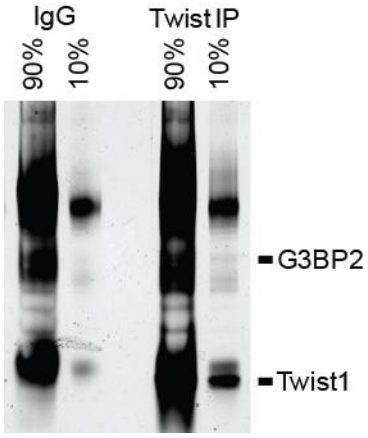

Figure 2-17. Co-immunoprecipitation of Twist1. Immunoprecipitation of endogenous Twist1 from MCF10A cell lysates resolved by SDS-PAGE and silver stained. Unique bands were identified, excised, and analyzed by mass spectrometry.

Table 2-1. Mass spectrometry identified novel Twist1 binding proteins. Proteins identified by mass spectrometry analysis of immunoprecipitates of endogenously expressed Twist1 in MCF10A cells and their respective known functions.

Gene ID	Name	Function
G3BP2	Ras GTPase activating protein binding protein 2	Cytoplasmic retention, RNA binding
C15orf44	C15orf44	NTPase, vWA domain
KPNA1	Karyopherin subunit alpha-1 (Importina subunit 1)	Nuclear import
TTBK2	Tau-tubulin kinase	Ser/Thr kinase
GRWD1	Glutamate-rich WD protein	ribosome component/biogenesis(50/80S)
DARS	Aspartyl tRNA synthetase	tRNA charging
TWIST1	Twist1	Transcriptional regulation, EMT
GFAP	Glial fibrillary acidic protein	Intermediate filament

G3BP1 was first identified via its interaction with the Ras GTPase activating protein p120(Parker et al., 1996). The murine homolog was soon identified thereafter, which is structurally similar and has 98% identity other than a 33 amino acid insertion(Kennedy et al., 1996). Subsequently, the *Drosophila* homolog, *rasputin* (*rin*), as well as the existence of two human G3BP orthologues, G3BP1 and G3BP2, were identified(Pazman et al., 2000). G3BP1 and G3BP2 share approximately 40% and 60% amino acid identity with Rin, respectively. G3BP2 is a 68 kDa protein with a highly conserved N-terminal nuclear transport factor 2 (NTF2) domain, proline-rich PXXP domain, and RNA recognition motif (RRM) domain containing two ribonucleoprotein (RNP) domains (Figure 2-18).

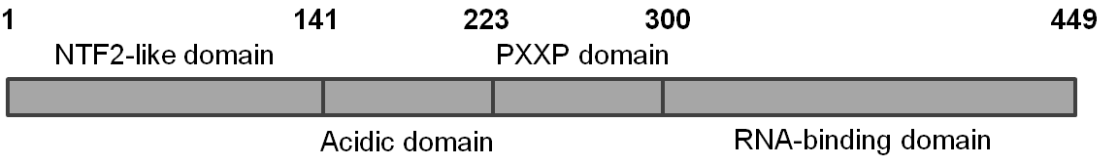

Figure 2-18. Schematic of G3BP2. G3BP2 contains four main domains which include a NTF2-like, acidic, PXXP, and RNA-binding domain. Figure is adapted from (Prigent et al., 2000).

Expression analysis suggests that G3BP may play a significant role during *Drosophila* development as it is highly expressed during the first three hours of development(Pazman et al., 2000). Genetic interaction analyses suggest that *rin* acts upstream of Ras in the signaling cascade. Interestingly, G3BP only binds p120 RasGAP when Ras is in the activated GTP-bound form(Parker et al., 1996). Furthermore, monoallelic loss of *rin* enhances the gain of function effects of RhoA on *Drosophila* eye photoreceptor and planar polarity(Pazman et al., 2000). This genetic interaction suggests that Rasputin, in addition to Ras signaling, plays a critical role in Rho signaling and thus connects two distinct and essential pathways.

G3BP1 and G3BP2, while retaining sharing significant overlapping functions have also been shown to have differential activity. For example, G3BP1 and G3BP2 contribute to formation of stress granules, which form in response to cellular stresses including hypoxia, chemical toxicity (e.g. arsenite), and heat shock (Matsuki et al., 2013; Tourriere et al., 2003). Other functions such as mRNA regulation specificity diverge. Regulation of peripheral myelin protein 22 (PMP22) mRNA G3BP1 but not G3BP2 is able to promote breast cancer cell proliferation(Winslow et al., 2013). Knockdown of G3BP2 using transient siRNA did not affect ^{3}H thymidine incorporation in Bt-549, MDA-MB-231, MDA-MB-468, and MCF-7 cells. Perhaps not surprisingly, the human paralogs,

which have 65.63% amino acid identity, have similar but distinct functions (Figure 2-19). So while G3BP1 and G3BP2 are relatively ubiquitously expressed, their roles in tissue homeostasis and cell behavior may be very different. Their expression in adult mouse tissue has been reported to be tissue specific(Kennedy et al., 2001). Furthermore, G3BP2 has two splicing isoforms, G3BP2a and G3BP2b which only differ in the excision of a short sequence in the proline-rich domain in the smaller 2b isoform. It is not clear, however, whether these isoforms have significantly distinct functions. Expression of the full length G3BP2 isoform is maintained at high levels post embryonic development while G3BP1 and the spliced G3BP2 isoform are downregulated, although seem to still be expressed in some adult tissues(Irvine et al., 2004; Kennedy et al., 2001). Some of the seeming discrepancies in reports concerning G3BP proteins may be due to differential isoforms however. For example the NTF2 domain has been crystallized and structure solved but the isoform that was reported as 52kDa while the main isoform that was originally reported and that we identified as a Twist1 binding partner is 68kDa(Parker et al., 1996). Much of the previous focus has been on the NTF2 domain of G3BP proteins because of its novel affinity for RasGAP. This interaction is surprising given that G3BP1 and G3BP2 contain proline-rich domains that are predicted to bind to the SH3 domain of p120 RasGAP. G3BP proteins appear to have many functions, which may be facilitated by their scaffolding function.

Figure 2-19. Homology of G3BP1 and G3BP2. Alignment of the amino acid sequences of human G3BP paralogs G3BP1 and G3BP2.

Among the diverse functions of G3BP proteins, their cytoplasmic retention of the transcription factors MDM2, p53 and IκBα was the most interesting in the context of our observations(Kim et al., 2007; Prigent et al., 2000). Given the putative interaction between Twist1 and G3BP2, this cytoplasmic sequestration activity provided a very enticing mechanism for Twist1 cytoplasmic localization. Consistent with this role, G3BP2 was observed only in the cytoplasm in MCF10A, Eph4Ras, and Bt-549 cells at all matrix rigidities (Figure 2-20 and 2-21). This concurs with previous data suggesting

that G3BP2 is only found in the cytoplasm(Parker et al., 1996; Pazman et al., 2000). We confirmed the mass spectrometry results biochemically and found that both endogenously and exogenously expressed Twist1 co-immunoprecipitated with endogenously expressed G3BP2 (Figure 2-22 and 2-23). However, because these immunoprecipitations were performed on lysates of cells grown on plastic, only a relatively low percentage of total Twist1 interacted with G3BP2. This is consistent with the hypothesis that signals at low stiffness allow for efficient Twist1-G3BP2 interaction and cytoplasmic sequestration.

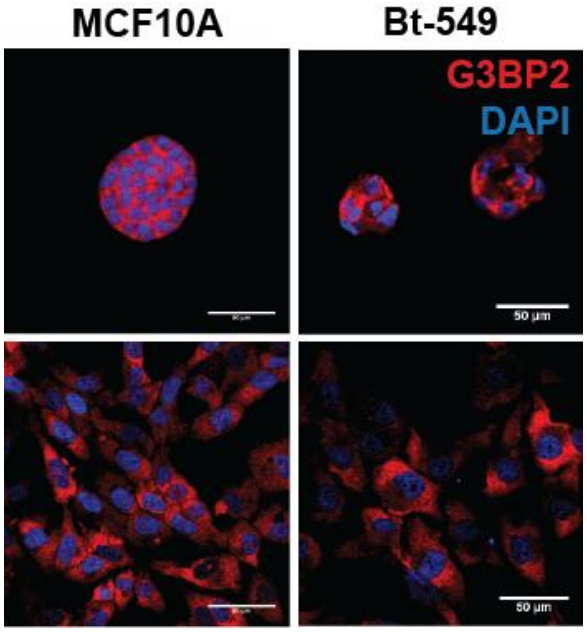

Figure 2-20. G3BP2 localization in normal and metastatic human mammary epithelial cells. Confocal images of MCF10A and Bt-549 cells grown in 3D culture stained for endogenously expressed G3BP2 (red) and DAPI (blue).

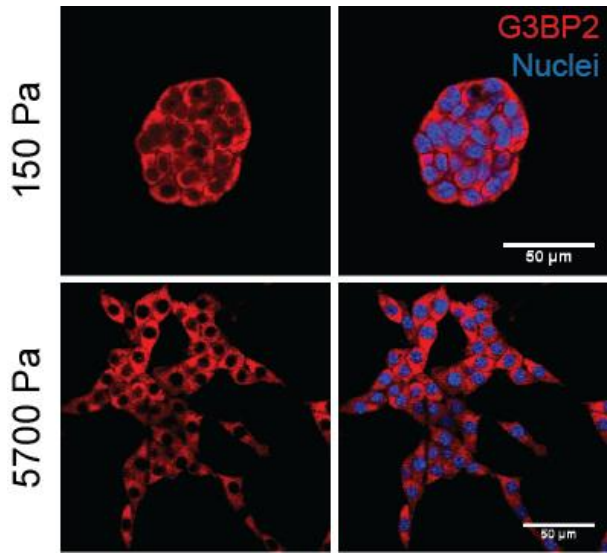

Figure 2-21. G3BP2 localization in Ras-transformed mammary epithelial cells. Confocal microscopy images of Eph4Ras cells grown in 3D culture stained for endogenously expressed G3BP2 (red) and DAPI (blue).

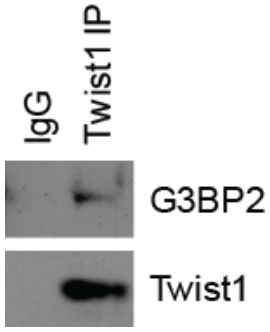

Figure 2-22. Co-immunoprecipitation of endogenous Twist1. Endogenously expressed Twist1 from MCF10A cell lysates was immunoprecipitated and analyzed by SDS-PAGE, and probed for G3BP2 and Twist1.

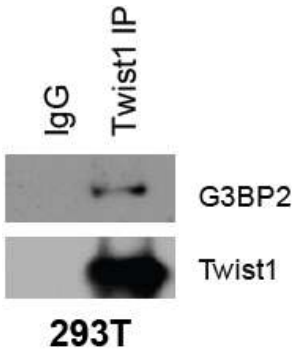

Figure 2-23. Co-immunoprecipitation of exogenous Twist1. Exogenously expressed Twist1 from 293T cell lysates was immunoprecipitated and analyzed by SDS-PAGE, and probed for G3BP2 and Twist1.

Previous studies identified IκBα amino acids 37-55 as being sufficient for interaction with G3BP2(Prigent et al., 2000). Interestingly, the domain of G3BP2 mapped by Prigent and colleagues to be sufficient to interact with IκB-α does not overlap with the N-terminal NTF2 domain that interacts with p120 RasGAP(Kennedy et al., 2001; Prigent et al., 2000). This may enable a single G3BP2 molecule to bind to multiple substrates and supports a scaffolding role for G3BP2(Kennedy et al., 2001). Taken together with its described functions, G3BP2 seemed like a potential mechanism for Twist1 cytoplasmic sequestration at low matrix stiffness. Sequence alignment of this G3BP2-interacting domain with Twist1 revealed a novel consensus G3BP2-binding motif, 101Q-X-X-X-E-L-Q-[ET]-X-[KR]-[LPV] (Figure 2-24A and 2-25). Interestingly, this G3BP2-binding motif is highly conserved among vertebrate Twist1 proteins, but not in *Drosophila* in which Twist expression rather than localization is regulated by mechanical cues(Desprat et al., 2008) (Figure 2-24B). Furthermore, this motif is located N-terminally from the bHLH domain of Twist1, suggesting it may be available for interaction even in dimers. Deletion of this motif (ΔQTmutant) in Twist1 abolished its interaction with G3BP2 (Figure 2-26). Together, these data show that cytoplasmic anchor G3BP2 binds

to Twist1 via the G3BP2-binding domain. We then investigated whether interaction is specific to Twist1. Strikingly, only five human genes contain the consensus G3BP2-binding motif (Q-X-X-X-E-L-Q-[ET]-X-[KR]-[LPV]). Three of them, Twist1, IκB-α, and MDM2 have already been discussed and were used in the identification of this motif. The transcription factor bicaudal and AKAP12 are the only two other proteins to contain this sequence, suggesting it is a highly specific interaction. Further investigation is required to determine if bicaudal and AKAP12 interact and have roles in G3BP2 mediated signaling.

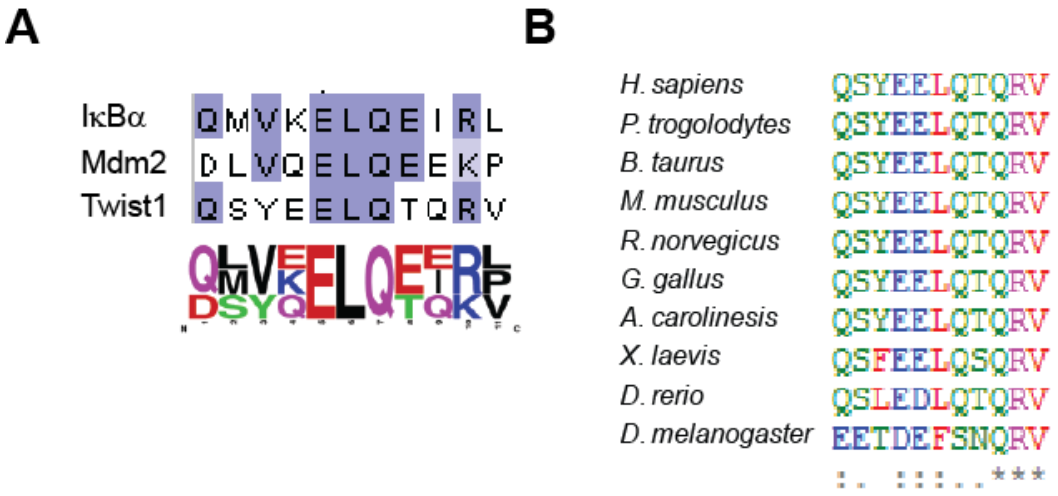

Figure 2-24. Identification of consensus G3BP2 binding motif. (A) Population plot of the putative G3BP2 binding domain motif. (B) Alignment of the putative G3BP2 binding domain in Twist1 homologs.

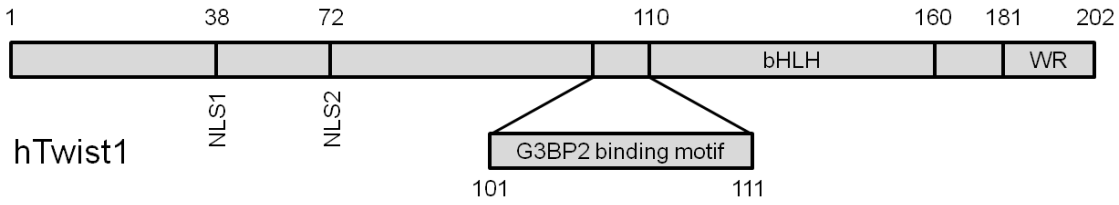

Figure 2-25. Schematic of the domains in human Twist1.

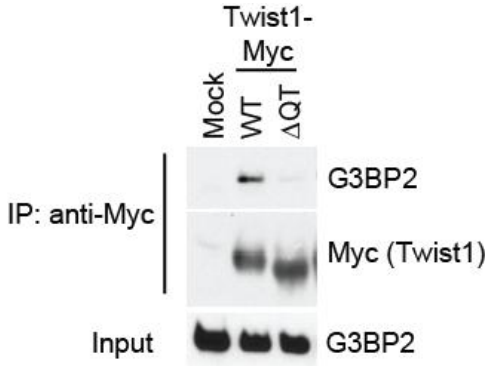

Figure 2-26. The G3BP2 consensus binding motif is required for Twist1-G3BP2 interaction. Exogenously expressed wild-type (WT) and Q105_T112del (ΔQT) Myc-tagged Twist1 from 293T cell lysates were immunoprecipitated and analyzed by SDS-PAGE, and probed for G3BP2 and Myc.

To directly test whether matrix stiffness regulates Twist1-G3BP2 interaction, we utilized *in situ* proximity ligation assay (PLA) to detect and localized the endogenous interaction of Twist1 and G3BP2 in 3D acinar cultures. Indeed, at 150Pa, a strong PLA signal, indicative of Twist1-G3BP2 association, was specifically enriched in the cytoplasm. In contrast, very little PLA signal was detected at 5700Pa, indicating the release of Twist1 from G3BP2 to allow Twist1 nuclear translocation at high matrix rigidity (Figure 2-27A and 2-27B).

To further understand whether Twist1/G3BP2 interaction is specifically regulated by matrix stiffness, but not changes in cell polarity or adherens junctions as a consequence of stiffness-induced EMT, we examined Twist1-G3BP2 interaction in single cells cultured on the PA hydrogels devoid of apical-basal polarity and mature adherens junctions. PLA analysis in single cells detected strong interaction of G3BP2 and Twist1 in the cytoplasm at low stiffness, but not at high stiffness (Figure 2-27C and 2-27D), identical to what we observed in mammary organoids. This result indicates that

changes in cell polarity or adherens junction stability do not affect Twist1/G3BP2 interaction. Together with our earlier findings, these experiments demonstrate that matrix stiffness directly regulates the interaction between Twist1 and G3BP2 to control Twist1 subcellular localization.

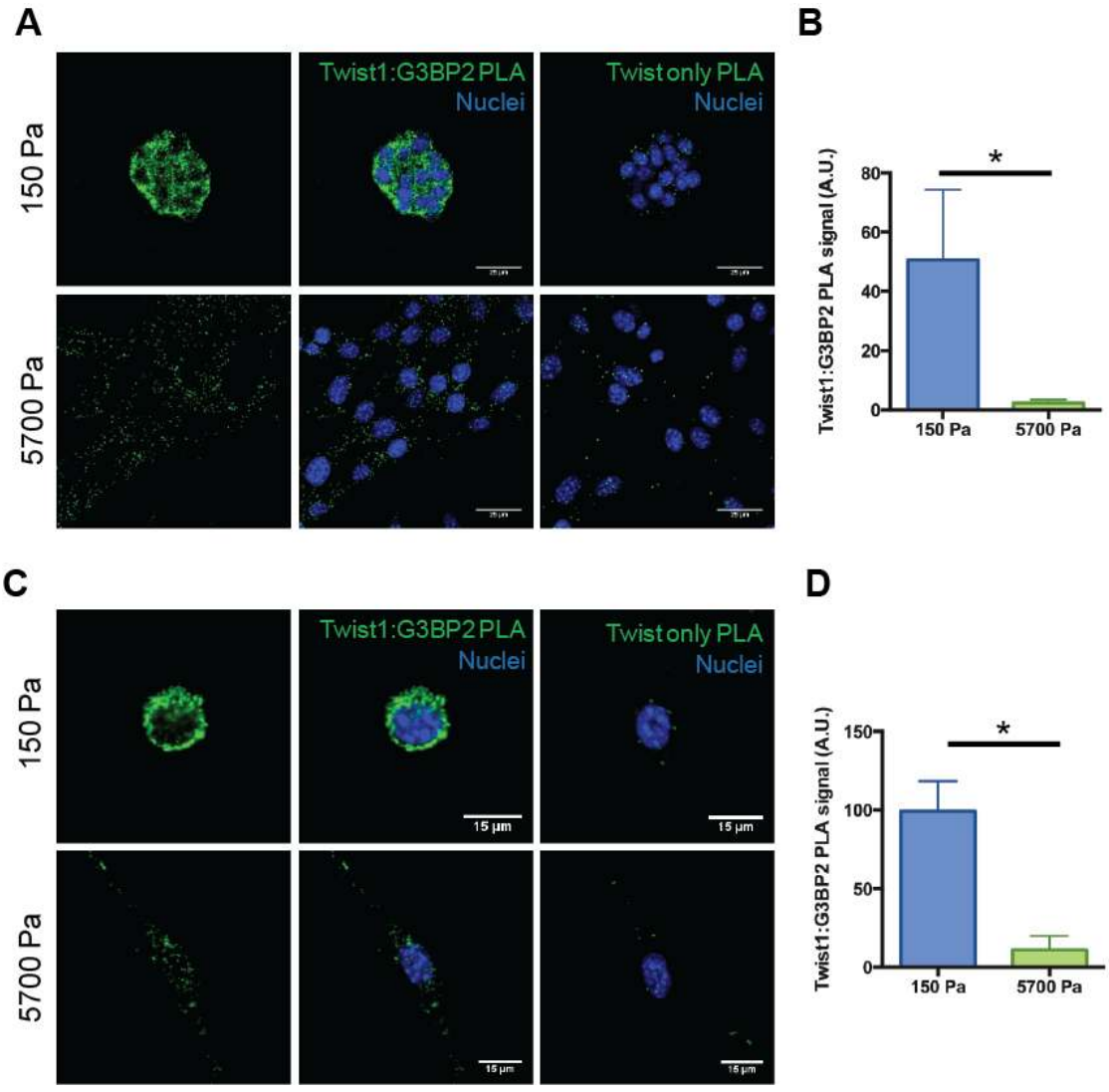

Figure 2-27. PLA analysis of the interaction between Twist1 and G3BP2. Eph4ras cells in 3D culture for 6 days (A) or 20 hours (C) at indicated rigidities were analyzed for Twist1 and G3BP2 interaction by in situ PLA assay, PLA signal (green) and DAPI (blue). (B, D) Quantification of PLA signal normalized to cell number in 3D cultures described in (A) and (C), respectively (*, $P<0.001$, representative experiments).

2.7 Cytoskeletal tension is not required for Twist1 regulation

How matrix stiffness induces release of Twist1 from G3BP2 was then the next logical question. Post-translational modification of Twist1 or G3BP2 might induce a conformation change or prevent interaction. Based on our findings that G3BP2 recognizes Twist1 via a consensus motif that is contained by multiple transcription factors, it seemed more likely that post-translational medication of Twist1 would be responsible for a specific mechanoregulation. Multiple post-translational modifications of Twist1 have been previously reported. Phosphorylation of serine 42 of human Twist1 by PKB/AKT mediates inhibition of apoptosis(Vichalkovski et al., 2010). This phosphorylation event results in inactivation of p53 but the molecular events that underlie this effect remain unknown. PKB/AKT is also able to phosphorylate human Twist1 at serine 123 *in vitro*, but this event could not be observed in cultured cells(Vichalkovski et al., 2010). The interaction between Twist1 and PKB/AKT does have significant consequences during tumor progression, facilitating Twist1-mediated metastasis(Xue et al., 2012). PKA can phosphorylate T125 and S127 of murine Twist1, corresponding to T121 and S123 in human Twist1, respectively(Firulli et al., 2005). Twist1 can also be phosphorylated by MAP kinases at serine 68 which results in stabilization of Twist1 protein(Hong et al., 2011). Expectedly with stabilization of Twist1 and thus an increase in effective Twist1 expression, breast cancer cells were rendered more invasive. However, because there was no significant change in Twist1 expression at the mRNA or protein level in response to matrix stiffness, S68 phosphorylation mediated mechanoregulation of Twist1 seems unlikely (Figure 2-11 and 2-12).

