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The role of Tyk2 in matrix stiffness-driven EMT and metastasis

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

in

Biomedical Sciences

by

Hannah Elisabeth Majeski

Committee in charge:

Professor Jing Yang, Chair
Professor Andrew Chisholm
Professor Adam Engler
Professor Kun-Liang Guan
Professor Tony Hunter

2020

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Chair

University of California San Diego

2020

DEDICATION

I would like to dedicate this dissertation to my family and friends for their unwavering support.

EPIGRAPH

"When we try to pick out anything by itself, we find it hitched to everything else in the universe."

- John Muir

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LIST OF ABBREVIATIONS

2D: Two dimensional

3D: Three dimensional

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

BM: Basement Membrane

CAFs: Cancer Associated Fibroblasts

CTC: Circulating Tumor Cell

DAPI: 4',6-diamidino-2-phenylindole

DIN: ductal intraepithelial neoplasia

DCIS: Ductal Carcinoma In Situ

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

DSP: Dithibis (Succinimidyl Propionate)

DTCs: Disseminated Tumor Cells

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

EMT: Epithelial-Mesenchymal Transition

ER: Estrogen Receptor

ERK: extracellular signal-regulated kinase

FAK: Focal Adhesion Kinase

FERM: Band 4.1 protein, ezrin, radixin and moesin

FGF: Fibroblast Growth Factor

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

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green Fluorescent Protein

gp130: Glycoprotein 130

HER2: Human Epidermal growth factor Receptor 2

IDC: Invasive Ductal Carcinoma

IFN: Interferon

IFNAR1: Interferon alpha/beta receptor 1

IF: Immunofluorescence

IHC: Immunohistochemistry

IL: Interleukin

IP/MS: Immunoprecipitation/Mass Spectrometry

JAK: Janus kinase

KLD: kinase-like domain

LCIS: Lobular carcinoma *in situ*

MET: Mesenchymal-Epithelial Transition

mRNA: Messenger ribonucleic acid

Pa: Pascal

PA: Polyacrylamide

PDGF: Platelet-Derived Growth Factor

PDGFRα: Platelet-Derived Growth Factor Receptor α

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-Kinase

PKC: Protein Kinase C

PLA: Proximity ligation assay

PR: Progesterone Receptor

rBM: Reconstituted basement membrane

RNAi: RNA interference

ROCK: rho-associated protein kinase

RTKs: Receptor Tyrosine Kinases

SDS: Sodium Dodecyl Sulfate

SFKs: Src family of non-receptor Tyrosine Kinases

SH2: Src homology 2 domain

SHG: Second harmonic generation

shRNA: Short hairpin ribonucleic acid

siRNA: Small interfering ribonucleic acid

SOCS: Suppressors of cytokine signaling

STAT: Signal transducer and activator of transcription

T-ALL: T-cell acute lymphoblastic leukemia

TAZ: Tafazzin

TNBC: Triple-negative breast cancer

TGF-β: Transforming growth factor β

Tyk2: Tyrosine kinase 2

Yap: yes-associated protein

ZEB: Zinc-Finger E-box binding

ZO1: Zonula Occludens

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ABSTRACT OF THE DISSERTATION

The role of Tyk2 in matrix stiffness-driven EMT and metastasis

by

Hannah Elisabeth Majeski

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2020

Professor Jing Yang, Chair

Breast tumors are often identified based on their apparent hardness compared to normal breast tissue, and in breast cancer patients an increase in tissue rigidity is often correlated with an increase in metastasis. When human mammary epithelial cells are grown in 3D culture with the matrix stiffness of breast tumors, they develop a more malignant phenotype. The link between increased tissue rigidity and invasion and metastasis at the

molecular level is not well described and is the focus of this dissertation. The transcription factor Twist1 is a key regulator of metastasis through its ability to induce Epithelial-Mesenchymal Transition (EMT), a developmental program also used by cancer cells to invade and metastasize. Previous research in the Yang lab has described a mechanosensing pathway in which Twist1 nuclear translocation is stimulated by increases in matrix rigidity. This nuclear translocation is controlled by phosphorylation of Twist1 on a tyrosine residue which decreases the interaction between Twist1 and its cytoplasmic binding partner G3BP2, allowing Twist1 to enter the nucleus where it can induce EMT. Tyrosine kinase 2 (Tyk2) was identified in a knockdown screen, where loss of Tyk2 was shown to increase nuclear Twist at lower rigidities. This dissertation characterizes the role of Tyk2 in regulating the Twist1-mechanosensing pathway. I describe how Tyk2 is affecting known components of the Twist1-mechanosesning pathway. I also show that Tyk2 activity and subcellular localization are regulated by rigidity. Tyk2 localizes to the plasma membrane at low rigidities and becomes cytoplasmic at high rigidities. This localization change for Tyk2 is recapitulated in human breast tissue samples. Tyk2 is membranous in normal breast tissue and low grade ductal intraepithelial neoplasia (DIN) lesions, which have softer ECM signatures. In ductal carcinoma in situ (DCIS) and breast tumors samples, which have stiffer ECM signatures Tyk2 is cytoplasmic. These findings indicate that Tyk2 is a negative regulator of Twist1 translocation in response to stiffness. This work also reveals a novel role for Tyk2 loss of function in breast cancer invasion and metastasis.