Tyrosine kinase and phosphatases are modulated by matrix rigidity; however the downstream sensor that transduces mechanical signals into biochemical signals is still unknown. Given that β1 integrin is necessary for matrix stiffness induced Twist1 nuclear translocation (Figure 2-14) and that integrin clustering can activate Src kinase, it was feasible that Src would mediate Twist1 mechanoregulation downstream of integrin activation. Tyrosine phosphorylation has been posited as a possible mechanism for this transduction(Giannone and Sheetz, 2006). Indeed, the Src family kinase substrate p130Cas is phosphorylated in response to mechanical signals(Sawada et al., 2006). The kinase responsible for this event is not known however. It was then of great interest that the consensus G3BP2-binding domain of Twist1 contained a Src family tyrosine kinase recognition motif. Indeed, a phospho-Y103 species of Twist1 has been previously identified using large scale mass spectrometry analysis of lung adenocarcinomas cells(Wu et al., 2009). This phospho-Y103 species of Twist1 will bind to Src SH2 domains. Twist1 contains only five tyrosines, of which only Y103 is known to be phosphorylated. Neither the biological effect of this phosphorylation event or the kinase that catalyzes the phosphorylation is known however. Y103 lies within the G3BP2 binding domain and thus its phosphorylation could regulation the interaction between the two proteins. To test this hypothesis, mammary epithelial cells were treated with small molecule inhibitors of Src and Src-family kinases. Inhibition of Src and other tyrosine kinases has no effect on Twist1 translocation however (Figure 2-28). This analysis was not exhaustive however and other tyrosine kinases may mediate this phosphorylation event. It also remains to be tested whether changes in matrix stiffness affect Y103 phosphorylation and whether changes in this site have functional consequences for Twist1-G3BP2 interaction.

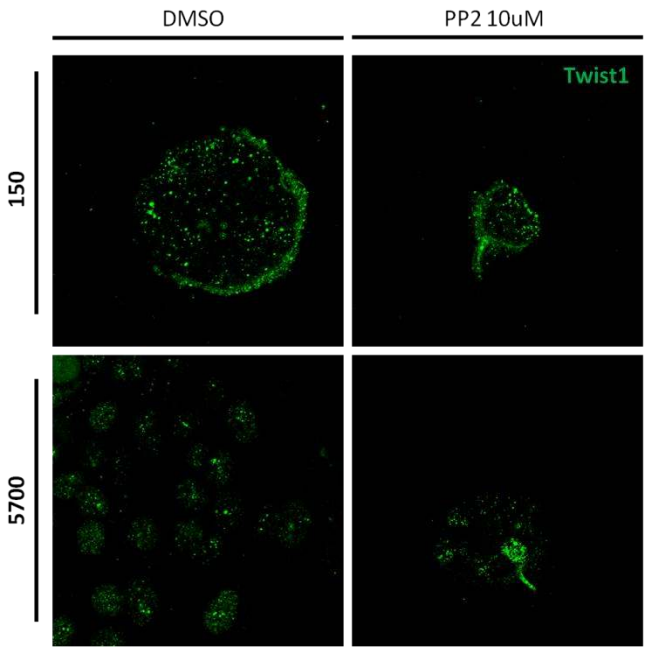

Figure 2-28. Src activity is not required for Twist1 mechanoregulation. MCF10A cells were grown in 3D culture at indicated rigidities and treated with either DMSO vehicle or PP2 constitutively and then stained for Twist1 (green).

Since Src activity was not required for Twist1 mechanoregulation we then were interested in whether canonical cytoskeletal signaling elements downstream of integrin activation were required. Surprisingly, inhibition of Myosin II and Rho kinase (ROCK) by blebbistatin and Y-27632, respectively, did not affect the localization of Twist1 in MCF10A cells cultured on glass compared to untreated and vehicle treated controls (Figure 2-29). The efficacy of the inhibitors was assessed by their clear effect on cytoskeletal organization and lack of stress fiber formation visualized by F-actin staining. This suggests that YAP and Twist1 are differentially regulated by integrin activation, as cytoskeletal tension is required for YAP nuclear localization(Dupont et al., 2011). The differential regulation of YAP and Twist1 may be due to the involvement of YAP in contact inhibition. To confirm that cytoskeletal tension was not required for Twist1 mechanotransduction we assessed Twist1 localization in MCF10A cells grown on PA

gels in 3D culture with and without inhibitors. Consistent with our results in MCF10A cells grown on glass, inhibition of cytoskeletal elements ROCK, Myosin II, and Focal adhesion kinase (FAK) by Y-27632, blebbistatin, and PF-228, respectively, did not affect Twist1 localization (Figure 2-30).

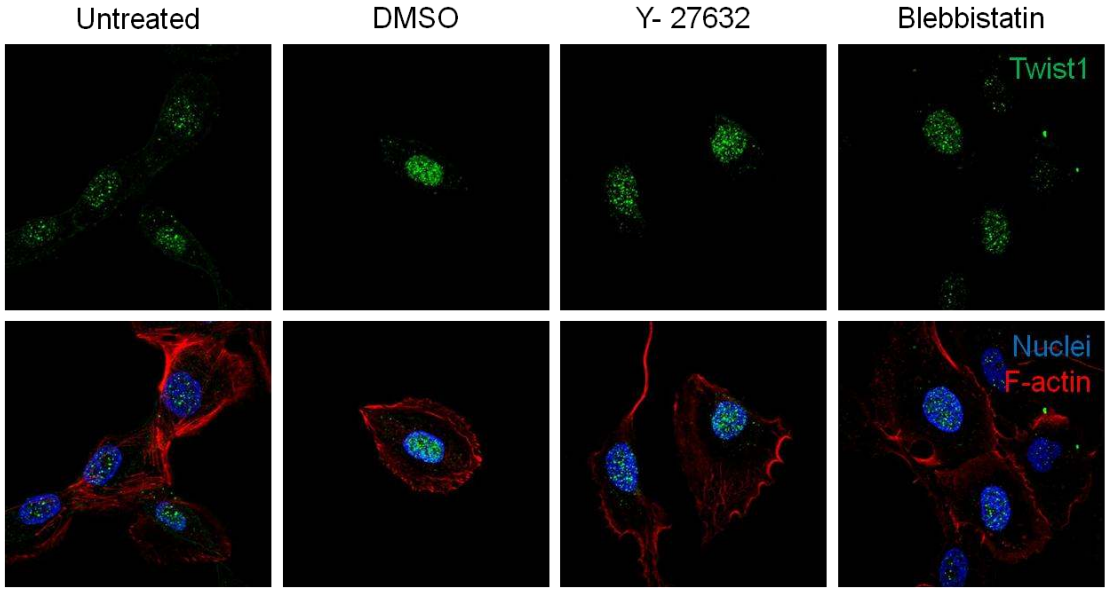

Figure 2-29. Cytoskeletal tension does not affect Twist1 localization. MCF10A cells were grown on fibronectin coated glass and treated with Blebbistatin (50 µM) and Y-27632 (50 µM) or DMSO and then stained for Twist1 (green), F-actin (Phalloidin, red), and DAPI (blue).

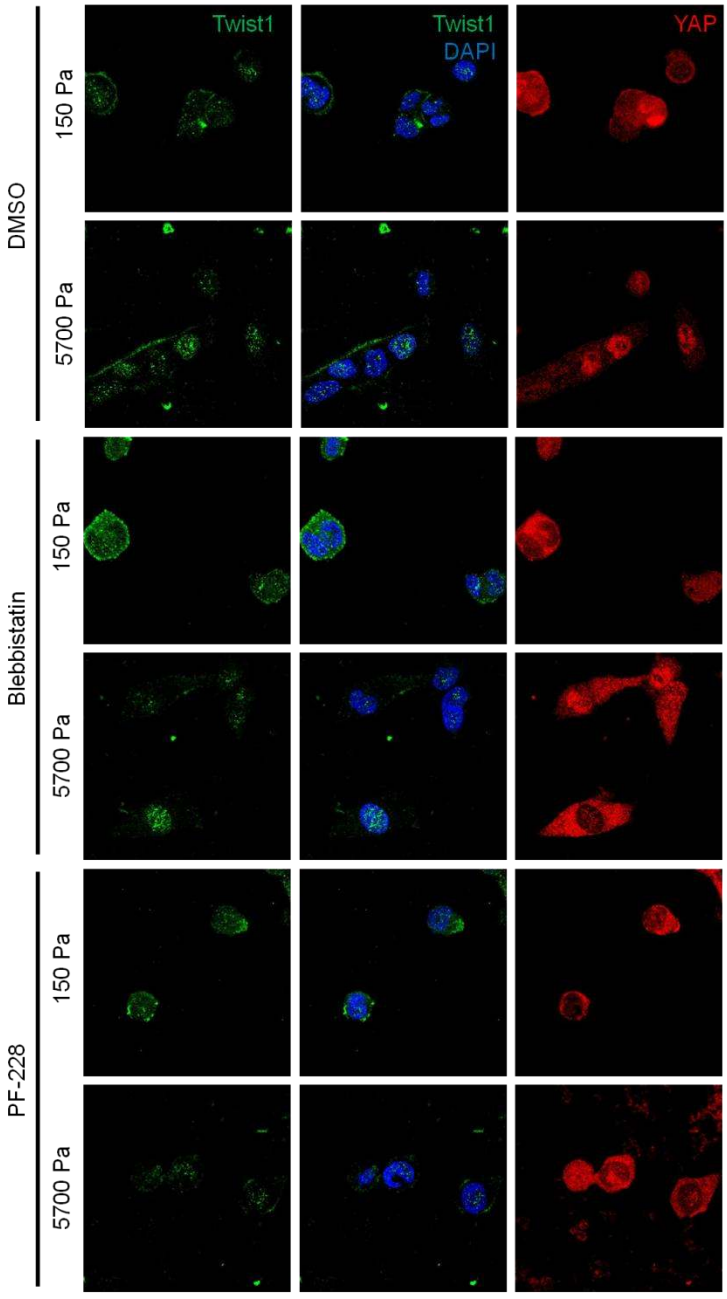

Figure 2-30. Cytoskeletal tension is not required for mechanoregulation of Twist1. MCF10A cells were grown on PA gels of indicated rigidities in 3D culture for 20 hours with treatments as indicated. Cells were then fixed and stained for Twist1 (green), YAP (red), and DAPI (blue).

The apparent dichotomy of YAP and Twist1 mediated mechanotransduction suggest the existence of multiple distinct mechanotransduction pathways. The observation that Twist1 is not dependent on cytoskeletal tension and perhaps more surprisingly, FAK activation, suggests that Twist1 is regulated very early and directly by matrix stiffness induced integrin activation. Further studies are required to identify the molecular mechanism of this regulation, however. Post-translational modification of Twist1 remains a probable mechanism for the regulation of Twist1 and G3BP2 interaction. In addition to the hypothesis that phosphorylation of Twist1 acts as a molecular switch that regulates Twist1-G3BP2 interaction, it also remains quite possible that other regulatory mechanisms exist. For example, modification of G3BP2, although unlikely as a specific mechanism to regulate Twist1, could induce dissociation of the two factors. Alternatively, acetylation of Twist1 could regulate its subcellular localization. Indeed, a diacetylated species of Twist1 has been recently reported. In fact, these acetylation sites, K73ac and K76ac, lie directly in the second NLS of Twist1. These lysine acetylations appear to not significantly affect exogenously expressed Twist1 nuclear localization in HMLE cells and are not in close proximity to the G3BP2 interaction domain, however. Further investigation is required to elucidate whether these post-translational modifications may still play a role in Twist1-G3BP2 mechanotransduction. Other post-translational modifications that have yet to be described, such as sumoylation and ubiquitination, could also regulate Twist1 activities. However, the absence of distinctly shifted species of Twist1 in Western blots does not support a role for sumoylated Twist1. Nonetheless, as many mechanisms remain feasible, further investigation is regulatory mechanism is clearly warranted as insight into this process will have significant impact. A potential future approach is mass

spectrometry analysis of cells grown at varying matrix stiffness conditions, either of whole cell lysates or immunoprecipitates.

2.8 Functional Consequences of Twist1-G3BP2 Signaling

If G3BP2 interacts with Twist1 in the cytoplasm to sequester it from nuclear import it would then follow that loss of G3BP2 would lead to constitutive Twist1 nuclear localization, independent of changes in matrix stiffness. Constitutive Twist1 nuclear localization would then promote EMT, thus disengaging regulation of EMT from regulation by this mechanosensory pathway. To test this hypothesis we first investigated whether G3BP2 is functionally required for Twist1 cytoplasmic retention on compliant matrices. We used shRNAs to knock down G3BP2 and determined its impact on Twist1 localization (Figure 2-31, 2-32, and 2-33). For both MCF10A and Eph4ras cells on compliant matrices, knockdown of G3BP2 resulted in nuclear accumulation of Twist1 (Figure 2-34 and 2-35). This is consistent with the hypothesis that G3BP2 sequesters Twist1 in the cytoplasm, as loss of G3BP2 would allow Twist1 to be trafficked into the nucleus by canonical import machinery via its *bona* fide NLS. These data strongly support a critical role of G3BP2 in regulating Twist1 subcellular localization in response to matrix stiffness.

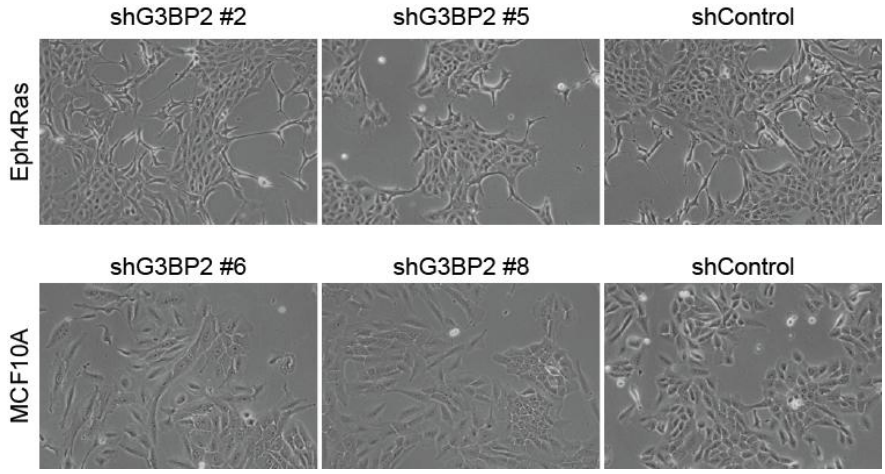

Figure 2-31. Generation of stable G3BP2 knockdown cell lines. Brightfield images of Eph4Ras and MCF10A shG3BP2 and shControl (shRFP) cells.

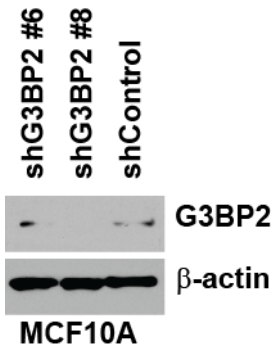

Figure 2-32. Loss of G3BP2 in stable MCF10A G3BP2 knockdown cell lines. Cell lysates from MCF10A cells expressing control or G3BP2 shRNAs were analyzed by SDS-PAGE and probed for G3BP2 and β-actin.

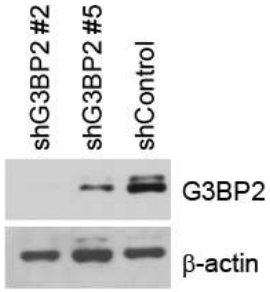

Figure 2-33. Loss of G3BP2 in stable Eph4Ras G3BP2 knockdown cell lines. Cell lysates from Eph4Ras cells expressing control or G3BP2 shRNAs were analyzed by SDS-PAGE and probed for G3BP2 and β-actin.

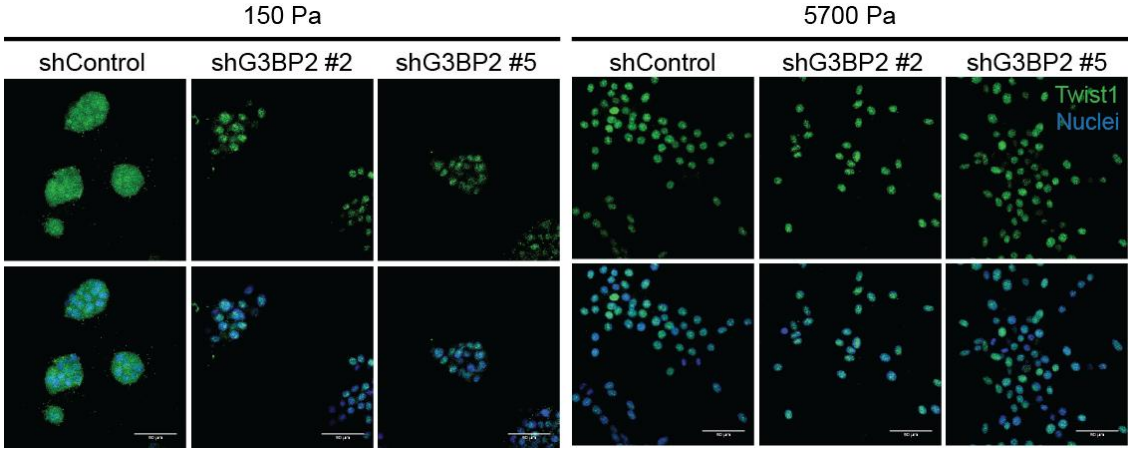

Figure 2-34. Constitutive Twist1 nuclear localization in G3BP2 knockdown mouse mammary epithelial cells. Eph4Ras cells expressing control or G3BP2 shRNAs were cultured in 3D culture at indicated rigidities for 5 days and stained for Twist1 (green) and DAPI (blue).

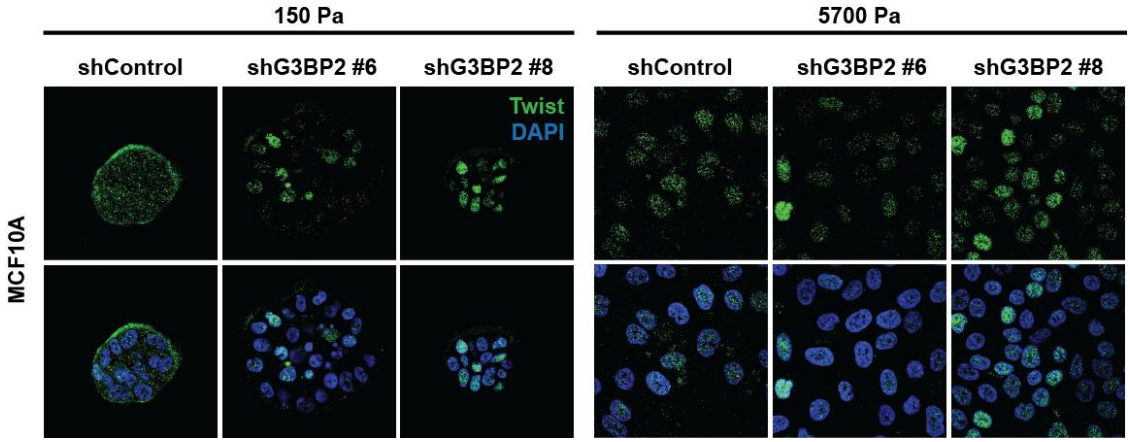

Figure 2-35. Constitutive Twist1 nuclear localization in G3BP2 knockdown human mammary epithelial cells. MCF10A cells expressing control or G3BP2 shRNAs were cultured in 3D culture at indicated rigidities for 5 days and stained for Twist1 (green) and DAPI (blue).

Because the transcription factor YAP has also been shown to be mechano-sensitive and translocate to the nucleus in response to increased matrix stiffness, we sought to determine whether G3BP2 also regulated YAP localization. In control cells YAP was cytoplasmic at low stiffness and translocated to the nucleus at high stiffness. Loss of G3BP2 did not affect this mechano-regulation of YAP localization, suggesting that G3BP2 mechano-regulation is specific to Twist1 (Figure 2-36). This is consistent with our finding that YAP does not contain the G3BP2 consensus binding motif identified in Twist1 and other G3BP2 targets.

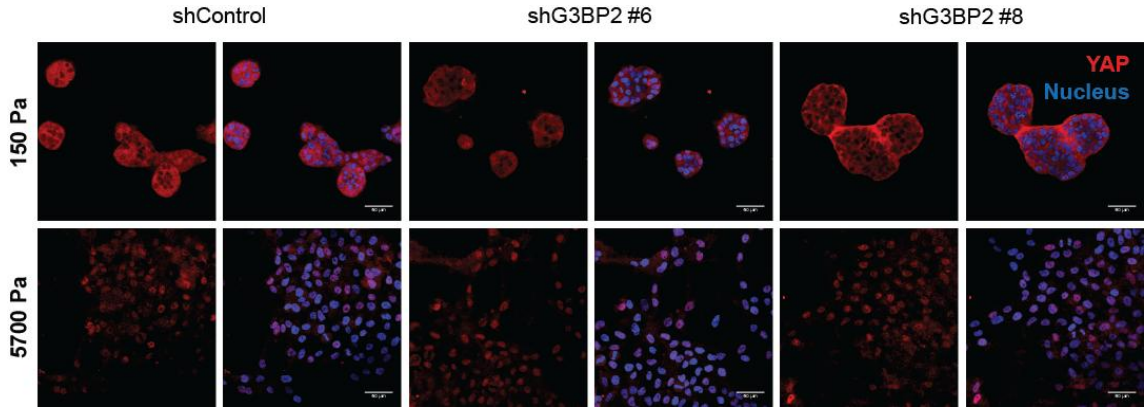

Figure 2-36. G3BP2 is not required for YAP mechanoregulation. MCF10A cells expressing control or G3BP2 shRNAs were cultured in 3D culture at indicated rigidities for 5 days and stained for YAP (red) and DAPI (blue).

Next, to test the impact of G3BP2 loss on EMT and invasion, we grew Eph4Ras and MCF10A mammary epithelial cells on a gradient of PA hydrogels in 3D culture. Curiously, while loss of G3BP2 did increase acinar invasion on compliant matrices, acini did not completely adopt an invasive phenotype despite Twist1 nuclear translocation (Figure 2-37 and 2-38). This suggests that Twist1 is not sufficient to induce EMT in low matrix stiffness conditions. This observation might be due to insufficient traction generated by cells on such a compliant substrate. To test this hypothesis we grew mammary epithelial cells on intermediate matrix stiffness conditions of 320 and 670 Pa. If the cells were primed to undergo EMT, but mechanically could not generate enough force to spread out when on 150 Pa, then as soon as their substrate was mechanically permissive the cells would undergo EMT. Loss of G3BP2 would thus effectively reduce the threshold of cells to undergo EMT in response to increasing matrix stiffness. At these intermediate matrix stiffness conditions loss of G3BP2 caused a dramatic increase in invasive acini. Thus, G3BP2 knockdown and subsequent Twist1 nuclear localization

significantly increased the percentage of invasive acini at matrix rigidities ranging from 150 to 670Pa (Figure 2-37 and 2-38).

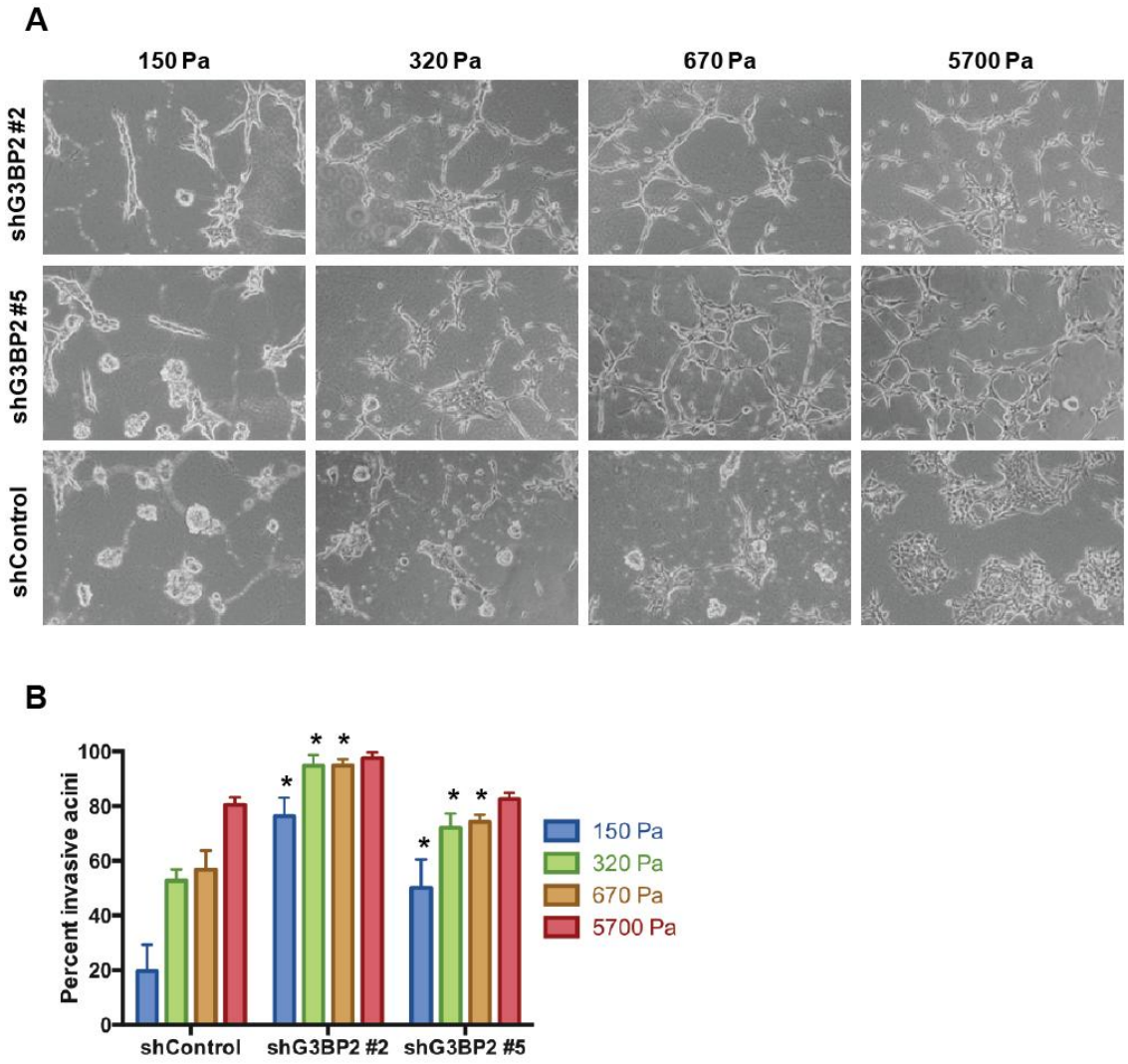

Figure 2-37. Loss of G3BP2 and increasing matrix stiffness induce an invasive phenotype. (A) Brightfield images of Eph4Ras cells grown for 5 days in 3D culture on varying matrix rigidities. (B) Quantification of invasive acini in 3D culture described in (A) from 3 independent experiments (*, P<0.001).

Invasive cell morphology was accompanied by down-regulation of E-cadherin and loss of basement membrane integrity as shown by Laminin V staining (Figure 2-38). These data indicate that G3BP2 directly impacts EMT and invasion in response to matrix

stiffness and that loss of G3BP2 sensitizes cells to changes in matrix stiffness. Given that Twist1 levels do not vary in response to changes in matrix stiffness, the effect of loss of G3BP2 is even more striking (Figure 2-12A). It remains to be seen whether changes in matrix stiffness could affect expression of G3BP2 as well. Mechanistically, G3BP2 expression may function as a molecular thermostat to regulate at what matrix stiffness cells will begin to respond. Without a large pool of G3BP2 molecules, a cell might not tolerate increases in matrix stiffness without allowing Twist1 nuclear translocation. Conversely, with high levels of G3BP2 expression a cell would be able to sequester more Twist1, and thus might act as a buffer from changes in matrix stiffness. This suggests that downregulation of G3BP2 expression in tumor cells could cooperate with increasing matrix stiffness in the tumor microenvironment to facilitate EMT and tumor invasion.

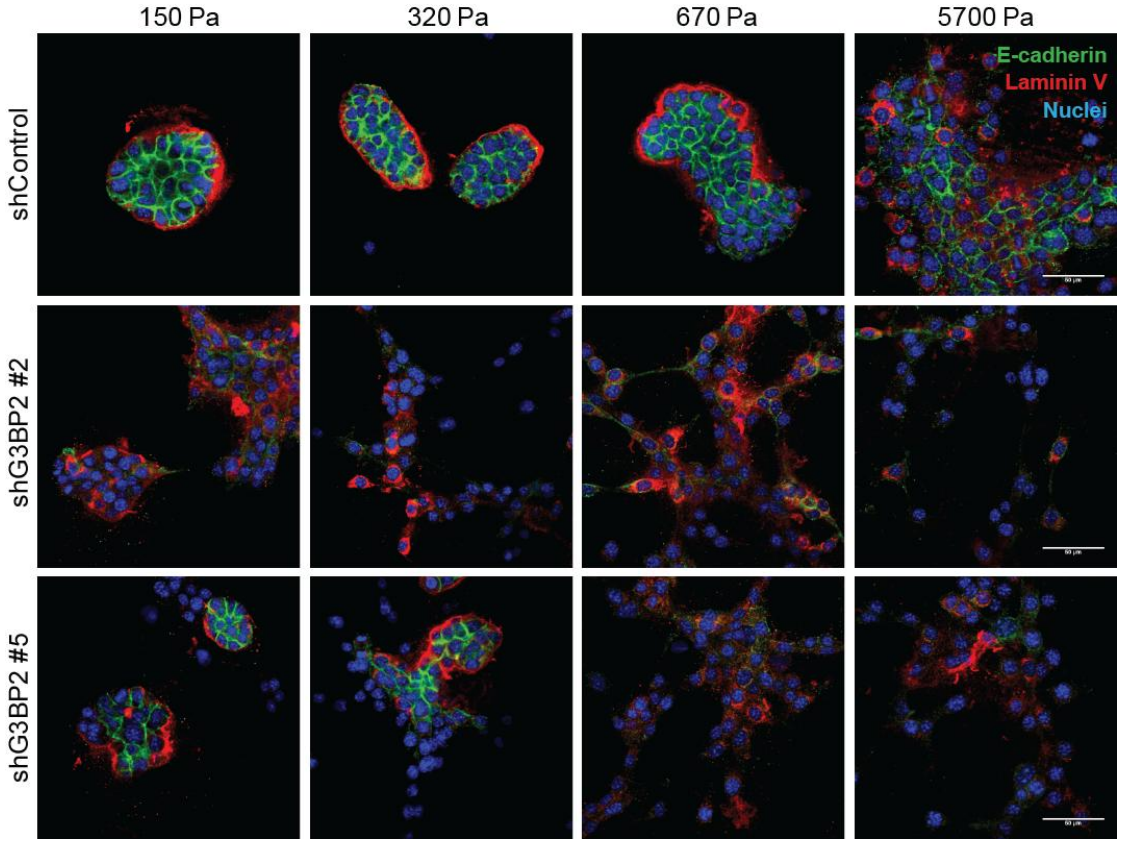

Figure 2-38. Loss of G3BP2 and increasing matrix stiffness collaborate to induce EMT and loss of basement membrane integrity. Eph4Ras cells expressing control or G3BP2 shRNAs were cultured in 3D culture with varying rigidities for 5 days and stained for E-cadherin (red), Laminin V (green) and DAPI (blue).

2.9 Matrix Stiffness and G3BP2 in Mouse Models of Breast Cancer

The involvement of G3BP2 in cancer remains unclear, both with respect to function and mechanism. G3BP proteins are upregulated in some cancers and has been posited as a potential drug target(Cui et al., 2010; Guitard et al., 2001; Vognsen et al., 2013). For example, molecular targeting of the NTF2 domain of G3BP1, which is nearly identical with that of G3BP2, has been proposed as a therapeutic approach to be used to specifically sensitize cells to cis-platin(Cui et al., 2010). The role of G3BP proteins is unclear as there are relatively few reports and those reports are conflicting in

some regards. G3BP1 has been reported to promote S phase entry and be upregulated in tumors(Guitard et al., 2001). However, another group has reported the G3BP1 is not upregulated in tumors but in fact G3BP2 is overexpressed in 88% of breast cancers(French et al., 2002). These findings are further complicated by the interesting observation that some tumor cells displayed nuclear expression of G3BP2, although in a minority of samples and a minority of cells within those samples(French et al., 2002). Our finding that matrix stiffness regulates Twist1 subcellular localization and EMT via G3BP2 warranted further investigation into the role of tissue rigidity and G3BP2 in breast cancer progression.

To validate this Twist1-G3BP2 mechanosensing pathway *in vivo* we utilized several breast cancer models. First, to assess the role of matrix stiffening during breast tumor progression we utilized a chemical inhibitor β-aminopropylnitrile (βAPN) of lysyl oxidase (LOX), the enzyme that catalyzes collagen crosslinking. Treatment with βAPN prevents collagen crosslinking and thus attenuates tissue stiffening. Previous studies have used βAPN for this purpose(Erler et al., 2006; Levental et al., 2009). To assess the effect of βAPN on tumor progression we utilized two orthotopic breast cancer models. In the first, Eph4Ras cells are syngeneic with Balb/C mice and were injected orthotopically into the mammary gland. Mice were treated with βAPN in their drinking water or by daily intraperitoneal injection (100mg/kg/day) and compared to a vehicle alone cohort of mice. Mechanical material measurements were taken on fresh tumor tissue to determine whether the treatment was effective in reducing tissue rigidity (Figure 2-39). The elastic modulus of tumors from βAPN treated was reduced approximately 40% indicative that the treatment was effective. This level of reduction is consistent with previous reports(Levental et al., 2009). βAPN treatment did not however affect primary tumor growth (Figure 2-40).

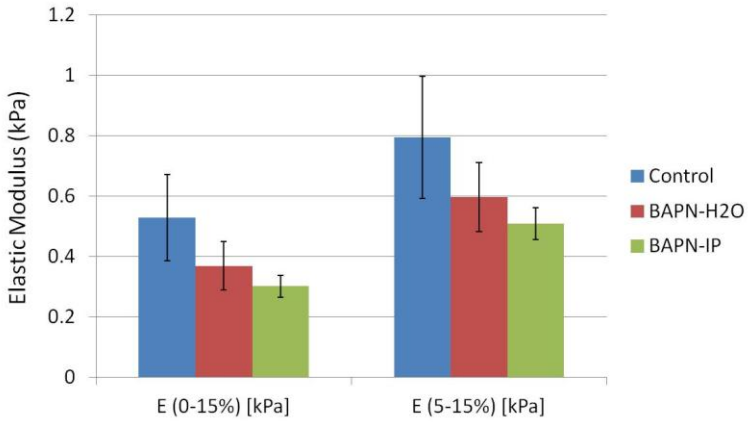

Figure 2-39. Mechanical analysis of Eph4Ras tumors. Mechanical analysis of Eph4Ras tumors from mice treated with PBS vehicle (control), βAPN treatment in the drinking water (BAPN H20), and βAPN treatment by intraperitoneal injection (BAPN IP), based on unconfined compression measurements.

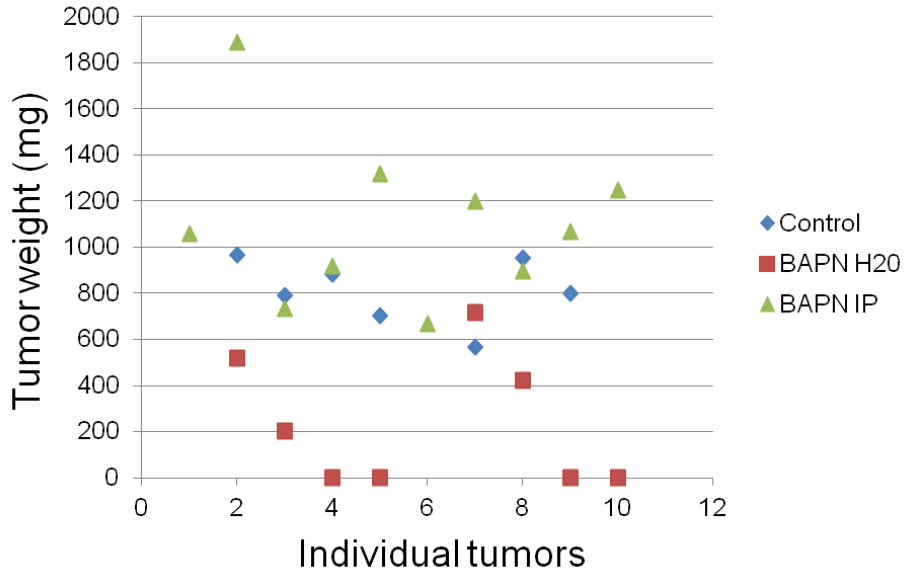

Figure 2-40. Primary tumor weight of control and βAPN treated Eph4Ras orthotopic tumors. Analysis of tumors from mice treated with PBS vehicle (control), βAPN treatment in the drinking water (BAPN-H20), and βAPN treatment by intraperitoneal injection (BAPN-IP). Tumors were excised from host Balb/C mice and weighed prior to mechanical analysis. Tumors 1-5 and 6-10 are from the left and right mammary glands, respectively.

Because the metastatic rate of Eph4Ras cells was relatively low and inconsistent we used 4T1, another murine tumor cell line derived from the Balb/C mouse strain. 4T1 cells are a highly invasive and metastatic cell line that has high expression of Twist1(Yang et al., 2004). 4T1 cells were implanted orthotopically into syngeneic Balb/C mice and separated into a βAPN or vehicle only (PBS) cohort. βAPN treatment did not significant affect either primary tumor growth or metastatic lung colonization (Figure 2-41 and 2-42). Our observation that Twist1 localization is not as effectively regulated in 4T1 cells may provide some clarity for this result. 4T1 cells express a high level of Twist1 which may be sufficient to overcome the pool of inhibitory G3BP2. This is consistent with the observation of nuclear and cytoplasmic Twist1 on compliant matrices compared to the exclusive nuclear localization at high matrix stiffness (Figure 2-43). Consistent with this observation Twist1 was predominantly localized in the nucleus in tumor tissue while G3BP2 was cytoplasmic (Figure 2-44). This result then might suggest that very high expression of Twist1 can bypass the requirement for increased matrix stiffness.

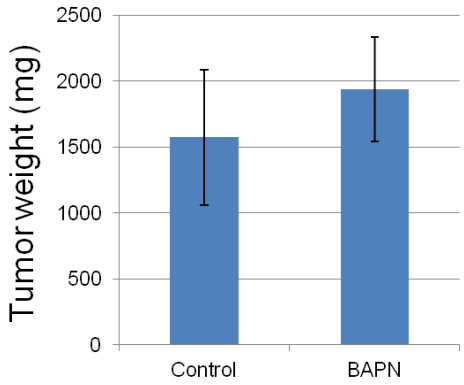

Figure 2-41. Primary tumor weight of control and βAPN treated 4T1 tumors.

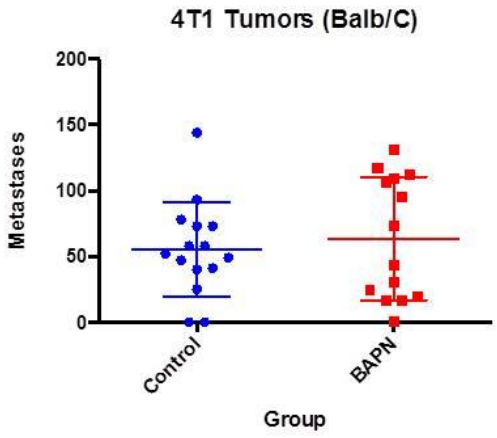

Figure 2-42. Metastases from control and βAPN treated 4T1 tumors. Quantification of the number of lung metastases in individual control and βAPN treated mice with orthotopically injected 4T1 tumor cells (n.s.).

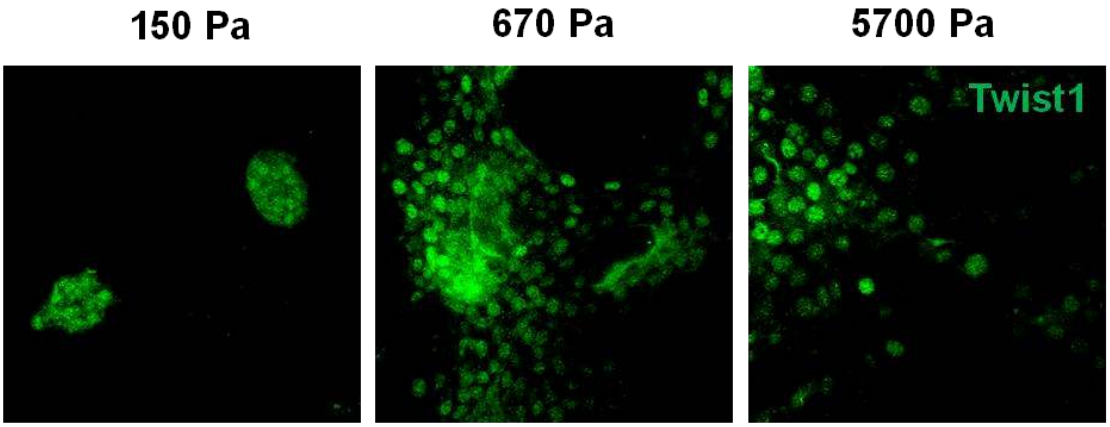

Figure 2-43. Twist1 localization in 4T1 cells on PA hydrogels. 4T1 cells were cultured on PA hydrogels of indicated rigidities in 3D culture and stained for Twist1 (green).

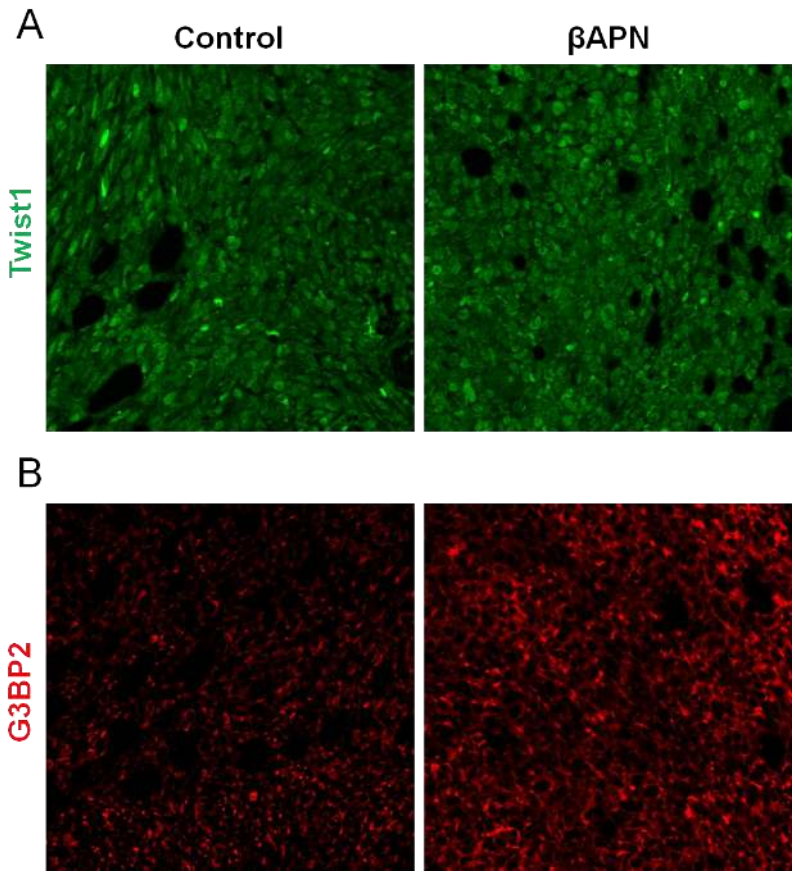

Figure 2-44. Localization of Twist1 and G3BP2 in 4T1 primary tumors. Confocal microscopy of FFPE primary tumor sections stained for either (A) Twist1 (green) or (B) G3BP2 (red).

To investigate whether this was a cell line dependent effect, we sought to confirm whether βAPN treatment could prevent lung metastasis of MDA-MB-231 cells as has been previously reported(Erler et al., 2006). In this human xenograft tumor model, MDA-MB-231 cells were injected sub-cutaneously into the flanks of nude mice. βAPN treatment did not affect primary tumor growth or metastatic lung colonization of MDA-MB-231 cells (Figure 2-45 and 2-46). This may be due the limited size of the cohorts which were not able to detect subtle differences. Also it is also unclear how dramatic the

effect of βAPN treatment is in subcutaneous models in which the tumor is not initially exposed to significant ECM.

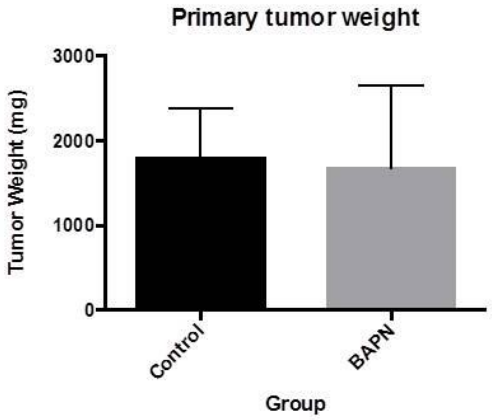

Figure 2-45. Primary tumor weight of MDA-MB-231 tumors in control and βAPN treated groups.