Chapter 1

Introduction

1.1 Breast Cancer

Breast cancer is the most common cancer diagnosed in women apart from skin cancers. Breast cancer is also the second leading cause of cancer death in women, with lung cancer being the first. Breast cancer incidence rate has increased by about 0.3% per year since 2004, although this increase is largely due to rising rates of local stage breast cancer diagnoses. In 2019, approximately 286,600 women were diagnosed with invasive breast cancer, and 48,100 women were diagnosed with ductal carcinoma in situ (DCIS), which is a more local stage disease (DeSantis et al., 2019). Approximately 41,760 women will die from breast cancer in 2019. The mortality of breast cancer has been steadily declining, although the rate of decline from 2011-2017 was 1.3% annual decrease compared to 1.9% from 1998-2011 (DeSantis et al., 2019). Although mortality has been declining, patients with metastatic disease still have poor survival rates. The 5-year survival rate for patients with localized disease is 99%, whereas for patients with regional spreading to lymph node- its drops down to 86%. Patients with distant metastasis have a 5 year survival of 27% (Noone AM, 2018; Wang et al., 2019a). This indicates that metastatic breast cancer is still a very deadly disease, largely due to the lack of treatment options that generate good increases in survival.

There are two major type of breast carcinomas originating from epithelial cells within two compartments of the breast – the ducts and the lobule. Ductal carcinoma arises from the cells lining the ducts and lobular carcinoma comes from the milk producing glands. Each of these subtypes can either be *in situ* or invasive. Ductal carcinoma *in situ* (DCIS) is characterized by hyperproliferation within the ducts. DCIS generally display a more normal cellular phenotype and are noninvasive. DCIS can give rise to invasive ductal carcinoma,

which is breast cancer also originating from the ductal cells, but has additionally invaded into the surrounding tissue. Invasive ductal carcinoma (IDC) are highly dysplastic lesions which invade through the basement membrane and can spread locally within the breast and to local lymph nodes, or metastasize to distant organs (Edge, 2010).

Due to increases in breast screening, there has been a large increase in the incidence of non-invasive breast lesions, including DCIS and pre-cancerous lesions that also can originate from the lobular or ductal cells. These pre-cancerous lesions have undergone a lot of name changes and diagnostic criteria changes over the years, primarily to try and identify proper courses of treatment. The ductal form of these lesions is now categorized into stages of ductal intraepithelial neoplasia (DIN). The first stage being DIN1a, also called flat epithelial atypia. This stage is generally characterized by changes in cell phenotype and the overall ductal structure of the mammary gland remains relatively normal (Said et al., 2015). DIN1b, previously called atypical ductal hyperplasia, is a benign growth with the ductal epithelial cells that displays an array of cytological features. DIN1b lesions, like DCIS, are localized within the ducts and do not display any invasive phenotypes (Tavassoli and Norris, 1990). These DIN1b lesions are separated from a DCIS diagnosis primarily due to lesion size. DCIS lesions can also be separated into three stages, simply termed stage 1, stage 2 and stage 3 DCIS (Bendifallah et al., 2012).

Invasive breast cancers are also separated into stages, and these stages are based on tumor size, lymph node positive and metastasis to distant organs. In stage I breast cancer, the tumor remains localized to the breast and relatively small (less than 2cm). In stage II, the tumor may have spread to regional lymph nodes (up to 3) and can be up to 5cm (but only 2cm if there are lymph node positive for tumor cells). Stage III breast cancer tumors have spread to regional lymph node and are larger than 5 cm (unless greater than 4 lymph

nodes are positive). These stage III tumors may have also spread locally from the breast into the chest wall or the skin. Stage IV tumors is metastatic and has spread to distant organs. Patient outcome, as mentioned earlier, is correlated with disease staging, and stage IV patients that are metastatic and the poorest survival (Edge, 2010).

Breast tumors are also be categorized molecularly based on the expression of several hormone receptors and growth factor receptors. Staging in this way is utilized to determine what type of therapy patients receive. The receptors include human epidermal growth factor receptor 2 (ERBB2, or HER2), estrogen receptor (ER), progesterone receptor (PR). Luminal A tumors are ER+ PR+ and HER2 negative. Luminal B tumors are ER+ PR+ and HER2+. There are additionally HER2 enriched, ER negative tumors and triple negative tumors which are negative for all three of these proteins. Luminal A type tumors are the most common and also have the best prognosis because they tend to be less aggressive and have the best targeted therapies. Triple negative type tumors constitute about 15-20% of all breast cancer, and they have the worst prognosis because they can't be treated with any hormonal or anti-receptor targeted therapy. Triple negative breast cancers are also referred to as 'basal-like' because they molecularly resemble the basal cells that surround the mammary ducts (Harbeck et al., 2019; Waks and Winer, 2019).