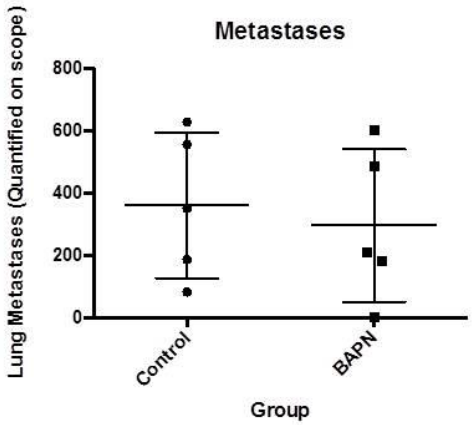

Figure 2-46. Metastases from control and βAPN treated MDA-MB-231 tumors. Quantification of lung metastases from MDA-MB-231 tumors (n.s.).

To investigate whether loss of G3BP2 would promote tumor invasiveness we employed the syngeneic murine Eph4Ras orthotopic breast cancer model. Eph4Ras cells stably expressing control or shRNA against G3BP2 were injected orthotopically into

Balb/C mice. To investigate whether loss of G3BP2 and TGF-β would synergistically promote tumor invasion, cells were injected with and without pre-treatment of TGF-β (Figure 2-47). This presumed that once exposed to soluble, active TGF-β Eph4Ras would establish an autocrine loop to further promote EMT(Oft et al., 1996). This approach also rested on our finding that matrix stiffness (and hence G3BP2) collaborates with TGF-β signaling to induce a complete EMT program.

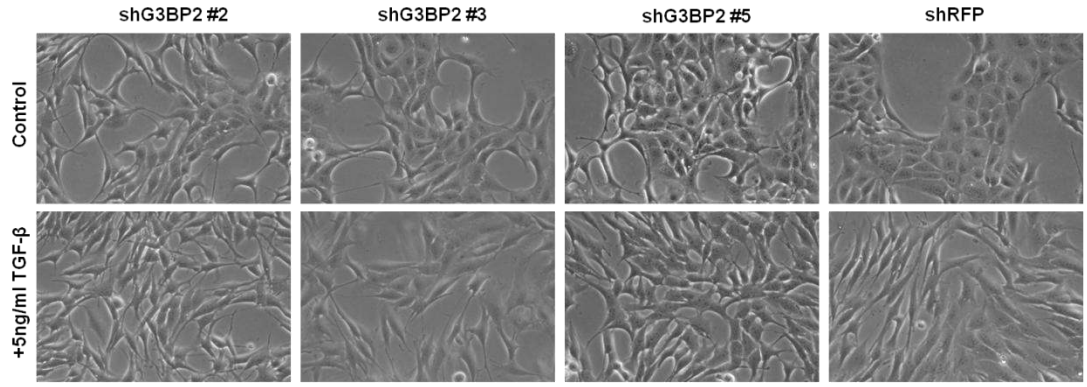

Figure 2-47. EMT induction by TGF-β treatment and loss of G3BP2. Brightfield images of Eph4Ras cells in the absence or presence of TGF-β for one week in tissue culture dishes prior to orthotopic injection into recipient mice.

We did observe a modest increase in either total number of lungs metastases and macroscopic lung metastases due to TGF-β treatment (Figure 2-48 and 2-49). There was no difference in tumor growth in control and #2 and #5 shG3BP2 lines (Figure 2-50 and 2-51). For unknown reasons the #3 shG3BP2 tumors were rapidly rejected. While an increase in micro and macro lung metastases was observed in shG3BP2 #5, a similar effect was not observed in shG3BP2 #2 Eph4Ras cells. Further complicating interpretation was the large amount of variation in metastatic rate. It is unclear whether a real effect of G3BP2 loss is obscured by high levels of variation in this model.

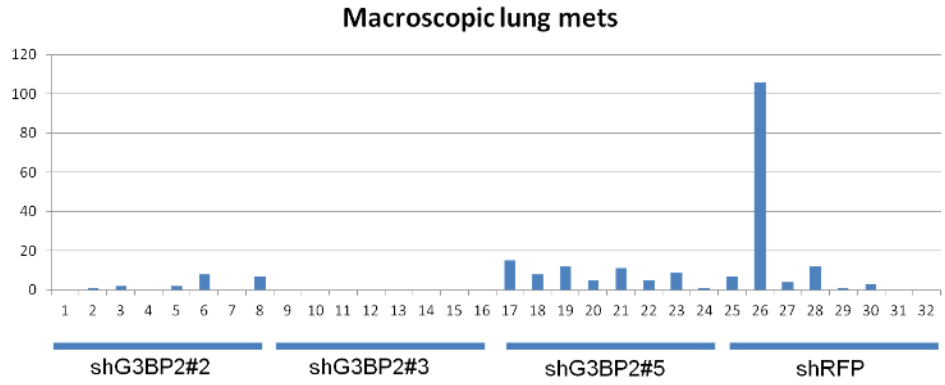

Figure 2-48. Metastases from control and G3BP2 knockdown Eph4Ras tumors. Quantification of lung metastases from individual mice bearing tumors from control treated Eph4Ras syngeneic cells with and without knockdown of G3BP2.

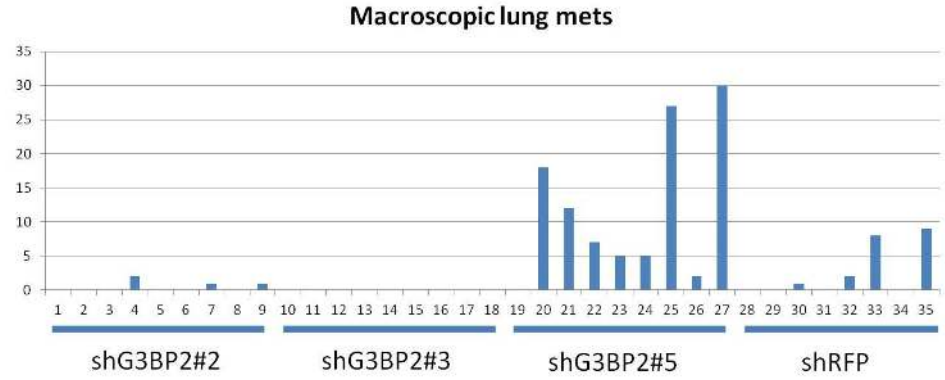

Figure 2-49. Metastases from TGF-β treated control and G3BP2 knockdown Eph4Ras tumors. Quantification of lung metastases from individual mice bearing tumors from TGF-β treated Eph4Ras syngeneic cells with and without knockdown of G3BP2.

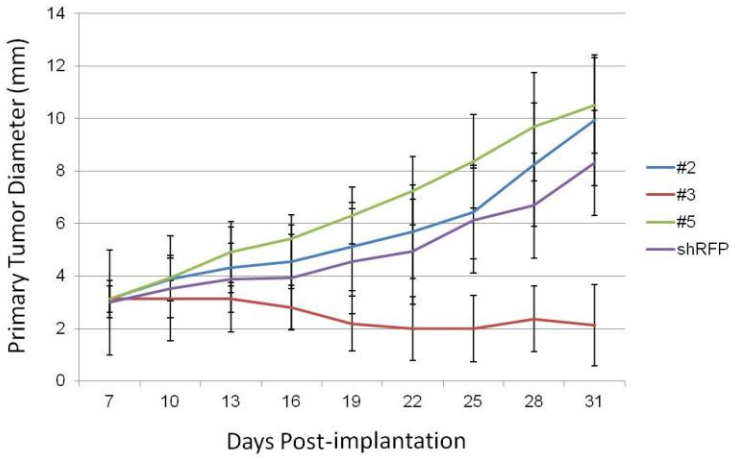

Figure 2-50. Growth curve of control and G3BP2 knockdown Eph4Ras tumors. Primary tumor growth of control (MEGM grown) Eph4Ras tumors with and without knockdown of G3BP2 (shG3BP2 lines #2, #3, and #5 compared to control shRFP).

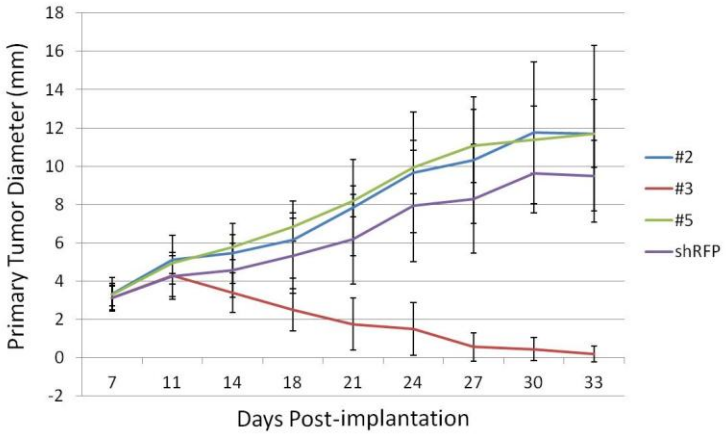

Figure 2-51. Growth curve of TGF-β treated control and G3BP2 knockdown Eph4Ras tumors. Primary tumor growth of TGF-β treated Eph4Ras tumors with and without knockdown of G3BP2 (shG3BP2 lines #2, #3, and #5 compared to control shRFP).

Thus, while these data partially support a role for G3BP2 in suppressing tumor invasion, the model system used does not appear to be well suited for this investigation. Furthermore, while βAPN treatment did produce a modest decrease in tumor rigidity, the effects were limited; also suggesting that these tumor models were not ideal for

investigating the role of mechanotransduction pathways during tumor progression. In support of this notion, ECM deposition and organization was relatively limited in orthotopic tumor tissue compared to normal mouse mammary gland as well as human tumor tissue (2-51 and 2-58). Second Harmonic Generation (SHG) imaging of orthotopic mouse tumors revealed a relative low amount of organized fibrillar collagen in both control and βAPN treated samples (Figure 2-52). This raised the possibility that even though βAPN has an effect on gross tissue rigidity, the rapidity at which these cell lines establish tumors may not be conducive for evaluating the role of matrix stiffness in tumor progression. Changes in ECM content and tissue rigidity are likely the result of a long process of ECM deposition and remodeling. Thus, analysis of human patient samples in which the tumor has developed over a long period time may be a more appropriate approach.

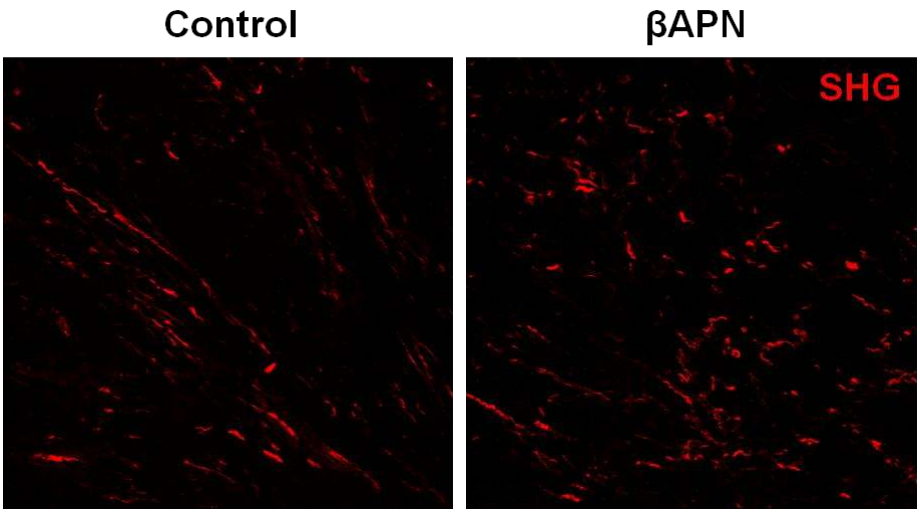

Figure 2-52. Second harmonic generation imaging of mouse orthotopic tumors. Rehydrated FFPE sections of Eph4Ras primary tumors were imaged by 2-photon SHG imaging (red).

βAPN mediated inhibition of LOX, while effective as demonstrated by mechanical testing, was not sufficient to attenuate tumor metastasis. This was surprising, as it has been previously reported that βAPN treatment can reduce metastasis by acting not only at the primary tumor site, but also the secondary site through modulation of the metastatic niche(Erler et al., 2009; Erler et al., 2006; Levental et al., 2009). The role of matrix stiffness in tumor progression may be more complex, however, and require a tumor that more accurately recapitulates human cancer. The time scale of growth in orthotopic and subcutaneous murine and xenograft tumor models is dramatically shorter than that of human tumors. To more fully understand how increasing tissue rigidity contributes to breast cancer progression genetic or spontaneous mouse models such as the mouse mammary tumor virus driven polyoma middle T (MMTV-PyMT) model, that develop slower, could provide significant advantages. Retrospective analysis of human patient samples could also allow for further insight into the roles of G3BP2 and matrix stiffness *in vivo*.

2.10 Matrix Stiffness and G3BP2 in Human Breast Cancers

To begin to understand the role of G3BP2 in tumor progression we first investigated whether tumor cells harbor alterations in G3BP2. Analysis of the TCGA datasets using the MSKCC cBioportal (http://www.cbioportal.org/public-portal/) revealed a relative alteration rate in breast cancer of 2.9%. Of these alterations most were actually amplications. The remaining alterations consisted of point mutants, however, there was not clear enrichment of mutations in functional NTF2, proline-rich, and RRM domains (Figure 2-53). Of note, there were relatively few identified mutations in the region sufficient for interaction with IκB-α(Prigent et al., 2000).

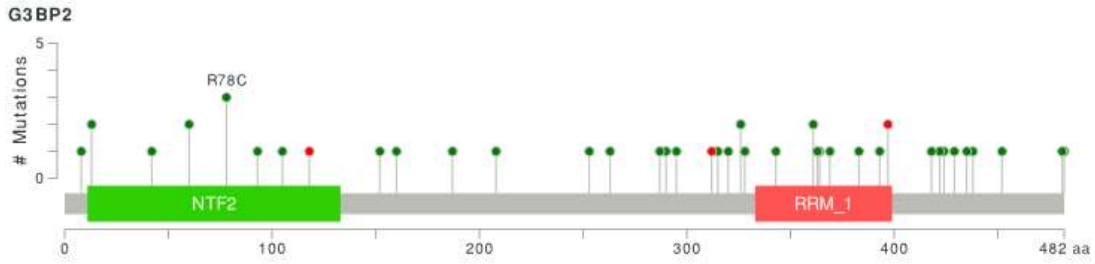

Figure 2-53. Diagram of mutations in G3BP2 identified in human cancers. Mutations identified in human cancers are displayed on G3BP2 with respect to their position in the protein. Data were retrieved from and are curated by the Memorial Sloan Kettering Cancer Center cBioPortal.

Analysis of other TCGA datasets also revealed a low mutation rate of G3BP2. Since the majority of alterations were amplifications and our findings suggest that loss of G3BP2 synergizes with increases in matrix stiffness to induce EMT and invasiveness, we were interested in whether changes in G3BP2 expression could correlate with patient outcome. Expression analysis of the TCGA breast cancer data set also using cBioportal identified 8% of tumors with disregulated G3BP2 mRNA expression using a Z=2 cutoff based on the expression of all genes, including both up and down regulated cases. Further analysis of the TCGA breast cancer (TCGA_BRCA_G4502A_07_3) dataset revealed a decrease in overall patient survival in the tumors with low G3BP2 expression (below mean compared to above mean expression), albeit this correlation was not statistically significant (Figure 2-54). Further analyses of this dataset also weakly supported a role for G3BP2 in breast cancer survival (Table 2-2). Because G3BP2 functions at the protein level to sequester Twist1, we surmised that G3BP2 protein expression would be more predictive of its function and relationship with changes in matrix stiffness.

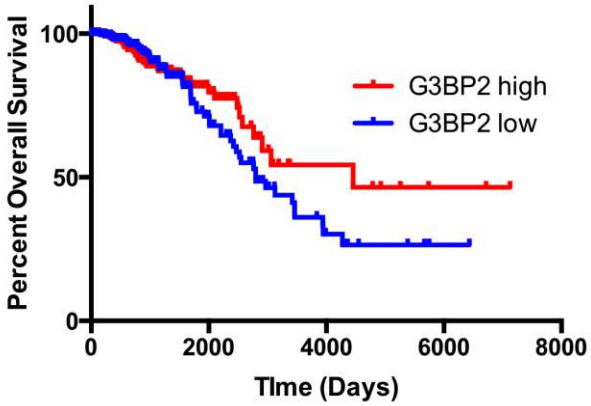

Figure 2-54. Kaplan-Meier survival plot of breast cancer patients stratified by G3BP2 mRNA expression. Kaplan-Meier survival curve of patients stratified by G3BP2 expression in the TCGA breast cancer dataset (TCGA_BRCA_G4502A_07_3) (Log-Rank P=0.2435).

Table 2-2. Stratification of breast cancer patients by G3BP2 expression. Statistics of overall survival of patients stratified by G3BP2 expression in the TCGA breast cancer dataset (TCGA_BRCA_G4502A_07_3).

	G3BP2 Low	G3BP2 High
Median Survival (Months)	91.99	146.50
Total Patients	291	273
Hazard ratio	0.7797	
Hazard ratio 95% CI	0.5155 to 1.181	

To understand whether loss of G3BP2 protein expression and increasing matrix stiffness synergistically impact human tumor progression, we next analyzed G3BP2 expression and matrix stiffness in a cohort of 398 Stage-2 and 197 Stage-3 breast tumors from the NCI cancer diagnosis program. These samples are analyzed in a tissue microarray with 0.6 mm cores and allow for the analysis of well annotated high quality samples. We used collagen fiber alignment, measured by SHG imaging, as a surrogate readout for tissue rigidity. As discussed previously in Chapter 1, increasing tissue rigidity can be used as a metastatic marker. Consistent with previous reports(Conklin et al., 2011; Hasebe et al., 2002; Provenzano et al., 2008), significantly more Stage-3

breast tumors present organized collagen structures than Stage-2 tumors (44.31% vs 29.88%, respectively; Fisher's Exact, P = 0.0027) (Table 2-3). Stage-2 breast cancers with organized collagen presented a trend towards poorer survival, with a decreased median survival, however these observations were not statistically significant (Figure 2-55). This dataset might not have enough power to detect these differences as it is not fully mature in that most of the samples are censored in the analysis. Alternatively, increasing matrix stiffness may represent a later event during tumor progression and have a more significant role in stage 3 or 4 breast cancers.

Table 2-3. Collagen organization in stage 2 and 3 breast cancer. Breast cancer tumor cores acquired from the NCI CDP were imaged by SHG and scored blindly.

	Disorganized Collagen Fibers	Straight Collagen Fibers
Stage 2	70.11494%	29.88506%
Stage 3	55.68862%	44.31138%

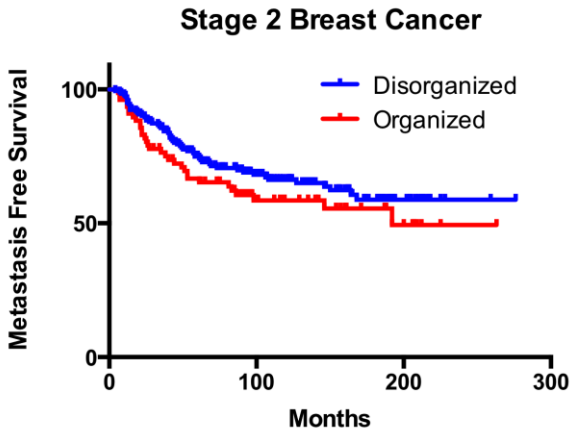

Figure 2-55. Metastasis free survival in stage 2 breast cancer stratified by collagen organization. Kaplan-Meier curve of metastasis free survival in stage 2 breast cancer patients based on SHG imaging (Log-rank P-value = 0.1894).

In agreement with previous publications(Colpaert et al., 2001; Conklin et al., 2011; Hasebe et al., 2002; Kakkad et al., 2012; Provenzano et al., 2008), Stage-3 patients presenting stiffer tumors (organized collagen structures) had poorer survival with a median of 31 months of recurrence-free survival time compared to 49 months in patients with more compliant tumors (P-value =0.0014) (Figure 2-56).

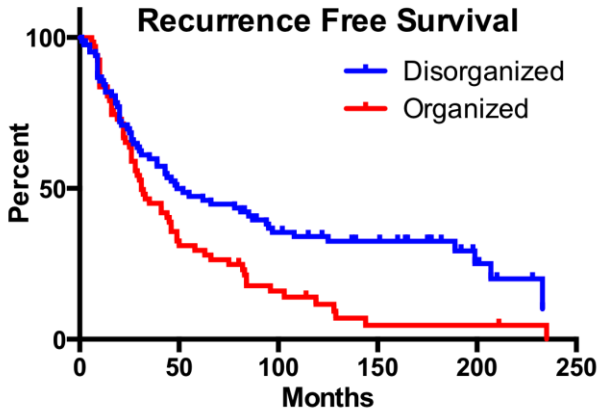

Figure 2-56. Collagen organization in breast tumors correlates with recurrence free survival. Kaplan-Meier curve of metastasis free survival in stage 3 breast cancer patients based on SHG imaging (Log-rank P-value = 0.0047).

Furthermore, consistent with a role in epithelial maintenance, we observed that G3BP2 expression was restricted to the luminal epithelial cells in normal human breast and colon tissues (Figure 2-57 and 2-58). This finding in some regards conflicts with a previous report that G3BP2 expression is undetectable in normal breast epithelium(Kennedy et al., 2001). This discrepancy may be caused by the use of different antibodies or sample preservation. Furthermore, the interpretation may be different since our findings suggest that G3BP2 opposes EMT and tumor invasiveness. Previous analyses of G3BP2 expression human tumor samples did not report the outcomes of the patients analyzed, which would provide additional clarity. The TMA

samples acquired from the National Cancer Institute's Cancer Diagnoses Program are annotated with receptor status, treatment course, and patient outcome among other parameters which enables effective retrospective analyses.

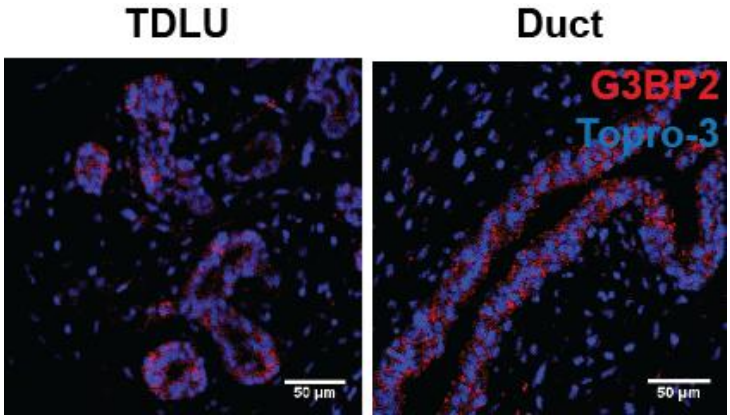

Figure 2-57. Expression of G3BP2 is restricted to epithelial cells in the mammary gland. Confocal microscopy of normal human breast terminal ductal lobular units (TDLU) and ducts stained for G3BP2 (red) and nuclei (blue).