In recent years there have been many developments in targeted therapies for cancer aimed at treating different 'hallmarks of cancer.' Advanced therapeutics targeting cell survival and DNA replication have been developed, taking advantage of specific mutations within certain cancers. In breast cancer specifically, targeted therapies have focused on specific oncogenic drivers, and treatment plans are split up based on the expression (or lack of expression) of hormone receptors and ERBB2. For tumors that are hormone receptor positive (ER+ or PR+) endocrine therapy is used. Tamoxifen, which is a selective estrogen

receptor modulator that competitively inhibits estrogen binding, is the primary systemic therapy used. This is usually used in combination with surgery and occasionally chemotherapy, depending on the tumor stage. Tamoxifen treatment is usually prolonged to prevent recurrence, and it is also used in patients with DCIS to lower the risk of developing IDC (Waks and Winer, 2019). For ERBB2 positive cancers, there is a monoclonal antibody against ERBB2 called Trastuzumab which inhibits ERBB2 activation and slows the growth of ERBB2 positive cancers. Trastuzumab has additionally been modified into an antibody-drug conjugate, the purpose being to target the therapies attached to the antibody to ERBB2 expressing cells – namely the cancer cells (Piccart-Gebhart et al., 2005; Wolff et al., 2013). There are additional targeted therapies that target CDKs to stop cell division, or drugs that target common mutations – like Alpelisib which is a PI3K inhibitor. The vast majority of these targeted therapies do not, however, work for triple negative disease. For most triple negative breast cancers the only therapeutic options are surgery, chemotherapy and/or radiation. For patients with germline mutations in the BRCA1 gene, PARP inhibitors, which target BRCA deficient cells due to synthetic lethality, can be used (Waks and Winer, 2019). For TNBC patients that have tumors which express PD-L1, immune checkpoint inhibitor therapies that target PD-L1 can be used (Schmid et al., 2018). Only 20% of TNBC patients have tumors that express PD-L1 however, which still leaves the vast majority of these patients with no specific therapies, which is one of the reasons why these patients have such a poor prognosis compared to other breast cancer subtypes. The lack of effective treatment for TNBC patients, particularly those with metastatic disease indicates that there is a clear need for improvements in this type of breast cancer. Understanding more of the molecular causes of tumor initiation and progression in this type of cancer will enable the production of more targeted therapies for these patients and hopefully increase survival rates in this group.

1.2 Breast cancer metastasis

Breast cancer metastasis is the leading cause of mortality for breast cancer patients, and 30% of patients diagnosed with an early stage of breast cancer will eventually develop metastasis later on. Metastasis is the process by which tumor cells from the original or ‘primary’ tumor (in this case the breast tumor) migrate to other organs and grow into new tumors within those distant sites. In order to metastasize tumor cells must gain the ability to migrate which generally involves the loss of cell-cell junctions, the loss of apical basal polarity and the loss of cell adhesion to the basement membrane. This is generally referred to as an epithelial-mesenchymal transition, which will be discussed in the next section of the introduction. This change to a more migratory phenotype is not the only challenge that tumor cells face in order to metastasize, and in fact metastasis is a very inefficient process. In order to colonize distant organs, breast cancer cells must locally invade into surrounding tissue and through the basement membrane. They must then intravasate into blood vessels or the lymphatic system, and then travel through the blood or lymphatic system to secondary sites (Scully et al., 2012). Tumor cells that are within circulation are called circulating tumor cells (CTCs), and CTC load can be an indicator of disease progression in patients. This is also the portion of the metastatic cascade that can be the most harsh on tumor cells – they must survive shear stresses within the vasculature and also become resistant to a specific type of cell death called anoikis which occurs when epithelial cells no longer are attached to the ECM (Guadamillas et al., 2011). This type of anchorage-independent growth has long been a marker for transformation, and is frequently seen in cancer cell lines. After arriving at a secondary site, tumor cells must then extravasate into that secondary tissue and establish within that tissue as a micro-metastasis by adapting to the new extracellular environment. These disseminated tumor cells (DTCs) can lay dormant for years as non-proliferating micro-metastases (Dasgupta et al., 2017). In order to grow out into a macro-metastasis tumor cells

generally need to undergo an EMT reversion (called mesenchymal to epithelial transition or MET) in order to become proliferative again (Brabletz et al., 2005; Tsai et al., 2012; Tsai and Yang, 2013). These cells also need to evade the immune system, and adapt to their new environment. The process by which these DTCs become dormant or leave dormancy many years later is still not well understood. It is also not well understood at which stage of tumor development tumor cells begin to disseminate. That is whether primary tumors grow and develop and then begin to invade, or whether tumor cells begin metastasizing from early on in tumor development.

1.3 Epithelial-mesenchymal transition (EMT)

Epithelial cells are characterized by a cobblestone-like morphology and form sheets within different tissue types within the body. Epithelial cells are held together laterally by forming tight cell-cell junctions including tight junctions, gap junctions, adherens junctions and desmosomes. The organization and function of these junctions is crucial for the structural integrity of the epithelial sheets. These sheets play a role in barrier formation and also in absorption, secretion and gradient formations. Adherens junctions are comprised of transmembrane cadherins (primary E-cadherin in epithelial tissues) and cytoplasmic catenins which connect the cadherins to the actin cytoskeleton (Harris and Tepass, 2010). Tight junctions are important for barrier function and are made up of occludin and claudin proteins (Schneeberger and Lynch, 2004). Gap junctions help mediate the diffusion of small molecules and are formed by connexin proteins (Giepmans, 2004). Epithelial cells also maintain interaction with the basement membrane though hemidesmosomes and specific integrin expression. Epithelial cells additionally express specific cytokeratins that stabilize desmosomal junctions (Lamouille et al., 2014; Shibue and Weinberg, 2017). Apical-basal polarity is another key characteristic for epithelial cells. Correct orientation is maintained by

apical basal polarity and this is critical for epithelial cells to determine the directionality of absorption or secretion. Polarity is defined by three main complexes within epithelial cells Crumbs, Par and Scribble (Moreno-Bueno et al., 2008).

Epithelial-mesenchymal transition (EMT) involves the conversion, or partial conversion, of epithelial cells into mesenchymal cells. Mesenchymal cells exhibit a spindle-like morphology, front-rear polarity, actin stress fiber formation and are generally more fibroblastic in nature with increased migratory and invasive capabilities. Mesenchymal cells express vimentin, an intermediate filament, fibronectin, and mesenchymal cadherins such as N-cadherin (Kalluri, 2009). Epithelial-mesenchymal transition (EMT) involves the repression of epithelial makers and the induction of mesenchymal markers. E-cadherin in particular is repressed, which leads to a disruption in cell-cell junctions. Additionally, cell polarity is lost, and there is an increase in the degradation of the basement membrane and a reorganization of the ECM. Mesenchymal markers such as N-cadherin, vimentin and fibronectin are upregulated (Lamouille et al., 2014; Shibue and Weinberg, 2017). The result is a more migratory, quasi-mesenchymal cell. EMT is a reversible process, governed completely through epigenetic changes discussed below. In many cases, full mesenchymal transition is not observed, and cells are thought to enter partially transitioned states which encompass some, but not all the molecular changes listed above.

EMT was first described as a critical process during embryogenesis and has since been shown to be an important program for several developmental processes. EMT is categorized into three types, each of which takes place in a certain context. Type I EMT occurs during gastrulation where cells invaginate from the primitive streak to form the mesoderm (Nieto, 2011; Thiery et al., 2009; Viebahn, 1995; Yang and Weinberg, 2008). EMT is also crucial for neural crest cell migration from the dorsal neural epithelium to form several

structures including craniofacial structures, peripheral nervous system and some endocrine cells and melanocytes (Shoval et al., 2007; Simoes-Costa and Bronner, 2015). Type II EMT occurs during wound healing and tissue regeneration. During wound healing, epithelial cells undergo EMT in order to migrate across a wound site, proliferate and then revert back to epithelial cells in order to re-instate epithelial barrier integrity (Savagner and Arnoux, 2009; Stone et al., 2016). Type III EMT is involved during the progression of carcinomas. EMT has been shown to be involved in tumor-initiating properties, increased motility and migration for dissemination and resistance to chemotherapy (Lambert et al., 2017; Mani et al., 2008; Morel et al., 2008; Shibue and Weinberg, 2017; Singh and Settleman, 2010).

EMT is controlled by a network of EMT inducing transcription factors (EMT-TFs). These include zinc finger E box binding homeobox factors such as ZEB1, ZEB2, Snail and slug. Basic helix loop helix factors including Twist1 and Twist2 are also important regulators (Dongre and Weinberg, 2019). These EMT-TFs can regulate each other, and different combinations can induce the hundreds of transcriptional changes which regulate the changes in cell behavior seen in EMT. Snail and Zeb1 can both repress E-cadherin expression, and Zeb1 can additionally induce mesenchymal genes such as vimentin and N-cadherin (Batlle et al., 2000; Cano et al., 2000; Herranz et al., 2008; Sanchez-Tillo et al., 2010). Snail and Zeb1 can also regulate polarity through repressing crumbs and other polarity complex proteins (Aigner et al., 2007; Spaderna et al., 2008). Other EMT-TFs like Twist1 can activate secondary EMT-TFs like Snail for example.