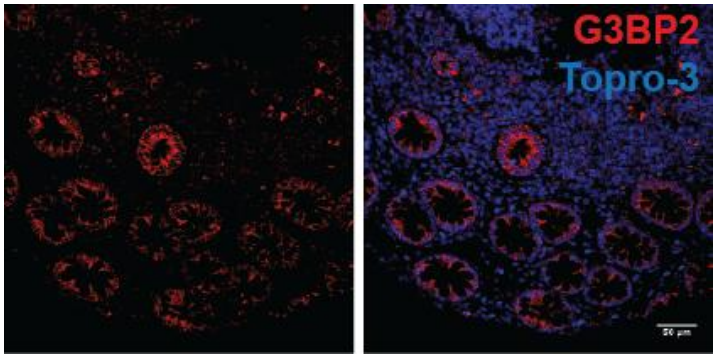

Figure 2-58. Expression of G3BP2 is restricted to epithelial cells in the colon. Confocal microscopy of normal human colon luminal epithelial cells stained for G3BP2 (red) and nuclei (blue).

In Stage-3 breast tumors, we found that the level of G3BP2 expression, together with matrix stiffness, could further stratify these patients to predict outcome (Figure 2-59 and 2-60). Specifically, patients with disorganized collagen/G3BP2^{high} tumors had

improved outcomes with dramatically increased 10 year recurrence free survival compared to those with organized collagen/G3BP2^{low} tumors (46.40% vs. 10.10%, respectively). Importantly, patients whose tumors presented either low G3BP2 or organized collagen fibers had intermediate survival outcomes (31.18% and 33.33% 10 year recurrence free survival, P-value = 0.0284), reflective of the cooperative effect from G3BP2 loss and increasing matrix stiffness. Concordant with data from the 3D culture, these results demonstrate that increasing rigidity in the tumor microenvironment, in concert with down-regulation of G3BP2, promotes tumor invasion and metastatic progression. Therefore, the Twist1/G3BP2 mechanotransduction pathway provides both novel biomarkers and therapeutic targets for tumor metastasis.

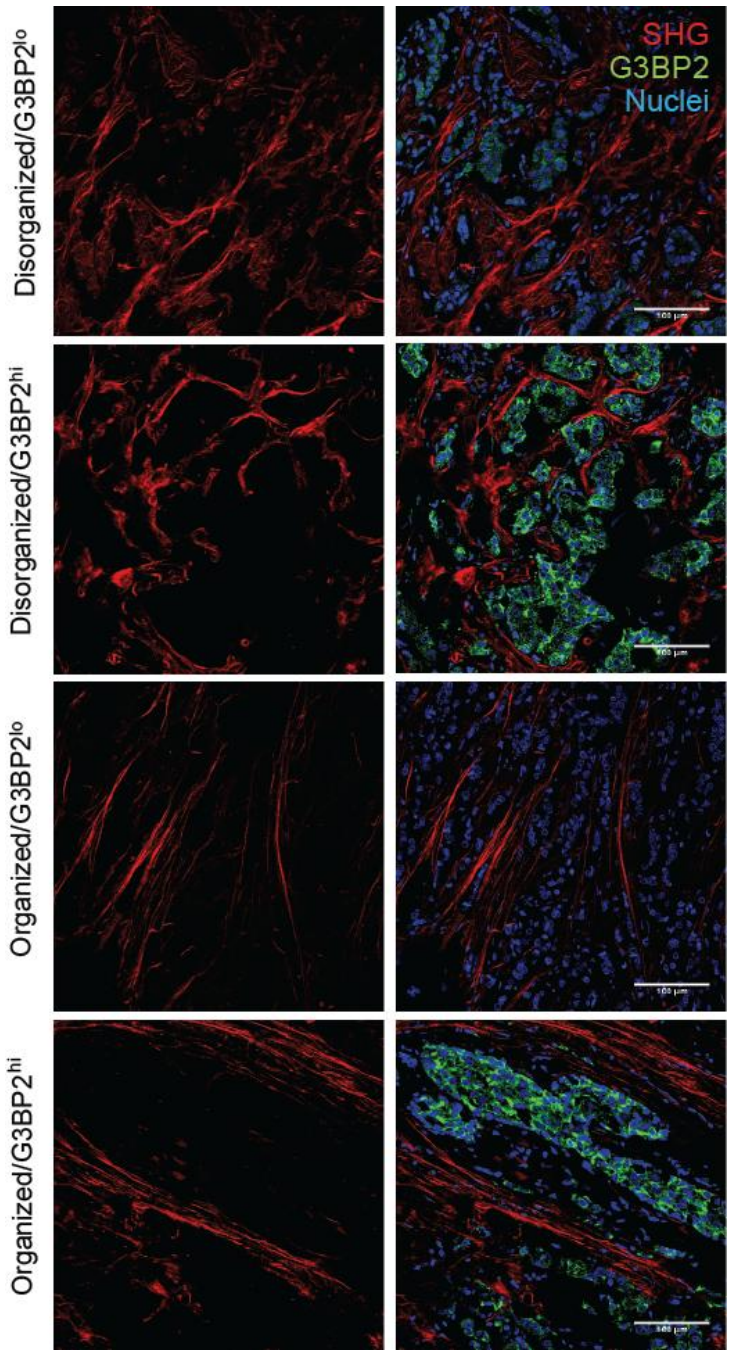

Figure 2-59. G3BP2 expression and collagen organization in human tumors. Representative images of Stage-3 human breast tumors analyzed for collagen organization (red) by SHG, and stained for G3BP2 (green) and Topro-3 for nuclei (blue) respectively.

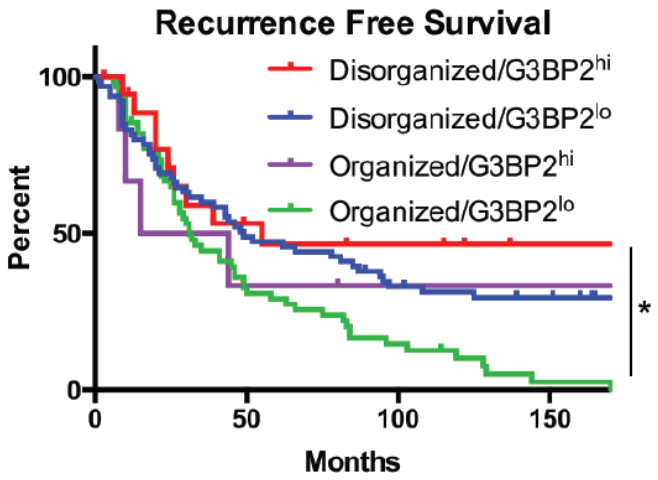

Figure 2-60. G3BP2 protein expression and collagen organization together correlate with recurrence free survival. Kaplan-Meier Curve of recurrence free survival for Stage 3 breast cancer patients, stratified by collagen organization (SHG) and G3BP2 expression (*, Disorganized collagen/G3BP2^{high} tumors vs. Organized collagen/G3BP2^{low}, Log-Rank P-value = 0.0135) (Disorganized collagen/G3BP2^{high} N= 19; Disorganized collagen/G3BP2^{low} N= 65; Organized collagen/G3BP2^{high} N= 6; Disorganized collagen/G3BP2^{low} N= 62).

Given our finding that G3BP2 sequesters Twist1 in the cytoplasm at low matrix stiffness to maintain an epithelial phenotype, and that these findings reflect a synergistic relationship observable in human tumors, we also investigated whether we could detect a change in localization of Twist1 in human tumor samples. This proved to be quite challenging for a number of reasons. First, tumor invasion can be mediated by a number of EMT transcription factors and pathways with which Twist1 is not involved so Twist1 is not highly expressed in all breast tumors. In fact, only approximately 20% of breast tumors express detectable levels of Twist1(Tsai et al., 2012). Second, we are limited technically by reagents to detect Twist1 in human tissues with a high signal to noise ratio. In an attempt to bypass this obstacle, at least in part, we employed tyramide signal amplication (TSA) which enzymatically deposits fluorescent molecules in a similar concept as in immunohistochemistry. While TSA was able to increase the detection

threshold, expression in stromal cells as well as background signal was also increased. We observed that Twist1 is expressed in the mammary gland fibroblasts at high levels (Figure 2-61). While this is consistent with some previous reports, it obscured our ability to identify Twist1-positive tumor cells that had undergone EMT. Additionally, we noted that Twist1 was localized to the nucleus in stromal cells despite being in the compliant mammary microenvironment, suggesting that fibroblasts and mammary epithelial cells may have differential responses to changes in matrix stiffness. Expression of Twist1 in fibroblasts also has significant implications, however, for interpretation of mRNA and protein expression arrays from whole mammary tissue as Twist1 from the stromal compartment will skew the expression results. While we were able to detect nuclear Twist1 in tumor cells in some samples, we were unable to score tumor cells with cytoplasmic Twist1 confidently, given relatively high levels of background signal from the tissue. Additional experimentation and investigation is required to validate whether increasing matrix stiffness and/or loss of G3BP2 expression induces Twist1 nuclear translocation *in vivo*.

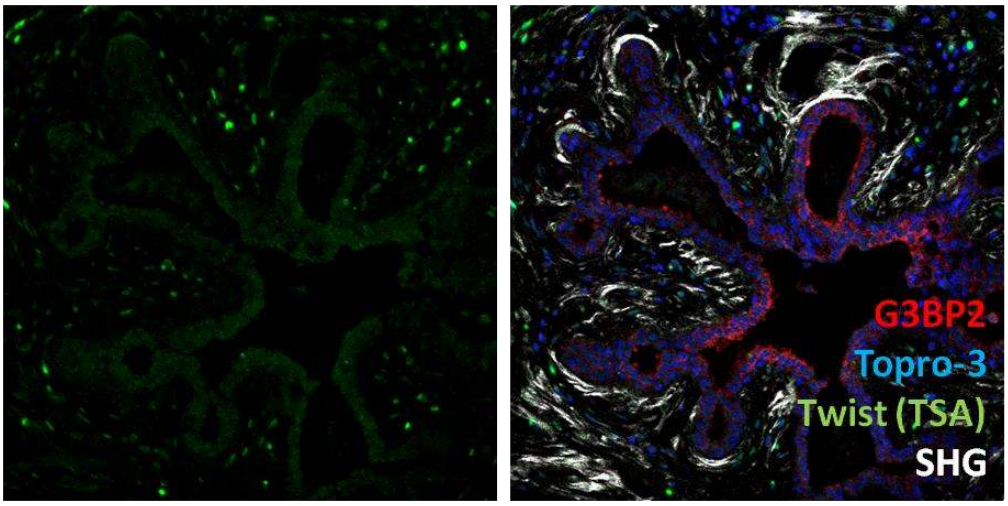

Figure 2-61. Twist1 expression in the normal human mammary gland. FFPE sections of normal human mammary gland were stained for G3BP2 (red), nuclei (blue), and Twist1 (green), which was detected using TSA. The samples were concurrently imaged using SHG.

Given that Twist1 and other EMT-inducing signals operate in complex signaling networks consisting of feedback and feed forward loops, we were interested whether Twist1 might regulate matrix stiffness once activated. Once released from G3BP2 Twist1 might induce transcription of ECM molecules or ECM modifying genes such as LOX. At first, this idea may seem at odds with the activation of matrix degradation programs enacted through invadopodia and diffusely secreted proteases during EMT. However, these invasive abilities are likely acquired to break through the local basal lamina, and tumor cell dissemination would subsequently facilitated by remodeling of the ECM to build fibrillar matrices upon which to migrate. Thus, the interaction between the ECM and the EMT program may consist of at least two stages including catabolism and anabolism. Many previous reports have documented the invasive component of EMT, which is consistent with a catabolistic phase(Eckert et al., 2011; Nieto, 2011; Yang and Weinberg, 2008). Consistent with the anabolistic phase, Twist1 is co-expressed with

many stromal genes in human cancers. Analysis of lymph-node negative breast cancer reveals that expression of Twist1 correlates significantly with many ECM remodeling components including collagens, matrix metalloproteinases and lysyl oxidase(Riaz et al., 2012). Furthermore, guilt-by-association (GBA) analysis using the online tool, Genefriends, of Twist1 expression in over 1,000 mouse microarrays revealed co-expression of ECM proteins including many collagens, versican, fibronectin, and tenascin-C as well as LOX and LOXL1(van Dam et al., 2012). Moreover, Cav1 is also highly co-expressed with Twist1. Cav1 mediates remodeling of tissue architecture by CAFs, and perhaps mediates a similar function in tumor cells(Goetz et al., 2011). These observations support a role for Twist-induced EMT in matrix remodeling and further support further investigation into the signaling networks that govern tumor cell mechanotransduction.

2.11 Conclusion

In summary, we report that Twist1 and G3BP2 form a mechanotransduction signaling axis that regulates EMT and tumor invasion in response to matrix stiffness in tumor microenvironment. This may have very important implications in breast tumors, as we found that G3BP2 loss and tissue rigidity act synergistically to promote tumor progression. Given the critical role of Twist1 and EMT in tumor metastasis, the Twist1/G3BP2 mechanotransduction pathway warrants further investigation as both novel biomarkers and therapeutic targets for metastatic cancers.

Because triple-negative breast cancers have poorer prognoses as well as fewer treatment options available, we were also interested in whether this mechanotransduction signaling pathway had a similar role in TNBC. Analysis of TNBCs in our stage 3 TMA dataset suggest that the synergistic effect of low G3BP2 expression

and high matrix stiffness has an even more potent consequence in TNBC, although with low statistical power due to sample number. Compared to both TNBC tumors with high G3BP2 expression and/or disorganized collagen as well as receptor positive breast cancers, TNBCs with low G3BP2 expression and organized collagen had significantly worse survival (Figure 2-62). This result, while underpowered due to our sample availability, suggests that further investigation into the role of the Twist1-G3BP2 mechanotransduction pathway in TNBCs may be worthwhile.

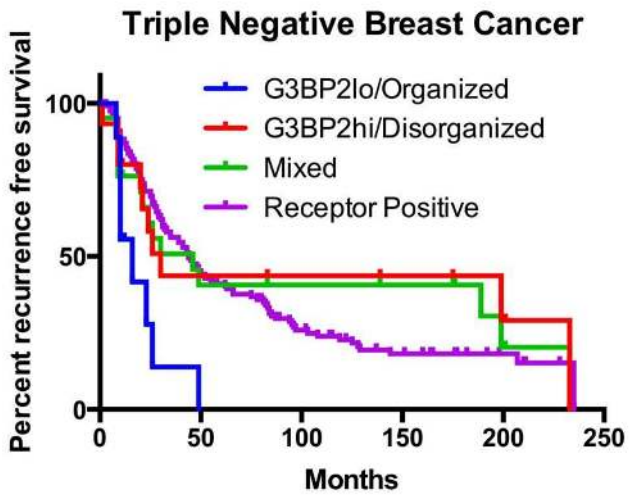

Figure 2-62. G3BP2 protein expression and collagen organization correlate with recurrence free survival in TNBC. Kaplan-Meier survival curve of TNBC stratified by G3BP2 expression and collagen organization. Mixed group denotes TNBC cases with either low G3BP2 expression or organized collagen but not both. Receptor positive denotes all cases with receptor positivity regardless of G3BP2 and collagen status. (G3BP2lo/Organized vs all other TNBC, Log-rank P-value = 0.037).

We were also interested in whether this mechanotransduction pathway was specific to breast cancer or might be a more generally applicable principle. Analysis of the TCGA PANCAN data set, which is the aggregation of all TCGA datasets demonstrates a significant increase in survival in patients with high G3BP2 expression (Figure 2-63). Thus, Twist1 and G3BP2 may constitute a more general

mechanotransduction pathway. We were then interested in whether particular tumor types might be particularly reliant on this pathway. Further analysis of the PANCAN dataset revealed a dramatic correlation between G3BP2 expression and survival in renal clear cell carcinoma(RCC) (KIRC TCGA dataset)(Figure 2-64).

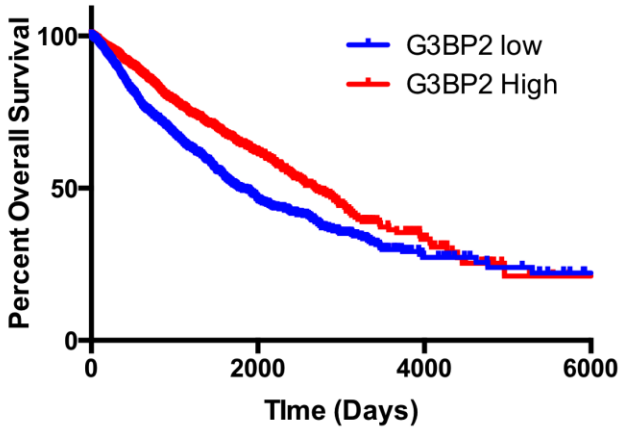

Figure 2-63. G3BP2 mRNA expression stratifies survival in the TCGA PANCAN dataset. Kaplan-Meier survival curve analysis of the TCGA PANCAN dataset based on G3BP2 expression (high, >mean expression; low, <mean expression; Log-rank P-value <0.0001; G3BP2hi N=2356, G3BP2lo N=2137).

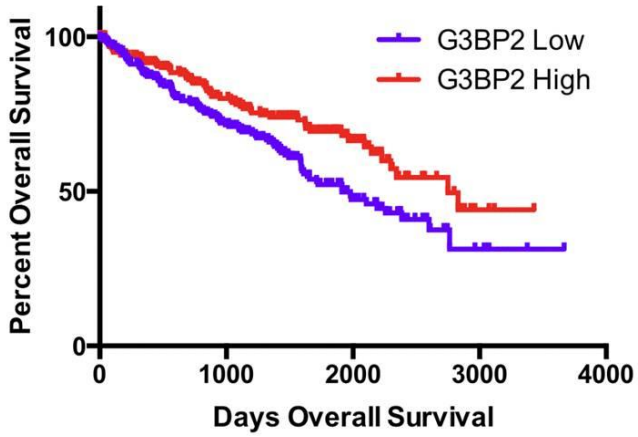

Figure 2-64. G3BP2 mRNA expression correlates with survival in RCC patients. Kaplan-Meier survival curve of RCC patients stratified by G3BP2 expression (high, > mean expression; low < mean expression; KIRC TCGA Kidney Renal Clear Cell Carcinoma dataset; Log-Rank P-value =0.0052).

Analysis of the expression profile of G3BP2 in normal tissue and primary RCC tumors revealed differential expression of G3BP2 in normal tissue compared to primary tumors (Figure 2-65). Normal tissue had consistently high expression while tumors have a wide distribution of expression levels. Interestingly, expression ranged from a high level similar to normal tissue to lower, but few tumors had higher expression than the normal tissue. This trend of variation of G3BP2 expression in the primary tumor was also reflected in the TCGA PANCAN dataset albeit in a less striking manner (Figure 2-66). This supports a model in which G3BP2 maintains epithelial identity and loss of its expression can promote EMT in conjunction with increasing matrix stiffness. Analysis of G3BP2 protein expression levels may identify an even more striking correlation as in our analysis of breast cancer TMAs since it function on the protein level. These studies warrant investigation into the involvement of the Twist1-G3BP2 mechanotransduction pathway in RCC.

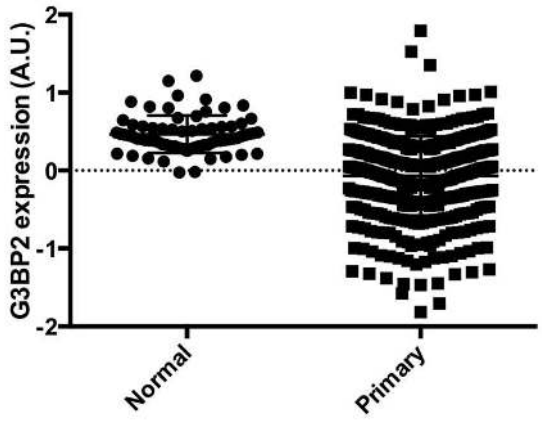

Figure 2-65. G3BP2 expression varies widely in primary RCC tumor tissue. Analysis of G3BP2 mRNA expression in RCC and normal tissues in the KIRC TCGA Kidney Renal Clear Cell Carcinoma dataset (two-tailed T-test, P<0.0001).

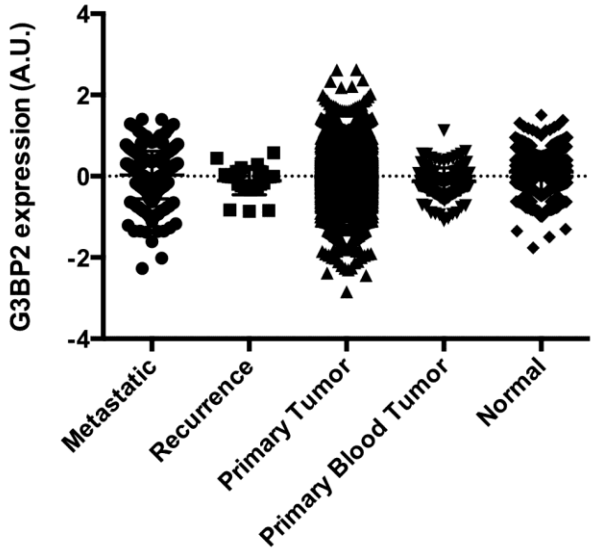

Figure 2-66. G3BP2 expression in subsets of the TCGA PANCAN dataset. Analysis of G3BP2 mRNA expression in the TCGA PANCAN dataset, normalized to mean expression value (two-tailed T-test, Normal vs Primary Tumor, P<0.0001).

Mechanistically, this study reveals a molecular pathway directly linking mechanical forces with transcriptional regulation of the EMT program. Our findings

suggest a model in which increasing matrix stiffness induces release of Twist1 from its cytoplasmic anchor G3BP2 to enter the nucleus and drive transcriptional events of EMT and invasion (Figure 2-67). Once transported into the nucleus via canonical nuclear import machinery, Twist1 is able to regulate transcription through a variety of mechanisms including heterodimerization with Class I and II bHLH transcription factors and interaction with epigenetic modifiers. This mechanism allows for dynamic positive and negative regulation of the EMT program.