Activation of these EMT-TFs can be induced by a wide-range of cues including soluble factors and changes within the composition of the microenvironment. During development EMT is activated primarily by signaling pathways mediated by the extracellular factors TGFβ, WNT and NOTCH. TGFβ signaling mediates the phosphorylation and

activation of SMAD complexes which act as transcription factors to induce a whole host of cell response including EMT. SMAD complexes can activate mesenchymal genes themselves, and can also activate EMT-TFs like SNAIL, SLUG, ZEB1 and Twist1 which coordinate to induce EMT both during development and in pathological contexts (Dhasarathy et al., 2011; Nawshad et al., 2004; Ramachandran et al., 2018). WNT and NOTCH signaling is very critical during development, but can also play a role in pathological EMT (Arwert et al., 2012; Savagner, 2001). Growth factors such as EGF, FGF and PDGF have also been shown to induce EMT (Grotegut et al., 2006; Shirakihara et al., 2011; Tashiro et al., 2016).

During cancer progression, there are a lot of changes to the microenvironment that can produce some of these factors that generate an EMT response. Changes that occur as tumors develop can induce EMT such as hypoxia and oxidative stress (Krishnamachary et al., 2006; Radisky et al., 2005). Cellular changes within the microenvironment can also affect the EMT status of cancer cells. Cancer associated fibroblasts (CAFS) remodel the extracellular matrix around a tumor, which can itself stimulate an EMT response (Wei et al., 2015). These CAFS can also secrete cytokines and growth factors, such as TGFβ and others listed above, which can induce EMT in the associated cancer cells (Soon et al., 2013; Yu et al., 2014). Additionally, there is evidence to suggest that components of the immune microenvironment, such as T cells and macrophages, can stimulate EMT within cancers (Bonde et al., 2012; Goebel et al., 2015; Sangaletti et al., 2016; Toh et al., 2011). There is debate about the true role of EMT in cancer progression in patients, and whether it is truly necessary or whether other cell migration mechanisms such as collective cell migration are more dominant. However, the data in the field clearly indicate that EMT can play a critical role and is sufficient to drive metastasis regardless of whether it is the only mechanism by which cancer cells metastasize.

1.4 The EMT transcription factor Twist1

Twist1, though acting indirectly, is potent inducer of EMT (Casas et al., 2011; Yang et al., 2004). Twist1 was originally identified as an essential regulator of embryogenesis in Drosophila (Thisse et al., 1987). Twist1 is also implicated in tumor development and dissemination in a variety of ways. NFκB can induce Twist1 expression during development and in certain cancers. Twist1 expression can be induced pathologically by a variety of upstream signaling including Stat3, which can activate Twist1 transcriptional activity in breast cancer cells (Cheng et al., 2008). TGFβ, in addition to all the other EMT-inducing effects already described, can induce Twist1 expression – primarily indirectly through upregulation of HMGA2 or Fibulin 5 which directly affect Twist1 transcription (Lee et al., 2008; Thuault et al., 2006). Twist1 is also subject to a number of post-translational modifications which affect its stability, and in turn its ability to induce EMT. Phosphorylation of Twist1 on serine 68, for example, prevents ubiquitin-mediated degradation. This phosphorylation event is another eventual downstream effect of TGFβ through MAPK pathway activation (Hong et al., 2011). There are several other serine phosphorylation events that regulate Twist1 stability, some mediated by casein kinase 2 which increase stability, and some mediated by AKT which are required for Twist1 ubiquitination and degradation (Li et al., 2016; Su et al., 2011). Twist1 has also been shown to be phosphorylated on tyrosine 107, which is critical for regulating Twist1 nuclear translocation (Wei et al., 2015).

Twist1 is a potent EMT promoter, and its most critical role in tumor progression is through EMT activation and the subsequent facilitation of tumor invasion and metastasis (Yang et al., 2004). Twist1 induces EMT primarily through the upregulation of N-cadherin and downregulation of E-cadherin. Twist1 can bind directly to elements in the first intron of the N-cadherin gene and induce transcriptional upregulation. Twist1 can also bind to the E-

cadherin promoter to downregulate promoter activity and repress E-cadherin expression (Alexander et al., 2006; Vesuna et al., 2008). In addition to inducing EMT, Twist1 is involved in a number of other tumor promoting signaling. Twist1 can promote invadopodia formation – leading to more local invasion. Twist1 is also involved in tumor initiation through aiding in the evasion of p53 mediated cell senescence. Some properties of cancer cell stemness can also be induced by Twist1 (Ansieau et al., 2008; Beck et al., 2015; Eckert et al., 2011). These studies show that Twist1 is not only an important regulator of EMT in developmental and disease settings, but is also a critical regulator of tumor progression and metastasis on many levels.

1.5 Mechanotransduction mechanisms

Recent studies have begun to reveal how mechanical forces are interpreted by cells to generate cellular responses. At the most basic level, a mechanotransduction pathway starts with the sensing of mechanical stimuli through force-induced conformation change of mechanically sensitive proteins, which leads to activation of downstream biochemical signaling pathways, effectively transforming a mechanical cue into a biochemical signal. While a few of these mechanically sensitive molecules have been discovered, a large number of them are likely still to be identified. Based on currently known mechanical sensors, these conformation changes usually occur in three modes: force-induced opening of ion channels, force-induced 'unfolding' of proteins exposing cryptic binding sites for other proteins, and force-induced alteration in enzymatic activity (Sawada et al., 2006; Wang et al., 2005).