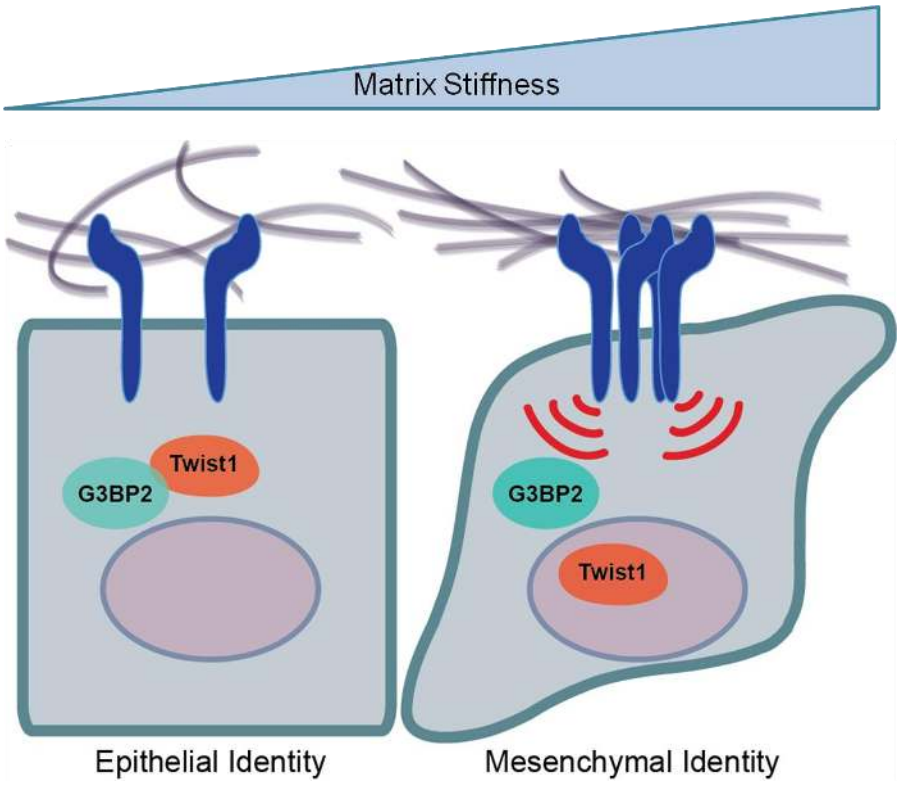

Figure 2-67. Model of the Twist1-G3BP2 mechanotransduction pathway. At low stiffness conditions integrins are not activated, allowing for cytoplasmic sequestration of Twist1 by G3BP2 and the maintenance of an epithelial identity. At high matrix stiffness, catalyzed by ECM deposition and modification, integrins are activated, leading to integrin clustering. This activation modulates an as yet unidentified signal that induces release of Twist1 from G3BP2, thus allowing for Twist1 nuclear translocation and the induction of a mesenchymal identity via EMT.

The role of G3BP2 in maintenance of epithelial identity via sequestration of Twist1 may differentiate it from G3BP1. G3BP1 has been reported to be overexpressed in a variety of cancers compared to their corresponding normal tissues, although it is not clear what the effect of this overexpression is(Guitard et al., 2001). Normal tissues did however, have significant detectable expression of G3BP1, supportive of previous reports that G3BP proteins seem to be relatively ubiquitously expressed. If the epithelial maintainance function is specific to G3BP2, then molecular characterization of the differential functions of G3BP1 and G3BP2 may yield important insights into tumor mechanotransduction. Furthermore, such approaches may inform specific approaches to modulate the interaction between Twist1-G3BP2 to positively or negatively regulate matrix stiffness driven EMT.

Together Twist1 expression, G3BP2 expression, and matrix rigidity form a regulatory circuit. As we observed that changes in both G3BP2 expression and matrix stiffness could induce invasion, a threshold may exist in this signaling pathway that is sufficient for EMT induction. This threshold is met by a combination of signals stemming from extrinsic changes in matrix stiffness and intrinsic changes in G3BP2 and Twist1 expression. This may be an important idea moving forward, as changes to each component will modulate the dependency of the cell on the other factors. In this way, G3BP2 may act as a molecular rheostat to buffer against changes in matrix stiffness.

To our knowledge, Twist1 is the first core EMT transcription factor found to be regulated by mechanical cues. The YAP/TAZ transcriptional co-activators are shown to be regulated by both matrix rigidity and cell shape(Dupont et al., 2011). Interestingly, we found that the Twist1/G3BP2 signaling axis is only responsive to matrix stiffness, but not cell shape, polarity, or junction formation. The interaction between Twist1 and G3BP2

may be regulated by several mechanisms, including post-translational modification, which will be further discussed in Chapter 3. Currently, the complete molecular pathways that transmit the mechanical signals from extracellular matrix to either the YAP/TAZ or Twist1 axis remain to be identified. Understanding the similarities and differences between the YAP/TAZ versus Twist mechanotransduction pathways would provide further insight on how different mechanical cues are interpreted into unique biological responses. Given the importance of mechanoregulation in embryonic morphogenesis, such information would have broad implications not only in tumor progression, but also in development.

2.12 Experimental Procedures

2.12.1 Cell Culture

MCF10A cells were grown in DMEM/F12 media supplemented with 5% horse serum, 20 ng/ml human EGF, 10 ug/ml insulin, 0.5 ug/ml hydrocortisone, penicillin, streptomycin, and 100ng/ml cholera toxin. Eph4Ras cells were cultured as previously described in MEGM mixed 1:1 with DMEM/F12 media supplemented with 10ng/ml human EGF, 10 ug/ml insulin, 0.5 ug/ml hydrocortisone, penicillin, and streptomycin(Eckert et al., 2011). Bt-549 cells were grown in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, 10% fetal bovine serum, and 1 ug/ml insulin. All cell lines were tested for mycoplasma contamination.

2.12.2 Antibodies

Primary antibodies include anti-beta-actin (Abcam, ab13822), anti-E-cadherin (BD, 610182), anti-E-cadherin (Abcam, Decma-1), anti-G3BP2 (Sigma-Aldrich , HPA018425), anti-Fibronectin (Sigma-Aldrich, F3648), anti-Integrin α6 (Millipore,), anti-

human Laminin V (Chemicon, D4B5), anti-mouse Laminin V (kind gift from M. Aumailley), anti-Twist1 (Santa Cruz, Twist2C1a), Rabbit anti-Twist1 (Sigma-Aldrich, T6451), 5b7 mouse anti-Twist1 hybridoma cell line, anti-YAP (Santa Cruz, H-125). Secondary fluorescent antibodies used included anti-mouse, anti-rat, and anti-rabbit conjugated with Alexafluor 488, 546, and 647 (Life Technologies).

2.12.3 Generation of Stable Knockdown Cell Lines

Stable cell lines were generated as previously described(Eckert et al., 2011). Briefly, shRNA target constructs were generated in 293T cells by transient transfection with target vector with packaging vectors (pCMV Δ8.2R and VSVG expression vectors for gag/pol and env, respectively). Filtered viral supernatants produced sans transfection reagents were applied to target cells with 6 ug/ml protamine sulfate. Transduced cells were then selected for with 2 µg/ml puromycin or blasticidin.

2.12.4 Polyacrylamide Hydrogel Preparation

Hydrogels were prepared as previously described on No. 1 12 mm and 25 mm coverslips were utilized(Chaudhuri et al., 2010). Briefly, No. 1 glass coverslips were etched using 0.1 N NaOH, functionalized using 3-Aminopropyltriethoxysilane, rinsed with dH20, incubated in 0.5% glutaraldehyde in PBS, dried, and then acrylamide/bis-acrylamide mixtures polymerized between the functionalized coverslip and a glass slide coated with dichlorodimethylsiloxane. Polyacrylamide coated coverslips were then washed twice with dH20, incubated with 1 mM Sulfo-SANPAH in HEPES buffer under 365 nm UV light for 10 minutes, rinsed twice with 50 mM HEPES pH 8.5 buffer, incubated at 37°C overnight with rat tail Collagen I (Millipore) in 50 mM HEPES pH 8.5 buffer, rinsed twice in HEPES buffer, and sterilized.

2.12.5 3D Cell Culture

MCF10A and Eph4Ras cells grown in 3D cell culture as previously described(Debnath et al., 2003). Briefly, Eph4ras cells were seeded on hydrogels in 2% Matrigel-MEGM mixed 1:1 with DMEM/F12. MCF10A cells seeded similarly in DMEM/F12 media supplemented with 2% horse serum, 5 ng/ml human EGF, 10 ug/ml insulin, 0.5 ug/ml hydrocortisone, penicillin, streptomycin, and 100 ng/ml cholera toxin.

2.12.6 Confocal Microscopy

Protocol adapted from method described previously(Debnath et al., 2003). In brief, cells were fixed with 2% paraformaldehyde (PFA) for 20 minutes at room temperature, permeabilized with PBS-0.5% Triton X-100, quenched with 100 mM PBS-glycine, and then blocked with 20% goat serum-immunofluorescence (IF) buffer. Samples were incubated with primary antibodies overnight in 20% goat serum-IF buffer, washed 3 times with IF buffer, incubated with secondary antibodies for 1 hour, washed 3 times with IF buffer, counterstained for nuclear for 15 minutes (5ng/ml DAPI or TO-PRO-3), washed once with PBS, and mounted with Slow Fade Gold (Invitrogen). Confocal images were acquired using an Olympus FV1000 with 405, 488, 555, and 647 laser lines. Images were linearly analyzed and pseudo-colored using ImageJ analysis software.

2.12.7 Second Harmonic Generation Microscopy

5 micron formalin-fixed paraffin embedded sections were re-hydrated and imaged using a multi-photon Leica SP5 confocal microscope using a TI-Sapphire light source and a 20x water-immersion objective at 880 nm. Fields were acquired using

resonant scanning mode, line averaging, and frame accrual. IF staining was sequentially imaged using scanning laser confocal microscopy.

2.12.8 Tumor Tissue Microarrays

National Cancer Institute Cancer Diagnosis Program Stage 3 breast cancer progression tumor tissue microarrays were stained for G3BP2 by immunofluorescence. TMAs were concurrently imaged by confocal microscropy and SHG. Missing, damaged, and cores without detectable tumor cells were omitted from analyses. G3BP2 was scored blindly according to the following rubrics. G3BP2 expression was scored 0 for no detectable expression, 1 for very weak expression, 2 for moderate expression in greater than 75% of tumor cells, and 3+ for strong expression in greater than 75% of tumor cells.

2.12.9 Immunoprecipitation

Cells were lysed using a 2-step protocol adapted from Klenova et al (Klenova et al., 2002). Cells were directly lysed with lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 10 mM MgCl2, 10 mM KCl, 2 mM EDTA, 1 mM NaF, 1 mM sodium orthovandate, 2.5 mM beta-glycerolphosphate, 10% glycerol, pH 7.5), scraped off the culture dish, sonicated, supplemented to 400 mM NaCl, sonicated, and diluted to 200 mM NaCl. Antibodies were conjugated to protein G beads (Invitrogen), crosslinked using disuccinimidyl suberate (Pierce) as per manufacturer's protocol, incubated with lysates overnight at 4°Celsius, washed eight times with IP lysis buffer supplemented with 200 mM NaCl, and eluted using 50 mM DTT LDS sample buffer at 95 degrees Celsius for 15 minutes. 5B7 mouse hybridoma concentrated supernatant was used. For immunoprecipitation of exogenously transfected Myc-Twist1, 293T cell lysates were harvested 48 hours after transfection and subjected to 2-step lysis protocol.

Immunoprecipitation was performed using anti-Myc antibody (9E10) crosslinked to protein A agarose beads (Invitrogen).

2.12.10 Mass Spectrometry

The gel bands were excised and cut into 1x1-mm pieces. In gel digestion and extraction were done as previously described(Guo et al., 2005). The peptides were separated on reversed-phase HPLC analytical column (360 µm O.D. x 50 µm I.D., ODS-AQ 5 µm, 10 cm) with an integrated tip (1-2 µm) with a gradient of 0-40%B in 30 min, 40-100% B in 5 min, 100%-0%B in 2 min, and 0% B for 15 min using Agilent 1100 quarternary pump and eluted into an LTQ Orbitrap. LTQ Orbitrap was operated in a data-dependent mode. MS was acquired in an LTQ Orbitrap with a resolution of 15000 and MS/MS was acquired in LTQ. Tandem mass spectra were searched against the IPI mouse database using Bioworks with the following modification: differential Methionine 15.9949. For peptides xcorr cut off filter of 1.5 for +1, 2.0 for +2 and 2.5 for +3 was applied, and identified peptides were confirmed by manually inspecting the MS/MS spectra.

2.12.11 Micropatterning

Micropatterned coverslips were designed with and produced by CYTOO (http://www.cytoo.com/). Square micropatterns were produced in blocks with a 90 µm pitch between each pattern with a block period of 1300 µm. Each pattern block was produced in duplicate on each coverslip. Activated coverslips were coated with 20 µg/ml rat tail collagen I for 2 hours at room temperature. Cells were then seeded for 6 hours and then fixed for analysis.

2.12.12 Motif Sequence Alignment

Nucleotide sequences were aligned using ExPASy SIB bioinformatics portal(Artimo et al., 2012).

2.12.13 Proximity Ligation Assay

Cells were 3D cultured on PA gels for 20 hours or 6 days and fixed and processed as described for immunofluorescence before performing PLA (Sigma-Aldrich) as per manufacturer's protocol. To quantify PLA signal, images were thresholded, the area of PLA signal quantified, and normalized to cell number in ImageJ.

2.12.14 Tyramide Signal Amplication

TSA was performed according to manufacturer's protocol on FFPE tumor tissue sections. Tissue sections were rehydrated using a xylene, ethanol series (Xylene 2x5 minutes, 100% EtOH 2x2 minutes, 95% EtOH 2x1 minute, 80% EtOH 1 minute, 70% EtOH 1 minute, PBS). Antigen retrieval was performed using a decloaking chamber in 10 mM Citric Acid pH 6 for 30 seconds at 125°C, and 10 seconds at 90°C (Biocare Medical). Tissue was blocked with with 20% goat serum. Following incubation with primary and secondary antibodies amplification was performed for 10 minutes and quenched. Samples were mounted using SlowFade and imaged.

2.12.15 Mouse Tumor Models

$1x10^5$ Eph4Ras cells were injected bilaterally into the mammary fat pad of BALB/C mice in 50% Matrigel medium. $1x10^5$ 4T1 cells stably labeled with cFu GFP were injected bilaterally into the mammary fat pad of BALB/C mice in 15 ul PBS. For MDA-MB-231 tumors $1x10^6$ cells were injected subcutaneously on the flank of nude mice in 50% Matrigel. βAPN treatments were administered daily by intra-peritoneal

injection in 100 ul PBS vehicle. Mice were sacrificed prior to tumor burden reaching 20% of total body weight or 2 cm in diameter. Tumors were then excised, samples snap-frozen for protein and RNA analysis, and the remaining samples fixed in 4% PFA-PBS for FFPE sectioning. Lung metastases were counted using a fluorescent dissection microscope (Leica). All animals were cared for in accordance with UC San Diego Animal Care Program and AALAC guidelines.

2.12.16 Tumor Mechanical Testing

Fresh tumor tissue was tested by unconfined compression. Tumors were excised from βAPN and control treated mice bilaterally. Discs of tumor tissue measuring 5mm in diameter and 2mm in thickness were prepared using a Vibratome and biopsy punch. Tumor disc thickness was measured using a laser micrometer at three locations in each disc in order to match similar samples, for at minimum two discs per sample. Samples were placed between stainless steel loading plates connected to a DynaStat uniaxial test system with a 0.50N load cell in PBS with calcium and magnesium. Samples were compressed to 5%, 10%, and 15% over 5s, allowing the pressure load to equilibrate. The elastic modulus at equilibrium was calculate as the slope between stress and strain using a linear fit model.

2.12.17 Tissue Immunofluorescence

Tumors were drop fixed in 4% PFA-PBS and subsequently embedded in paraffin. 5µm sections were cut using a microtome (Leica). Tissue sections were rehydrated using a xylene, ethanol series (Xylene 2x5 minutes, 100% EtOH 2x2 minutes, 95% EtOH 2x1 minute, 80% EtOH 1 minute, 70% EtOH 1 minute, PBS). Antigen retrieval was performed using a decloaking chamber in 10 mM Citric Acid pH 6 for 30 seconds at

125°C, and 10 seconds at 90°C (Biocare Medical). Tissue was blocked and stained in a protocol similar as discussed in section 2.12.6.

2.12.18 Expression Array Analysis

Publicly available expression datasets were access and analyzed using the Cancer Genome Browser *(*https://**genome**-**cancer**.ucsc.edu/*)*. Samples were assigned to G3BP2 high and G3BP2 low groups based on G3BP2 expression values above and below mean expression of the entire cohort. Datasets include the TCGA breast cancer dataset *(TCGA*_BRCA_G4502A_07_3), PANCAN, and RCC cohorts.

2.12.19 Statistical Analysis

All p-values derived from two-sided Student's T-test unless otherwise noted. Error bars denote standard deviation. Kaplan-Meier survival curves were analyzed by Cox-Mantel Log-rank analysis. Contingency tables were analyzed using Fisher's Exact analysis.

2.12.20 Real-time PCR

RNA was extracted from cells using RNeasy Mini and Micro Kit (Qiagen). cDNA was generated using random hexamer primers and cDNA Reverse Transcription Kit (Applied Biosystems). Expression values were generated using ddCt values normalized to GAPDH. Experiments were performed in biological and technical triplicate. Target mRNA were amplified by validated primers with amplicon lengths of between 100 to 200 nucleotides (Table 2-4).

Table 2-4. Real-time PCR primers.

Target mRNA	Primer	Sequence (5' to 3')
hTwist1	hTwist exon 2 forward	AAGAGGTCGTGCCAATCAG
	hTwist exon 2 reverse	GGCCAGTTTGATCCCAGTAT
hGAPDH	hGAPDH forward	GACCCCTTCATTGACCTCAAC
	hGAPDH reverse	CTTCTCCATGGTGGTGAAGA
hCDH1	hE-cadherin Forward	TGCCCAGAAAATGAAAAAGG
	hE-cadherin Reverse	GTGTATGTGGCAATGCGTTC
hFN1	hFibronectin Forward	CAGTGGGAGACCTCGAGAAG
	hFibronectin Reverse	TCCCTCGGAACATCAGAAAC
hVimentin	hVimentin Forward	GAGAACTTTGCCGTTGAAGC
	hVimentin Reverse	GCTTCCTGTAGGTGGCAATC
hSnail1 and mSnail1	hSnail Forward	AAGATGCACATCCGAAGCC
	hSnail Reverse	CGCAGGTTGGAGCGGTCAGC
mSnail2	mSlug Forward	ATGCCCAGTCTAGGAAATCG
	mSlug Reverse	CAGTGAGGGCAAGAGAAAGG
mZeb1	mZeb1 Forward	TGATGAAAACGGAACACCAGATG
	mZeb1 Reverse	GTTGTCCTCGTTCTTCTCATGG
mZeb2	mZeb2 Forward	TGAAGAGAACTTTTCCTGCCCT
	mZeb2 Reverse	ATTTGGTGCTGATCTGTCCCT
mTwist1	mTwist1 Forward	CAGCGGGTCATGGCTAAC
	mTwist1 Reverse	CAGCTTGCCATCTTGGAGTC

2.12.21 shRNA sequences

pSP108 lentiviral target sequences: Twist1 #3, AAGCTGAGCAAGATTCAGACC. Twist1 #5, AGGTACATCGACTTCCTGTAC. shControl (shGFP) does not target any endogenous coding DNA. pLKO.1 (Sigma-Aldrich) lentiviral target sequences: shG3BP2 #2, AGTTAAATTGAGGTGGACATT. shG3BP2 #5, TTCGAGGAGAAGTACGTTTAA.

shG3BP2 #6, CGGGAGTTTGTGAGGCAATAT. shG3BP2 #8, CCACAAAGTATTATCTCTGAA.

2.13 Acknowledgements

This work, in part, has been submitted for publication as an article titled “Matrix stiffness drives Epithelial-Mesenchymal Transition via a Twist1-G3BP2 mechanotransduction pathway”. I thank my coauthors Jeff H. Tsai, Laurent Fattet, Yurong Guo, Albert Chen, Robert Sah, Susan Taylor, Adam Engler, and Jing Yang. I also thank members of the Yang lab, especially Mark Eckert for helpful discussions. We thank the Shared Microscope Facility and UCSD Cancer Center Specialized Support Grant P30 CA23100 and the NCI Cancer Diagnosis Program (CDP) for providing breast tumor tissue microarrays. The shRFP control pLKO.1 plasmid was a kind gift from S. Stewart. This work was supported by grants from ACS (RSG-09-282-01-CSM), NIH (DP2 OD002420-01), NCI (1RO1CA168689) and DOD Breast Cancer Program W81XWH-13-1-0132 to J.Y., from DOD W81XWH-13-1-0133 to A.J.E., and from NIH (DK54441) and HHMI to S.S.T. S.C.W. was supported by a NIH Cancer Cell Biology Training grant (2T32CA067754), NIH Molecular Pathology of Cancer Training grant (5T32CA077109), and is an ARCS Foundation Scholar. L.F. was supported by a postdoctoral fellowship from Fondation pour la Recherche Médicale (SPE20130326547).

Chapter 3

Conclusion

3.1 Mechanical Properties as Drivers of Metastasis and Embryogenesis

Mechanical properties of tumors have been increasingly recognized as drivers of tumor progression(Butcher et al., 2009; Jaalouk and Lammerding, 2009). This reflects just one aspect of the microenvironment which has enormous impact on tumorigenesis and tumor progression(Bissell and Hines, 2011). Physical aspects of the cellular microenvironment play critical roles in embryogenesis, tissue homeostasis, and disease progression(Eyckmans et al., 2011). There remain many unanswered questions surrounding the role of matrix stiffness and other mechanical cues during tumor progression. When do these ECM modifications occur and by what cell populations are they carried out? Are there opposing signals that balance ECM anabolism and catabolism? Furthermore, do different mechanical cues influence each others' generation or responses? It is clear that tissues utilize distinct mechanotransduction pathways to recognize and respond to unique physical signals(Hoffman et al., 2011). How similar are the mechanotransduction pathways that are aberrantly activated during tumor progression to those that are employed during embryogenesis to specify germ layers? Lessons from the role of tissue mechanics and architecture as well as developmental signal transduction pathways have informed our understanding in the tumor. Going forward it will be important to relate information derived from all contexts to more fully understand how the variety of mechanical cues define cellular behavior independently and in concert with each other as well as biochemical signals.

As discussed in Chapters 1 and 2, increased tissue rigidity of primary tumors can be used as a metastatic marker and correlates with poor patient survival(Conklin et al., 2011; Provenzano et al., 2008; Yu et al., 2011). Tissue rigidity is defined by aggregate mechanical properties of the extracellular matrix (ECM) as well as the cellular component of tissue. The actinomyosin cytoskeletal plays a key role in both these processes, both acting as an effector molecule in mediating cell contractility as well as a signaling molecule to transducer mechanical signals from transmembrane mechanosensors. Understanding the molecular mechanism by which tumor tissue rigidity increases and how tumor cells respond to increased matrix stiffness will have implications in cancer biology as well as for tissue homeostasis and development. In cancer biology the mechanical properties of the tumor microenvironment is a topic is of intense interest. Despite observations that clearly indicate a critical role for mechanical signals in tumor progression, it remains to be completely resolved what this role is.

We and other have identified molecular links between mechanical signals in the tumor microenvironment and EMT that indicate that increased tissue rigidity promotes tumor invasivness. For example, increased matrix stiffness induces a malignant phenotype in tumor cells via engagement and activation of integrins(Levental et al., 2009; Paszek et al., 2005). This cellular mechano-response dovetails with biochemical signals from the microenvironment that also regulate tumor behavior such as TGF-β(Leight et al., 2012). As described in this dissertation, I have identified a novel Twist1-G3BP2 mediated mechanosensing pathway that induces EMT in response to increasing matrix stiffness in collaboration with TGF-β signaling. Other mechanical cues such as intercellular forces and tissue geometry can also induce EMT, a process which reflects regulation of EMT during embryogenesis(Gomez et al., 2010). However, there is a seemingly opposing paradigm in which cancer cells, at the single cell level are more

compliant, and that this property facilitates their dissemination and the eventual formation of distant metastases. This dichotomy may be in part explained by the observation that cell compliancy affects migration through confined spaces. In a general sense, this is logical as a compliant cell will be able to move through tight spaces in the ECM, which requires less remodeling of the ECM. Interestingly, it has been recently demonstrated that oncogenic transformation of MCF10A cells enabled them to migrate more effectively through narrow passages(Pathak and Kumar, 2013). Increasing matrix stiffness also facilitated migration of MCF10A cells. Furthermore, channel width and matrix stiffness can independently modulate cell migration(Pathak and Kumar, 2012). The effect of these inputs together was different between ErbB2 expressing and parental cells, suggesting that intrinsic cell signals modulate how cells respond to mechanical cues including matrix stiffness and channel width(Pathak and Kumar, 2013).

The differences in matrix stiffness and cell compliance in each microenvironment and tissue also likely play a significant role. Force mapping of human and mouse breast tumors indicates that while benign lesions have markedly increased rigidities compared to normal mammary tissue, invasive tumors have heterogenous mechanical properties(Plodinec et al., 2012). Interestingly, the tumor core was soft while the periphery was stiff, perhaps consistent with the observation that cells from the invasive front of tumors undergo EMT prior to dissemination(Brabletz et al., 2005). However, force mapping of matched mouse primary and metastatic lesions indicate that metastases have low rigidity, similar to that of the primary tumor core(Plodinec et al., 2012). Taken together with the observation that metastatic tumor cells are more compliant than regular cells at the single cell level, this may suggest that metastases might arise from this tumor core population(Cross et al., 2007; Lekka et al., 1999). These variations may depend on genetic background and environmental factors as

isolated tumor cells have also been reported to be stiffer than corresponding isolated normal cells(Lopez et al., 2011). Alternatively, dynamic regulation of EMT and MET may also explain this apparent contradiction, in which the stiffer tumor periphery induces EMT which is then reversed at secondary sites, which give rise to compliant, epithelial metastases. This mechanism relates well to the observation that primary and secondary tumors are both histologically epithelial, which either can be explained by induction of MET at secondary sites or dissemination of epithelial cells. Additional studies are clearly warranted to further investigate how tumor cell compliance, tissue rigidity, and ECM modifications are regulated and how changes in each of these properties affect tumor progression and metastasis.

Many of the same mechanical signals regulate EMT in the developmental context as in the disease context. For example, extrinsic tensile forces and intracellular mechanical properties regulate keys process during both embryogenesis and tumor progression, suggesting that mechanotransduction is both a conserved cell intrinsic and extrinsic phenomenon. Tumor cells can have increased intercellular tension, the ECM and extracellular mileau contribute to tissue rigidity as isolated tumor cells are significantly more compliant than *in vivo* tumor tissue(Lopez et al., 2011). In fact, matrix elasticity affects cell stiffness, as increasing substrate rigidity also increases the cellular stiffness of individual mammary epithelial cells(Alcaraz et al., 2008). Similar modes of regulation via intercellular tension occur during development in epithelial sheet movement and EMT. For example, tensile forces regulate germ band extension prior to gastrulation and large scale cell movement during *Drosophila* embryogenesis(Butler et al., 2009). These changes in tissue architecture and tension modulate epithelial cell shape and polarization to regulate migration and drive changes in cellular identity. Epithelial polarization can also facilitate changes in cell shape through regulation of

adherens junctions positioning to facilitate epithelial sheet folding(Wang et al., 2012). Polarity, cell shape, and extrinsic tensile forces likely work in concert to regulate cell migration and cellular programs such as EMT in both the developmental and disease context. In fact, adherens junctions can thus act as cellular mechanosensors, using vinculin as a molecular bridge to the actinomyosin cytoskeleton(le Duc et al., 2010). Tissue tension can drive tumor progression through destabilization of adherens junction and nuclear translocation of β-catenin(Samuel et al., 2011). During development cells undergoing EMT may also feedback to regulate contractility, as *twist* mutant embryos, which cannot form mesoderm, are unable to generate sufficient force in order to gastrulate. Twist also mediates the generation of cell contractility during embryogenesis which is required for proper development(Martin et al., 2010). Together, these findings demonstrate a two-way regulation of cell intrinsic and extrinsic tensile forces and highlight a deeply intertwined relationship between cellular identity and tissue homeostasis.

3.2 EMT and Remodeling of the Tumor Microenvironment

How then is tissue rigidity in turn modulated by tumor cells that have undergone EMT during tumor progression? Invasive cells can produce increased contractile forces. Twist1 induces tumor cell invasion through Rac1, which mediates an increase in cell tension, contributing to tissue rigidity(Yang et al., 2012b). This ideology contrasts with the paradigm that epithelial cells attach to their matrix with higher affinity through focal adhesions; however mesenchymal cells require cell contacts to invade as well. Analysis of lymph-node negative breast cancer reveals that expression of Twist1 correlates significantly with many ECM remodeling components including collagens, matrix metalloproteinases and lysyl oxidase suggesting that EMT induces remodeling of the

tumor microenvironment en route to tumor cell dissemination(Riaz et al., 2012). Tumor cells that have undergone EMT degrade local basement membrane utilizing secreted matrix metalloproteinases and invadopodia among other mechanisms(Eckert et al., 2011). Tumor cells, however, still require an organized ECM to efficiently migrate and disseminate.

Generation and modification of the ECM is consistent with the normal function of mesenchymal cells in connective tissues. Accordingly, highly metastatic breast cancer cells have high expression of lysyl oxidase, which can be further induced by stromal cells(El-Haibi et al., 2012; Kirschmann et al., 2002). Fibronectin and other ECM molecules are also upregulated following induction of EMT(Nieto, 2011; Yang and Weinberg, 2008). Interestingly, fibronectin is critical for the formation of microfibrils and the deposition of collagen I and thrombospondin-I, suggesting that EMT can influence ECM remodeling at multiple levels(Sottile and Hocking, 2002). In fact, EMT induces the expression and deposition of fibrillin, the main component of the elastic fiber microfibrils(Baldwin et al., 2014). Surprisingly, EMT alters the requirements for microfibril formation, as TGF-β bypasses the necessity for the heparan sulfate proteoglycan syndecan-4. The EMT program regulates not only the expression but also the secretion of ECM molecules. Ras transformed mouse mammary epithelial cells express tenascin-C but only secrete it upon undergoing TGF-β induced EMT(Maschler et al., 2004). The specific role of tenascin in specifying matrix stiffness may be complicated as it has very elastic molecular properties itself(Oberhauser et al., 1998). Interestingly, cells that have undergone EMT produce and modify ECM components such as fibrillin, suggesting that tumor cells can remodel the ECM to facilitate their migration and invasion(Baldwin et al., 2014). Tumor cells may lay down these ECM

substrates to use as *de novo* substrates for migration as they are remodeling the existing ECM.

Tumor cells can also hijack the normal function of stromal cells to facilitate this remodeling process. Lysyl oxidase (LOX) from the stromal compartment can drive matrix stiffening through collagen crosslinking to facilitate tumor dissemination and metastasis(Levental et al., 2009; Pickup et al., 2013). Matrix stiffening can also be mediated by CAFs through caveolin-1 (Cav1) to promote tumor invasion and metastasis(Goetz et al., 2011). Loss of Cav1 leads to disorganized stromal architecture while expression of CAF expression of Cav1 promotes directional migration of tumor cells. Mechanical remodeling of the tumor microenvironment also regulates CAFs in turn, feeding forward to maintain CAF abundance and function(Calvo et al., 2013). Conditioned media from tumor cells also induced CAF mediated ECM remodeling, suggesting that paracrine signaling also plays a significant role. Consistent with this notion, myofibroblast contraction can induce the release and activation of latent TGF-β from the ECM(Wipff et al., 2007). This response could generate a feed forward regulation between matrix stiffness and growth factors. Fibroblast polarization in response to increased matrix stiffness also supports these results(Prager-Khoutorsky et al., 2011). Furthermore, this study indicates that a network of protein tyrosine kinases regulates fibroblast cell contractility and focal adhesion formation, suggesting that CAF mediated tissue stiffening could be a feasible therapeutic target. The interaction between CAFs and matrix stiffening likely interact to induce EMT and facilitate tumor dissemination. Consistent with hypothesis the presence of fibrotic foci and atypical stromal fibroblasts identifies are breast cancer population with poor outcome(Hasebe et al., 2011). These observations suggest that increasing matrix stiffness likely precedes EMT, and plays a role in inducing tumor cell invasion. Together, these and many other

observations discussed lead to the generation of a complex model governing tumor cell invasion and metastasis (Figure 3-1). This model will most certainly require modification as we learn more in the future about the dynamic relationship between tumor cells and their environment.

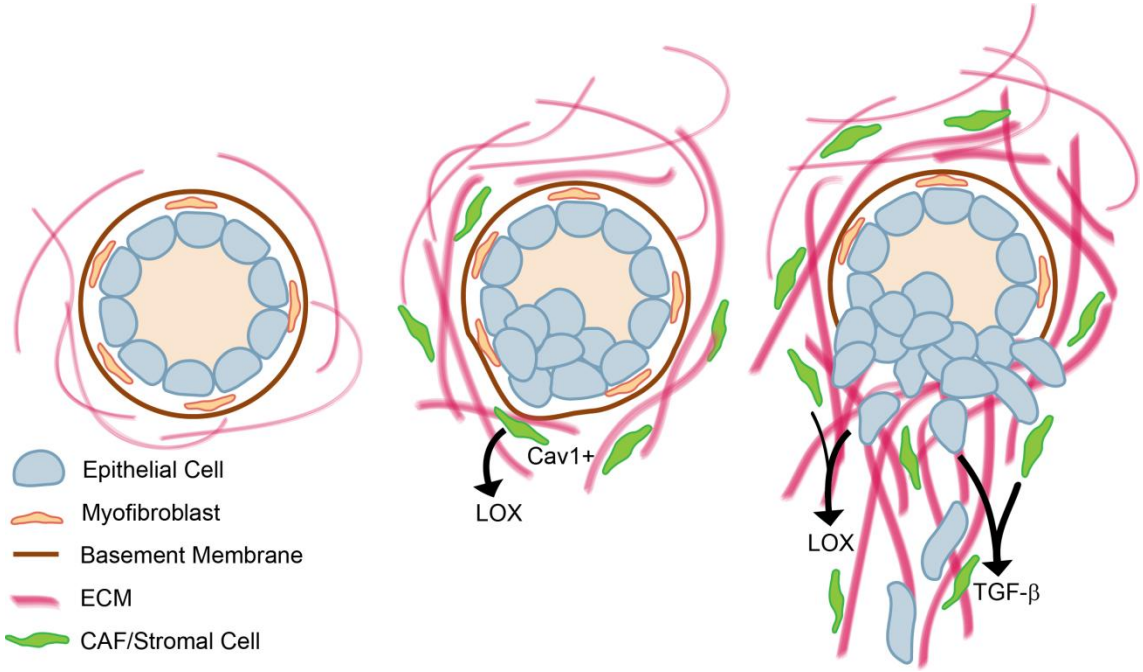

Figure 3-1. Model of progression in the primary breast tumor. Metastasis is facilitated by remodeling of the ECM by stromal and tumor cells. The normal mammary acinus (left) is composed on a single later of luminal cells surrounded by basal myoepithelial cells and surrounded by an intact basement membrane. Collagen fibers are disorganized in this state which engenders a compliant microenvironment. A non-invasive breast tumor forms subsequent to genetic and epigenetic alterations (middle). The primary tumor begins to break down the local basement membrane and can recruit stromal cells such as CAFs and bone marrow-derived cells which can remodel the ECM, resulting in a stiffer microenvironment. In combination with biochemical signals and additional genetic and epigenetic alterations, increased matrix stiffness drives these tumor cells acquire invasive traits through EMT and degrade the local basement membrane. Tumor cells that undergo EMT can further remodel the ECM and secrete additional factors and induce the release of latent TGF-β from the ECM through contractility to form a positive feed-forward signaling loops.

3.3 EMT and Collective Cell Migration

Cellular migration and invasion can be mediated through multiple distinct cellular programs including amoeboid movement, collective cell migration, and EMT. Each has distinct functions and regulation, but how each program might cooperate and interact with the others during tumor metastasis remains unclear. Are hypotheses that EMT and collective cell migration can drive tumor cell dissemination and metastasis even compatible? If so, how does a tumor cell decide which program to enact and how does that determine its metastatic potential?

Changes in the ECM can also regulate collective cell migration of normal and malignant human mammary epithelial cells independent of gross changes in EMT markers(Nguyen-Ngoc et al., 2012; Wicki et al., 2006). For example, collective cell migration of MMTV derived mammary tumor explants is dependent on interaction with collagen I in the microenvironment(Cheung et al., 2013). In fact, collective cell migration is enriched in areas containing organized collagen fibers(Cheung et al., 2013). These findings suggest that matrix stiffness and more generally, the ECM, have critical roles in regulating cellular migration and invasion regardless of the cellular program used. At a larger level, this makes sense if the mechanical and biochemical properties of ECM can provide either a permissive or non-permissive environment for migration, regardless of cellular mechanism. Are the hypotheses of EMT and collective cell migration mediated breast cancer metastasis compatible though? The idea that EMT underlies tumor metastasis has been contentious because it disseminated tumor cells migrating away from the primary tumor had not be observed by pathologically(Tarin et al., 2005). Furthermore, the observation that both the primary tumor and metastases are often of epithelial morphology seems to argue against the involvement of EMT in tumor dissemination. However evidence that EMT occurs *in vivo* during disease progression has been produced to augment the wealth of reports that define a role for EMT in tumor

invasion and metastasis using cell and molecular biology approaches(Kalluri, 2009; Trimboli et al., 2008). Moreover, it has been recently demonstrated that reversion of EMT is required for tumor metastasis, further supporting EMT's critical role(Ocana et al., 2012; Tsai et al., 2012). This observation provides a mechanism by which both the primary and secondary tumor have epithelial morphology, and supports the hypothesis of an opposing MET program, as posited by Thomas Brabletz and Jean Paul Thiery. Thus, EMT seems to be, at the very least, one of several mechanisms by which tumor cells can disseminate.

EMT and collective cell migration seem in theory to contradict, however there may be circumstances in which they collaborate during tumor metastasis. During collective cell migration epithelial characteristics including cell polarity and adherens junctions are transiently lost, reminiscent of an EMT process(Ewald et al., 2012). Epithelial components E-cadherin and β-catenin remain present between leader and follower cells during collective cell migration, however, suggesting that tumor cells retain at least some epithelial characteristics(Cheung et al., 2013; Ewald et al., 2012). It has been previously proposed that a transient EMT could drive collective cell migration, potentially regulated by dynamic growth factor signaling(O'Brien et al., 2002). As extracellular cues from the ECM or epithelial lumen can induce polarization, it stands to reason that pathological modifications of the ECM such as increased matrix stiffness could specifically and locally regulate cell behavior, perhaps even down to the single cell level. Such cues could alter cellular plasticity of leader cells in collective cell migration, acutely and transiently imbuing invasive characteristics while downregulating epithelial attributes. Thus, leader cells could remain connected to follower cells by adherens junctions on their trailing edge, but acquire the necessary functions for invasion through the microenvironment. Consistent with this hypothesis intercellular junctions attenuate

3D membrane protrusions of epithelial cells(Ewald et al., 2012). This hypothesis may also allow for the coexistence of EMT and collective cell migration. Indeed, in podoplanin mediated collective cell migration, podoplanin is most highly upregulated in cells directly in contact with the ECM while E-cadherin, while expressed, is mainly localized to the rear of the tumor cell(Wicki et al., 2006). Thus, forward-to-rear polarization of leader cells may allow for a plastic and dynamic role for EMT in collective cell migration. Furthermore, this phenomenon may be conserved as polarity also defines collective cell migration during *Drosophila* embryogenesis in which apical specific degradation of the ECM by secreted AdamTS-A mediates development of the salivary gland(Ismat et al., 2013). Perhaps contrary to this hypothesis, however, is the observation that leader cells do not express EMT markers including Twist1, Snail2, and vimentin while maintaining expression of E-cadherin, albeit only at cell-cell contacts with following cells(Cheung et al., 2013). This observation may provide evidence that EMT does not play a role in collective cell migration leader cell invasion. Alternatively, transient, single-cell EMT may be mediated through other EMT markers such as Zeb1/2 and Snail1. Yet another possibility is that a cell population, distinct from the tumor, act as leader cells to form invasive channels for tumor cells. Indeed, CAFs can fulfill this role, utilizing both proteolytic and structural deformation of the ECM to allow for tumor cell collective migration(Gaggioli et al., 2007). Alternatively macrophages could also fulfill this role. A similar process could underlie the observation that luminal tumor cells can transdifferentiate to a basal-like state to fulfill leader cell functions(Cheung et al., 2013). Of interest is how this luminal-basal transdifferentiation is regulated, whether it occurs if other cell types such as CAFs are available to fulfill the role of leader cell, and what EMT plays in collective cell migration, if any. Nonetheless, these and other remarkable observations clearly indicate the need for further investigation of regulation,

interplay between, and roles of distinct mechanisms governing tumor cell invasion and dissemination.

3.4 EMT and Intrinsic Cellular Mechanotransduction Pathways

Whether EMT changes how cells respond to changes in matrix stiffness is relatively unclear. A few recent reports have indicated however that such a mode of regulation could exist. Expression of cell surface receptors of ECM ligands could be a potential mechanism to regulate the cellular response to matrix stiffness. For example, collagen can be recognized by integrins as well as discoidin domain receptor tyrosine kinases (DDR). Expression of both can be modulated during tumor progression. Integrin expression and dimerization is regulated during tumor cell transformation and EMT, which in turn modulates tumor cell adhesion and ECM recognition(Maschler et al., 2005). Likewise, discoidin domain receptors DDR1 and DDR2 are expressed exclusively in epithelial and mesenchymal tissues, respectively, and are regulated during EMT(Valiathan et al., 2012). This dichotomy allows for cell type specific ECM recognition and interaction. In fact, overexpression of Twist1 in HMLE and T47D cells results in a dramatic upregulation of DDR2 (GSE53222)(Shi et al., 2014). Furthermore, DDR2 which facilitates tumor metastasis by inducing the stabilization of Snail1 is induced by TGF-β(Walsh et al., 2011; Zhang et al., 2013). DDR proteins are uniquely situated to respond to cues from the ECM as they are the only receptor tyrosine kinases that specifically recognize ECM ligands. Crosstalk between integrin and DDR signaling opens the possibility that tumor cells may utilize both collagen receptors in concert to modulate cell-matrix interactions and responses(Xu et al., 2012). It may be interesting to explore whether DDR2 cooperates or signals into the Twist1-G3BP2 or other mechanotransduction pathways.

Physical changes in cell shape during EMT may also affect the cellular response to ECM. Interestingly, epithelial sheets generate more force at their leading edge than single epithelial cells(du Roure et al., 2005). It is of great interest then, how single cells that have undergone EMT generate sufficient force to move through the ECM. Both cellular tension and extrinsic mechanical forces can activate molecular mechanotransduction switches such as integrins(Geiger et al., 2001). In mesenchymal stem cells, cell adherence and spreading induces a change in the folding of vimentin as shown by a chance in the surface availability of cysteines(Johnson et al., 2007a). This suggests that changes in cell shape can induce changes in the molecular fold of intracellular proteins, thus regulating their function through binding site and post-translational modification site accessibility. Inside-out integrin signaling may also play a role in feedback regulation in response to changes in matrix stiffness. Once engaged, the β integrin subunit recruits talin, which in turns induces conformational changes that increase integrin affinity(Tadokoro et al., 2003). Thus engaged to a ligand and activated, integrins induce a feed-forward mechanism to engage further ligands and form focal adhesions. This observation is supported by other evidence that mechanical strain can induce conformational changes in integrins(Friedland et al., 2009; Zhu et al., 2008). Modulation of integrin affinity and avidity by mechanical forces underlie the regulation of cell shape by matrix stiffness. On compliant matrix integrin attachments are not stabilized and thus focal adhesions are not allowed to mature(Jiang et al., 2006). In the absence of stabilizing signals, focal adhesions become destabilized and are resolved, preventing effective cell spreading and movement. Mechanical regulation of focal adhesion formation and cell attachment contributes to durotaxis, cell migration towards substrates of higher rigidity. Indeed, focal adhesions are able to sense minute changes in matrix rigidity which directs focal adhesion polarization and cell movement(Plotnikov

et al., 2012). The actual physical organization of high stiffness matrices also promotes cell migration. Migration of fibroblasts along 1-dimensional surfaces (i.e. fibers) closely resembles movement in 3D environments(Doyle et al., 2009). These observations support a model in which increased matrix stiffness promotes tumor cell invasion, which has been previously corroborated in 2D linearly migration(Pathak and Kumar, 2012). However, once disseminated, do tumor cells interact in a similar fashion with the more compliant surrounding connective tissue? In breast cancers, for example, to what extent does the tumor remodel the surrounding mammary fat pad and through what molecular mechanism? Furthermore, do the same rules apply in secondary sites of metastatic dissemination as in the primary tumor?

3.5 Mechanical Properties of the Metastatic Niche

Remodeling of the tumor microenvironment and metastatic niche clearly play a significant role during metastasis. The network of signals that induce this remodeling and the identity of cells that carry out this process remain to be fully elucidated. Both stromal and tumor cells themselves can contribute to ECM deposition and remodeling. For example, the tumor secretome can be regulated by miR200s to promote metastasis via Sec23a secretion(Korpal et al., 2011). Sec23a is responsible for secretion of suppressive ECM molecules such as TINAGL1 as well as collagens(Korpal et al., 2011; Townley et al., 2008). What effect downregulation of Sec23a or other elements of the secretory pathway has on rigidity of the tumor microenvironment and metastatic niche remains to be determined. Whether and how the primary tumor remodels the metastatic niche and how that remodeling determines metastatic tropism remain topics of great interest. There is already evidence that the breast tumors begin to remodel the lung microenvironment prior to metastatic colonization(Erler et al., 2009). Tumor derived lysyl

oxidase facilitates recruitment of CD11b+ stromal cells to facilitate metastatic outgrowth of disseminated tumor cells. Moreover, inhibition of the LOX family member, LOXL2, attenuates development of the metastatic niche as demonstrated by reduced stromal fibroblast activation, ECM deposition and modification, and growth factor availability(Barry-Hamilton et al., 2010). Other ECM molecules have also been implicated in the formation of the premetastatic niche. Interestingly, fibronectin is deposited in the lung premetastatic niche following tumor implantation but prior to recruitment of stromal bone marrow derived cells(Kaplan et al., 2005). Fibronectin may serve as ECM scaffold to facilitate the deposition and organization of additional proteins such as collagen. Fibronectin deposition may be a function of resident fibroblasts responding to as yet unknown signal from the primary tumor. Placental growth factor has been identified as a potential candidate to mediate this ECM deposition(Kaplan et al., 2005). Regulation of the biochemical and mechanical properties of the premetastatic niche clearly plays a role in metastatic colonization and further to identify the inducing and effector molecules is clearly warranted.