The first cases of mechanosensitive ion channels were discovered in bacteria, such as the MscL and MsCs channels that open in response to membrane stretch in E. coli (Martinac et al., 1987; Sotomayor and Schulten, 2004; Sukharev et al., 1994). These

mechanically sensitive channels are also prevalent in sensory cells, such as the hair cells discussed above. The mechanosensory mechanisms in non-sensory cell types have proven to be more complicated and involve a wider variety of protein structures. The focal adhesion complex, serving many roles in the adhesion and migration of cells, has also been shown to be a major mechanosensing structure. Its key components, integrins, are transmembrane proteins that bind to various ECM proteins to sense mechanical properties of the matrix and also associates with a number of intracellular proteins (Jaalouk and Lammerding, 2009). Among them, talin and vinculin both bind actin, serving as a link between integrins and the actomyosin cytoskeleton network (Humphrey et al., 2014). Actin, non-muscle myosin, and various other associated proteins, which make up the actomyosin network in a cell, transmit mechanical loads within the cell (Jaalouk and Lammerding, 2009). Importantly, some of these adaptor proteins act as mechanosensory molecules through conformational changes. Talin, for example, exhibits force-induced unfolding and exposure of cryptic binding sites (Elosegui-Artola et al., 2016). This unfolding occurs in response to a threshold of force, usually in the form of matrix rigidity, needed to induce subsequent activation of downstream signaling pathways leading to focal adhesion reinforcement.

In addition to the adaptor proteins, there are a plethora of focal adhesion proteins that are recruited to this complex upon formation, and this review does not intend to address each of them in detail. One essential protein that will become important later, when discussing mechanosensing targets in cancer, is focal adhesion kinase (FAK). This kinase is recruited to focal adhesions after integrin engagement, and upon auto-phosphorylation, creates an SH2 docking site for Src kinase, which then recruits other adhesion proteins such as p130Cas, and paxillin (Hanks et al., 2003; Nojima et al., 1995; Panetti, 2002; Sawada and Sheetz, 2002; Sawada et al., 2006). This in turn, leads to activation of GEFs for Rho and subsequent activation of Rho effectors such as the ROCK kinase, thus increasing myosin

activity and actomyosin (Carisey et al., 2013; Lessey et al., 2012; Pasapera et al., 2010). Notably, p130Cas has also been shown to undergo force-induced conformation change exposing tyrosine phosphorylation sites (del Rio et al., 2009; Hotta et al., 2014; Margadant et al., 2011; Moore et al., 2010; Sawada et al., 2006; Wang et al., 2011), and Src itself has been shown to undergo force-induced kinase activation (Arias-Salgado et al., 2003; Sawada et al., 2006; Wang et al., 2005).

Although these, and several other effectors, have been shown to change conformation, or become activated in response to force, it is important to note that mechanical sensing differs significantly among various cell types. Most published studies on focal adhesions and matrix rigidity were conducted in fibroblasts, which respond to a certain rigidity level. This 'threshold' for sensing rigidity seems to be a common theme among different cell types, whether through focal adhesions or not, however the threshold force differs depending on the cell type. Fibroblasts and endothelial cells appear to activate their cytoskeleton and increase spreading at about 3000 pascals (Pa, unit of force measurement), while pre-osteocytes do not respond until about 60,000Pa. Neutrophils on the other hand, respond to rigidities as low as 2Pa (Kong et al., 2005; Yeung et al., 2005) and mammary epithelial cells start to change morphology around 300-500Pa (Paszek et al., 2005). This indicates that there are a variety of different 'threshold sensing' mechanisms, the majority of which have yet to be identified.

One additional mechanosensing mechanism that is largely in its infancy involves the force-induced nuclear organization. What is known is that this involves connecting the cytoskeleton to the nucleus through Nesprin molecules. Nesprins bind to both cytoskeleton and nuclear membrane proteins, which then interact with nuclear envelope proteins such as lamins to form stable structures with DNA. This link between the cytoskeleton and the

nucleus transmits mechanical cues to changes in chromatin structure or movement of DNA and chromatins (Haque et al., 2006).

1.6 Mechanotransduction in cancer

In addition to regulating normal physiology, mechanotransduction is recognized to play important roles in tumor progression. The extracellular environment of tumors, or tumor stroma, constantly changes as tumors progress. The ECM around tumors is found to stiffen progressively in a variety of human cancer types. Perhaps the best example is in breast cancer, which is often first detected by direct palpitation due to its increased stiffness compared to surrounding tissues. The presence of a fibrotic focus, which is an accumulation of collagen and fibroblasts, is correlated with an increase in metastatic disease and a decrease in recurrence-free survival in patients (Boyd et al., 2002; Colpaert et al., 2001; Hasebe et al., 2002; Mujtaba et al., 2013). In 3D cultures mimicking stiffness changes during breast cancer progression, altering ECM rigidity could induce an invasive, malignant phenotype in mammary epithelial cells (Levental et al., 2009; Paszek et al., 2005). While cells normally exist in their physiological environment with certain rigidities, pressure, and strain, alterations in this environment can aberrantly activate certain mechanotransduction pathways, leading to a variety of tumorigenic processes such as sustained proliferation, resistance to cell death and EMT, invasion and metastasis.

One of the hallmarks of cancer is sustained proliferation. Mechanical cues are shown to increase cell proliferation, and when aberrantly activated by a deregulated extracellular environment, can facilitate cancer development. Mechanistically, increasing stiffness can promote growth factor signaling and lead to enhanced proliferation. In a glioblastoma model, increased ECM stiffness enhanced activation and expression of EGFR and its downstream mitogenic factors such as PI3K and Akt (Umesh et al., 2014). Increases in rigidity could also

promote transition through G1/S phases of the cell cycle. In mouse embryonic fibroblasts, an increase in FAK activation and p130Cas signaling led to activation of ERK and PI3k signaling and subsequently Rac, which induced Cyclin D1to increase cell proliferation (Bae et al., 2014; Chambard et al., 2007; Provenzano et al., 2008; Provenzano and Keely, 2011; Pylayeva et al., 2009). Matrix stiffening is also shown to induce the expression of microRNAs, such as miR18a to inhibit the expression of tumor suppressor PTEN. This increases PI3k/AKT activities and cell growth and survival (Mouw et al., 2014). Transcription co-activators YAP/TAZ, which promote cell growth and inhibit apoptosis, are also sensitive to mechanical cues from the ECM. Increasing matrix stiffness promotes YAP/TAZ nuclear localization to drive proliferation gene expression and overcome growth suppression (Dupont et al., 2011).

In addition to promoting cell proliferation, alterations in ECM mechanics can also enhance tumor growth through promoting resistance to cell death. TGFβ is known to induce both apoptosis and EMT, depending on biological contexts. When cells are cultured on a soft, compliant ECM, TGFβ induces apoptosis. However, on stiff ECM, TGFβ switches its role to promote EMT, leading to a decrease in cell death and an increase in invasiveness (Leight et al., 2012). Increased rigidity can also promote integrin-mediated cell survival (Paszek et al., 2014). Adhesion to ECM inactivates pro-apoptotic molecule Bax, and induces anti-apoptotic gene Bcl2 (Frisch et al., 1996; Gilmore et al., 2000; Ruoslahti and Reed, 1994). ECM stiffening can also promote anchorage-independent cell survival through Rac and resistance to anoikis (cell death resulting from lack of attachment) through FAK signaling (Zahir et al., 2003; Zhang et al., 2004).

As mentioned earlier, through various mechanisms, increased rigidity appears to induce a malignant phenotype (Leight et al., 2012; Paszek et al., 2005). Several recent

studies show that increases in rigidity can promote invasion and metastasis – by enhancing the activity of MMPs to aid in degradation and invasion through ECM (Haage and Schneider, 2014), via promoting invadopodia formation for ECM degradation (Parekh et al., 2011), and by inducing EMT. Two mechanistic studies directly linked increasing matrix stiffness to two major EMT-inducing transcription factors. Our study found that increasing matrix rigidities could induce EMT and tumor invasion and metastasis in mammary tumor cells by activating the EMT-inducing transcription factor Twist1. Increasing rigidities led to nuclear translocation of Twist1 by releasing Twist1 from its cytoplasmic anchor proteins G3BP2 (Wei et al., 2015). A recent study found that increasing matrix stiffness could promote protein stability of another EMT transcription factor Snail1 through enhanced collagen/DDR2 binding. Activation of DDR2 stimulated ERK activity via Src, and ERK subsequently phosphorylates Snail1, leading to enhanced nuclear accumulation and decreased ubiquitination (Zhang et al., 2013). Together, these studies provide direct links between matrix stiffening and the molecular machinery of tumor invasion and metastasis.

Interestingly, ovarian cancer cells were shown to present opposite responses to matrix rigidities– these cancer cells preferentially adhere to softer ECM and present an enhanced invasive phenotype when cultured on softer substrates through RHO/ROCK signaling pathways (McGrail et al., 2014). This supports the notion that multiple mechanosensing mechanisms exist among different cell types possibly due to the different mechanical environments of their tissues of origin. The same alterations in ECM could yield vast different biological responses, thus targeting mechanotransduction pathways needs to be tailored for specific cancer types.

Currently, our understanding on how mechanical signals are sensed and transmitted to generate specific cellular responses is still rudimentary. In most cases, the

mechanotransduction pathway linking the mechanical sensors all the way to the transcriptional or translational responses in response to specific mechanical cues are far from completely defined. As described above, more and more cellular studies are carried out under physiological stiffness of the tissue of origin, instead of simply on plastic dishes with extremely high rigidities out of physiological ranges. Future studies incorporating physical properties of matrix into experimental conditions could uncover many hidden mechanical regulatory mechanisms for therapeutic targeting.