Regulation of the premetastatic niche may also contribute to the dynamic regulation of EMT and MET cellular programs (Figure 3-1). Metastatic colonization of secondary sites is dependent on the dynamic regulation of EMT(Ocana et al., 2012; Tsai et al., 2012; Tsai and Yang, 2013). Constitutive activation of the EMT program prevents metastatic outgrowth as tumors must reacquire proliferative properties. Thus, tumor cells undergo the reverse process, mesenchymal-epithelial transition (MET) which facilitates metastatic colonization and explains why primary and secondary tumors have similar histology. Interestingly, PRRX1 induces EMT which facilitates tumor dissemination but loss of PRRX1 induces MET and stemness(Ocana et al., 2012). This is partially contrary to current ideology, in which EMT induces stemness and the

generation of cancer stem cells(Mani et al., 2008; Morel et al., 2008). How and when cancer stem cells are generated and maintained remains to be fully resolved. The stem cell niche undoubtedly plays a critical role in this process though. Equally perplexing is how EMT and MET programs are turned off and on, respectively, to mediate metastatic outgrowth. We still do not know the cues that induce the reversion of EMT to MET, however as we identify the effector molecules such as Twist1 and PRRX1, we can begin to understand the process. Given that Twist1 and PRRX1 collaboratively regulate EMT and MET and that Twist1 is regulated by mechanical cues, it is possible that tissue rigidity could play a significant role. Consistent with this hypothesis, inhibition of lysyl oxidase attenuates metastatic lung colonization by preventing remodeling of the metastatic niche(Erler et al., 2009; Erler et al., 2006). Remarkably, force mapping of PyMT-MMTV tumors reveals that the metastases are compliant and have a rigidity that matches the lowest rigidities identified in the heterogeneous primary tumor(Plodinec et al., 2012). This supports the notion that low matrix stiffness which maintains an epithelial phenotype in mammary epithelial cells, could induce MET at secondary sites (Figure 3-2). Furthermore, ECM molecules including collagen and laminin were highly enriched the invasive front of the tumor where the matrix stiffness was highest, supporting a model in which EMT is induced at the edge tumor to facilitate dissemination(Brabletz et al., 2005; Plodinec et al., 2012). If involved, matrix stiffness likely operates in conjunction with other signals and context to regulate EMT/MET. Characterization of the Twist1-G3BP2 mechanotransduction pathway suggests that dynamic regulation of EMT might be achieved through cooperative regulation of matrix stiffness and G3BP2 expression. Other biochemical signals such as TGF-β also contribute to this regulation of EMT and tumor invasion. Indeed, TGF-β derived from perivascular endothelial cells in the metastatic niche has been shown to induce breast

cancer cell proliferation(Ghajar et al., 2013). Tumor cell dormancy may actually be a phenomenon caused in part by the requirement for an additional signal to induce MET in disseminated tumor cells. This final 'hit' during the metastatic cascade may present a therapeutically targetable bottleneck.

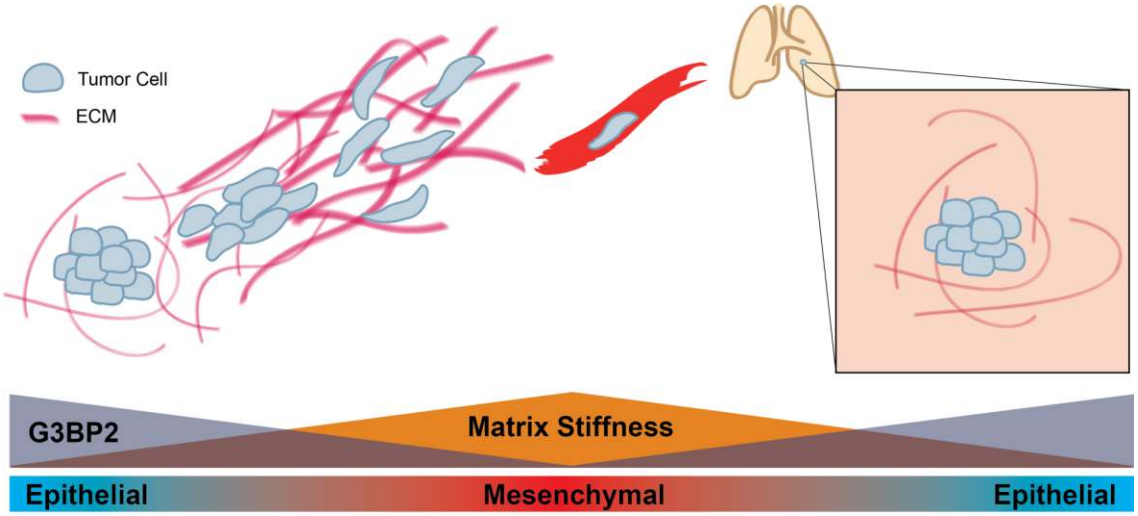

Figure 3-2. Model of the involvement of matrix stiffness during the metastatic cascade of breast cancer. Early during tumorigenesis the primary tumor resides in a compliant microenvironment. Matrix stiffness increases as a result of ECM remodeling by stromal and tumor cells. Increasing matrix stiffness induces EMT in tumor cells in concert with other factors such as TGF-β. Tumor cells disseminate through vascular and lymph systems to secondary sites such as the lung, where the microenvironment is again compliant. This mechanical cue may induce MET(or passively allow for the reversion of EMT), thus facilitating metastatic colonization.

3.6 Molecular Functions of G3BP2

As discussed in Chapter 2, G3BP2 can function as a molecular cytosolic scaffold to sequester transcription factors. In other contexts G3BP2 has also been shown to have several other molecular functions including nuclear transport, RNA binding, and stress granule formation. Might the other described G3BP2 functions feed into or affect mechanosensing? G3BP1 and to a lesser extent, G3BP2, can regulate breast cancer

cell proliferation via binding PMP22 mRNA(Winslow et al., 2013). Regulation of mRNA is likely not specific to PMP22, as G3BP proteins have been shown to bind other mRNAs including c-myc (Tourriere et al., 2001). G3BP2 has also been identified as a critical factor in Wnt signaling. G3BP2 interacts with the cytoplasmic transducer protein Disheveled to mediate phosphorylation of the downstream effector molecule LRP6(Bikkavilli and Malbon, 2012). Loss of G3BP2 attenuated Wnt3a stimulated nuclear accumulation of β-catenin and downstream TCF/Lef mediated transcription. Thus, modulation of G3BP2 expression levels may also an effect through β-catenin signaling. Moreover, the Drosophila homolog, Rasputin, likely links Rho and MAPK signaling via Ras(Pazman et al., 2000). While this signaling mechanism has not been fully explored in either fly or mammalian systems, it is intriguing to speculate whether G3BP2 could act as a molecular hub to join Rho-mediate cell contractility and MAPK-mediated cell survival and proliferation.

3.7 Metastatic Colonization and Mechanotransduction: Roles for G3BP2 and Twist1

It has been proposed, now almost twenty years ago, that the epithelial state is the default and only through active regulation of epithelial and mesenchymal characteristics can EMT be induced and maintained(Frisch, 1997). If this is true, then once a tumor cell disseminates from the primary tumor and loses EMT inducing signals, such as TGF-β or increased matrix stiffness, it begin to re-epithelialize. This would be consistent with observations of differentiated metastases that correspond to the similarly differentiated tumors. However, this predicates that the metastasis is of a type I (environmentally induced) origin versus a type II (genetically induced)(Brabletz, 2012). Thus, future therapies that aim to inhibit tumor metastasis via modulation of matrix

stiffness or other environmental factors might be best targeted to differentiated primary tumors with differentiated metastases, indicative of involvement of type I EMT. One mechanism for such a therapeutic strategy would be to induce tumor re-epithelization through upregulation of E-cadherin. E-cadherin is increased at low matrix stiffness in both normal and metastatic breast cancer cells and has been shown to be sufficient to regulate β-catenin and NF-κB transcriptional regulation (Figure 2-6, 2-8, and 2-9)(Solanas et al., 2008). Alternatively, modulation of G3BP2 expression could provide a mechanism through which tumor cells re-epithelialize at secondary sites since G3BP2 can regulate epithelial identity and behavior as discussed in Chapter 2.

In addition to modulation of extrinsic properties of the premetastatic niche, cell intrinsic changes may also permit the induction of MET. As downgregulation of G3BP2 in tumor cells collaborates with changes in matrix stiffness to induce malignancy, it is quite possible that upregulation of G3BP2 could promote an epithelial phenotype. Thus in the metastatic niche, disseminated tumor cells that upregulate G3BP2 might re-epithelialize, thus facilitating the metastatic colonization. Although there is not a described molecular mechanism that regulates G3BP2 expression levels, there are some reports characterizing the regulation of G3BP1 expression. G3BP1 expression is down-regulated by PTEN activity and appears to be more sensitive to loss of PI3K activity by wortmanin treatment. If this regulation also affects G3BP2, this provides a potential mechanism for dynamically modulating G3BP2 expression and thus Twist1 mediated EMT. G3BP2 localization can be modulated in some contexts via phosphorylation. While unlikely given our observations thus far, mechanotransduction via G3BP2 post translational modification remains a viable possibility(Tourriere et al., 2001). Interestingly, G3BP2 has been reported to be arginine methylated(Bikkavilli and Malbon, 2012). This post-translational modification disrupts the interaction between

G3BP2 and Dishevelled. It is unlikely that G3BP2 arginine methylation regulates the interaction between G3BP2 and Twist1, however, since the modifications are on the arginine-glycine rich C-terminal of G3BP2 which is relatively distant from the putative Twist1-interaction domain. These hypotheses require thorough investigation, however, as regulation of the Twist1-G3BP2 mechanotransduction pathways has implications in both primary tumor invasion and metastatic colonization. Specifically, regulation of G3BP2 has the potential to define a cellular mechanism to escape from tumor cell dormancy. Activation or upregulation of G3BP2 by cell extrinsic or intrinsic signals may be sufficient, for example, to overcome signals from the presmetastatic microenvironment suppressing MET in disseminated, dormant tumor cells.

3.8 Hydrogel and 3D Culture Systems

The PA hydrogel and rBM based 3D culture system utilized in the studies described herein has many advantages but also has some disadvantages. The use of a biologically inert PA gel in combination with Matrigel allows for the use of native ECM; however, this allows the cells to dynamically modify their own microenvironment. User-defined ECM could allow more specific questions to be asked, however, a number of other variables must be addressed in such systems including growth factor availability, oxygenation, matrix heterogeneity, and applicability of standard cell and molecular biology approaches(Tibbitt and Anseth, 2009). Furthermore, the PA hydrogel-Matrigel 3D culture system only provides mechanical cues in a unidirectional manner. This raises the question of how mechanical signals are transmitted within an acinus if only several cells are in contact with the substrate with defined mechanical properties. I reason that this issue is resolved to some degree in that the mammary epithelial cells are seeded as single cells. Every cell, and thus every acinus, is exposed to the rigidity

of the PA gel substrate. Matrix stiffness then perhaps 'reprograms' the cell and the cellular response is defined early during acinar development at the single- or several-cell stage. Another potential weakness of this culture system is that it is static. A tunable hydrogel system would allow one to investigate the effect of dynamic modulation of matrix stiffness. This has obvious advantages in probing the effect of matrix stiffness in vivo in which the mammary gland environment is compliant but stiffened during tumor progression. There are several tunable hydrogel systems that could support such an application such as DNA polymer-based hydrogels (Jiang et al., 2008). These gels satisfy the required dynamic range of the gel system as the stiffness required for acinar formation is very low, but then must be stiffened by one to two orders of magnitude. Similarly, methacrylated hyaluronic acid (MeHA) based hydrogels can be inducible tuned using UV-inducible crosslinking(Marklein and Burdick, 2010).

Hydrogel systems may also be used to investigate durotaxis by generating stiffness gradients within a gel. The combination of photo-activated crosslinking and photomasks allow for the fabrication of such gels(Tse and Engler, 2010). Using such a system would allow one to ask whether acini respond to fluctuations in stiffness and whether cells on a higher stiffness might signal in a paracrine fashion to those on lower stiffness. An important caveat to this system is that the step-size of the gradient must be sufficiently large that individual cells will be able to sense the change in stiffness from end-to-end in order to assay processes such as durotaxis. These and other hydrogel systems will allow for time-based and cue-based dynamic modulation of matrix stiffness.

For the 3D culture aspect of this system, while rBM/Matrigel offers many biological advantages, it is also provides an undefined ECM. The concentration of each component can vary widely as well as the overall protein concentration between

production lots. This requires that biological observations be repeated in multiple lots to ensure that the effect is not lot-specific. Hydrogel systems that embed cells in matrices of defined components may partially solve this issue; however lack the diversity of ECM ligands that EHS-derived rBM offers. Each system offers distinct advantages and disadvantages and will likely be best employed in conjunction with each other in addition to biochemical and animal model approaches.

3.9 Therapeutic Implications of Tumor Mechanotransduction

These findings may also have implications for diagnostics and therapeutics for breast cancer. Elastography and sonography are emerging as non-invasive diagnostic approaches to that can identify and risk-stratify breast tumors(Thomas et al., 2006). Other approaches such as atomic force microscopy (AFM) to directly measure the material elasticity of fresh tumors may also be employed to identify malignant lesions in combination with traditional histological and gross examination(Plodinec et al., 2012). These approaches each have strengths and weaknesses. AFM is biopsy-based and potentially limited by sample acquisition and is carried out *ex vivo*, but has the potential to be more accurate. Elastography is a non-invasive technique that can be used in concert with mammography but potentially is limited by its sensitivity to differentiate malignant versus benign lesions due to image quality and observer variability(Burnside et al., 2007). Risk stratification by these methods may be augmented by the use of biomarkers. As discussed in Chapter 2, because changes in G3BP2 expression can sensitize tumor cells to changes in matrix stiffness, G3BP2 may have value as a metastatic biomarker. Evaluating tumor tissue rigidity in parallel with quantitative expression analysis of G3BP2 and ECM components such as collagens, lysyl oxidases, and TGF-β may provide a more accurate risk assessment and allow for a more

appropriate treatment plan. This will rely however on a more complete understanding of how these factors interact and affect tumor growth and metastasis.

EMT, in addition to imbuing epithelial cells with stem-like characteristics, can also induce chemotherapeutic resistance. It is possible that stiffer tumors might also be more resistant to chemotherapy. Activation of β1 integrin by collagen induces expression of the transmembrane transporter ABCC1 in T cells to attenuate the cellular response to doxorubicin(El Azreq et al., 2012; Naci et al., 2012). ABCC1 can facilitate the export of chemotherapeutics such as doxorubicin. Whether a similar mechanism is active in epithelial tumors remains to be seen. As EMT has been reported by multiple groups to increase tumor chemoresistance in other contexts, it is tempting to speculate that matrix stiffness induced EMT could also induce chemoresistance. Or perhaps more therapeutically applicable, is the notion that by reducing tissue rigidity it would be possible to increase the efficacy of chemotherapeutic agents.

Because, as discussed in previous sections, modulation of matrix stiffness and G3BP2 regulates EMT, it is possible that these factors affect dynamic regulation of EMT and thus metastatic colonization. Modulating G3BP2 expression or matrix stiffness will affect Twist1-G3BP2 mechanotransduction in the context of the primary tumor and perhaps also the secondary site. However, because of the opposing effects in the primary versus secondary site, any therapeutic approach will require exquisite specificity and/or balance. The inherent flaw in all therapies hoping to prevent metastasis by inhibiting EMT is that approaches to prevent induction of EMT will likely also induce MET collaterally. This may be highly problematic if tumor cells are already disseminated to distant tissues but are unable to make the transition to macrometastases. By therapeutically intervening and inducing MET one might facilitate metastatic colonization

and outgrowth thus defeating the purpose of preventing further tumor cell dissemination. Approaches to prevent EMT by reducing matrix stiffness and/or increasing G3BP2 expression might be effective if applied sufficiently early during tumor progression so that there is minimal tumor dissemination present. Accurate and specific diagnostics to detect disseminated tumor cells will be required however as dissemination may occur extremely early during tumorigenesis(Husemann et al., 2008).

Interestingly, inhibition of G3BP1 by monoclonal antibody to induce tumor cell apoptosis and use of anti-G3BP1 monoclonal antibody based diagnostics has been patented by Aventis Pharma, now Sanofi-Aventis (U.S. Patent 7001980). The proposed mechanism is through prevention of interaction with RasGAP, and thus through modulation of Ras signaling. Whether G3BP1 shares any functions related to mechanotransduction or otherwise will also determine the effect of any such treatment or diagnostic technology. Targeting of intracellular proteins by biologics such as monoclonal antibodies will be problematic however since they will not be cell permeable. To my knowledge there has not been any significant advance or clinical trial partaken with respect to this monoclonal antibody however.

3.10 Matrix Stiffness and Cancer Stem Cells

Given that EMT regulates epithelial cell plasticity and can generate cancer stem cells it might follow that increasing matrix stiffness would foster the generation of CSCs(Mani et al., 2008; Morel et al., 2008). It is unclear whether mechanical signals contribute directly to the induction and maintenance of cancer stem cells. Surprisingly, in co-culture experiments neither overexpression or inhibition of lysyl oxidase affected bone marrow derived mesenchymal stem cell induced generation of breast cancer cancer stem cells(El-Haibi et al., 2012). While the effect of MSCs on breast cancer cell

malignancy is LOX dependent, generation of cancer stem cells in the systems utilized may be dependent on other mechanisms. MSCs can regulate breast cancer stem cell generation through paracrine cytokine signaling networks(Chaturvedi et al., 2013; Liu et al., 2011). The inducing signals of CSCs may be induced through multiple parallel pathways depending on the unique microenvironment and genetic background of each tumor. These mechanisms include, but are certainly not limited to, recruitment of bone marrow derived MSCs, genetic aberrations, or microenvironment signals. As discussed, matrix stiffness increased matrix stiffness induces EMT and may contribute to CSC formation but this hypothesis remains to be tested. Other EMT-inducing environmental signals such as Wnt may also signal cooperatively with mechanical to induce CSCs. Stromal-derived periostin (POSTN) can induce Wnt signaling in cancer stem cells to promote maintenance of stemness(Malanchi et al., 2011). POSTN is an ECM protein and has been recently implicated in defining the mechanical properties of connective tissues(Norris et al., 2007). Mechanistically, POSTN may contribute to matrix stiffening by acting as a scaffolding protein, bridging interactions between other ECM molecules including collagen I, tenascin-C, and fibronectin through multiple interaction domains(Kii et al., 2010). Furthermore, POSTN expression is required in the secondary site to facilitate metastatic outgrowth(Malanchi et al., 2011). In the secondary site POSTN is derived from tumors cells, rather than the stromal cells as in the primary tumor. Cells that have undergone EMT may secrete more POSTN as TGFβ2 and TGFβ3 can induce POSTN expression. This suggests that mechanical properties might also play a critical role in the metastatic niche. One can imagine a scenario in which the rigidity of different tissues would influence metastatic tropism. Matrix stiffness might also influence the ability of disseminated tumor cells to colonize and grow in secondary sites.

In summary, mechanical signals from the tumor microenvironment can drive tumor progression at each step of the metastatic cascade. I pose a model in which following the initial genetic aberrations leading to the formation a non-invasive primary tumor, the tumor generates a desmoplastic stromal response (Figure 3-1). This response is mediated through a variety of factors including stromally-derived LOX and upregulation of Cav1 in fibroblasts among factors that have yet to be fully described. This desmoplastic response results in increased matrix stiffness and release of growth factors such as TGF-β which induces EMT in tumor cells allowing for local invasion. These EMT cells feed forward to enhance matrix remodeling through deposition of additional ECM molecules, modification of the ECM, and increasing the pool of available growth factors. Together this positive feedback loop facilitates tumor dissemination to secondary sites. Once at the secondary site, the local microenvironment attenuates this signaling cascade, allowing for the re-epithelialization of tumor cells and the colonization of the premetastatic niche (Figure 3-2). This model presumes a type I environmentally induced cell plasticity(Brabletz, 2012), and will likely require revision as we learn more about the role of the mechanical and biochemical contributions of the primary and metastatic tumor microenvironment. Nonetheless, it is clear that mechanical properties have a significant role during tumor progression and additional insight into mechanotransduction pathways will have implications for cancer biology, developmental biology and therapeutics.

3.11 Concluding Remarks

In this dissertation I have discussed the role of mechanical cues during tumor progression. The identification of a novel Twist1-G3BP2 mechanotransduction pathway is, to my knowledge, the first connection between matrix stiffness and a core EMT

transcription factor (Figure 3-1). This link highlights a role for matrix stiffness in facilitating tumor invasion and dissemination and provides a mechanistic explanation for the correlation between increasing matrix stiffness of tumors and poor patient prognosis. Of great interest moving forward will be to understand how the association between Twist1 and G3BP2 is modulated in response to changes in matrix stiffness. The identification of a Src-family kinase tyrosine phosphorylation motif within the Twist1 G3BP2 recognition sequence raises the possibility that direct modification of Twist1 downstream of integrin activation might regulate the molecular interaction between Twist1 and G3BP2. If Twist1 phosphorylation regulates this interaction, it will present an attractive therapeutic target to inhibit matrix stiffness mediated EMT. Such an approach certainly has significant merit as low matrix stiffness as well as loss of Twist1 prevented TGF-β induced EMT, suggesting that inhibition of Twist1-G3BP2 mechanotransduction might also prevent induction of EMT by other cues such as TGF-β. Careful dissection of the molecular mechanisms regulating Twist1-G3BP2 mediated mechanotransduction pathways will shed additional light on this topic.

Also of significant interest is whether Twist1-G3BP2 mechanotransduction is an evolutionary conserved process and furthermore whether this process plays a role during embryogenesis. Given the key role that Twist1 has during development in the generation of germ layers and large scale migration, it would stand to reason that multiple layers of regulation affect Twist1 activity. Thus, Twist1 expression as well as localization may be modulated, although changes in Twist1 localization during embryogenesis have not yet been described. This may be a regulatory mechanism specific to mammals since the identified G3BP2 interaction motif begins to degenerate in *Xenopus* and is not present in *Drosophila* (Figure 2-23). Moreover, given that EMT-transcription factors signal most often in concert within complex feedback networks, it

will be interesting to investigate whether other key EMT transcription factors have any role in mechanotransduction signaling pathways. Mechanical cues could either signal to other factors including Snail1/2, Zeb1/2, and FOXC1/2 in parallel to or as a part of the Twist1-G3BP2 mechanotransduction signaling cascade. Additionally, taking into account the close association of NF-κB signaling and Twist1 regulation and that TGF-β signals cooperatively with matrix stiffness, it is worth investigating whether NF-κB (or perhaps more generally, inflammation) and matrix stiffness might signal in concert. Of note, it will be of great interest how, when, and by what cell type matrix stiffness is modulated in the tumor microenvironment and which biochemical cues are involved in the induction of mechanical changes in the ECM.

In conclusion, this study adds to the rapidly developing and burgeoning field of mechanobiology. Specifically, it provides a direct mechanistic link between mechanical cues from tumor micronenvironment and tumor cell transcriptional regulation which mediates induction of EMT and cellular invasion. Moreover, it highlights the notion that mechanical signals can interact with biochemical factors to regulate cellular processes. The mechanical properties of the developing embryo, adult tissues, and tumor are not merely passive byproducts of cellular processes, but active participants in cellular and tissue processes. Moving forward, we will undoubtedly discover new and exciting modes of regulation and roles for mechanical signals in all aspects of biology.

3.12 Acknowledgements

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