1.7 Tyk2 structure and function

Tyrosine kinase 2 (Tyk2) is a member of the Januse kinase (JAK) family of proteins. There are four proteins in this family: Jaks 1-3 and Tyk2. These kinases are structurally similar – they all have an N-terminal FERM domain followed by an SH2 domain. The SH2 domain of these proteins are missing key residues for p-Tyr binding, which is the normal function of SH2 domains. The FERM and SH2 domains are, however, both critical for Jak protein binding to transmembrane receptor proteins. These kinases also contain a kinase-like domain (KLD) followed by a functional kinase domain at the C-terminus. Tyk2 was cloned in 1990 (Firmbach-Kraft et al., 1990), and was shortly thereafter determined to be important for interferon signaling (Velazquez et al., 1992). Tyk2, like Jak1 and Jak2, is ubiquitously expressed, but has been largely studied in the hematopoietic system as it plays an important role in relaying cytokine signaling. Tyk2 binds to a variety of different cytokine and growth factor receptors including interferon α receptor 1, interleukin 10 receptor 2, interleukin 12 receptor β1, interleukin 13 receptor α 1 and gp130 (Strobl et al., 2011). Tyk2 has been implicated in binding to a large number of additional receptors, however the data to support those interactions in less robust and has not been as well investigated as the more canonical Tyk2 signaling that involves the receptors listed above. Tyk2 is activated upon ligand binding

– that is the binding of various interferons that interact with the receptors listed above. The specific molecular events that occur to induce activation of his pathway are still contested. One hypothesis is that ligand binding induces dimerization of cytokine receptors, and it is this dimerization that brings the associated Jak kinases in close enough proximity to trans-phosphorylate (Saka et al., 2012). There is also evidence that these cytokines may exist as pre-formed dimers, and that binding of ligands to these receptors induces a conformational change which mediates the transphosphorylation of associated Jaks (Constantinescu et al., 2001; Lupardus et al., 2011; Malka et al., 2008). Regardless, once the cognate Jak kinases are phosphorylated, they become hyperactive and instigate downstream signaling. The pair of Jak proteins involved are dependent on the receptors involved, but are not generally the same Jak protein. Tyk2, for example, is generally paired with Jak1 or Jak2 because these are the Jaks that associate with the other cytokine receptor chain.

Activated Jak proteins then phosphorylate receptor chains, which create docking sites for SH2 domain containing proteins that are recruited and phosphorylated. These SH2 domain containing proteins are called STAT proteins. There are 7 proteins in the Stat family, and which Stat proteins are recruited is controlled primarily by the receptor and Jak proteins involved, although the specificity mechanisms are not well understood. Tyk2 has been shown to be able to activate all STAT proteins. Multiple STAT proteins may be activated by one stimulus, however only some tend to be important for mediating cell phenotypic changes. The STAT proteins activated are determined by type of cytokine stimulation and the cell type. Stat proteins are transcription factors, and once phosphorylated and activated these proteins dimerize in a phosophotyrosine/SH2 dependent manner and enter the nucleus to induce the expression of a host of interferon response genes. These gene signatures are too expansive and too dependent on context to list out here but include genes important for modulating

immune system function and regulating activation of Jak-Stat pathway itself as a form of negative feedback (Becker et al., 1998; Darnell, 1997).

There are approximately 60 different cytokines that bind to type 1 and type 2 cytokine receptors, which include the majority or receptors which Tyk2 is found to associate with. These cytokines are important for initiating a whole host of different cellular response. Cytokine signaling can regulate the immune system, both innate and adaptive, hematopoiesis, development and metabolism (O'Shea and Plenge, 2012). Tyk2 specifically is integral for relaying IFNα and IFNβ signaling – primarily because it is required to maintain IFNAR1 surface expression in human cells. The kinase activity of Tyk2 is only required to relay some IFNα and IFNβ signaling – for example IFNβ induced Stat3 phosphorylation required Tyk2 kinase activity, and does IFNα induced Stat5 phosphorylation (Fish et al., 1999; Rani et al., 1999). On the other hand, Tyk2 kinase activity is not required for IFNβ induced Stat1 or Stat2 phosphorylation. Additionally, there are many cytokine signals, such as those that stimulate gp130 and OSM, for example, which induce the phosphorylation of Tyk2, but for which Tyk2 is not required for signal transduction (Strobl et al., 2011). Tyk2 deficient mice are viable and fertile, unlike Jak1 or Jak2 deficient mice, which are both lethal either embryonically (Jak2) or perinatally (Jak1) due to serious developmental defects (Neubauer et al., 1998; Rodig et al., 1998). Tyk2 deficient mice do, however, show serious immunological deficiencies. They are prone to infectious diseases – viral, bacterial and protozoan pathogens. The predominant causative effect of increased infection in Tyk2 deficient mice is an impaired or delayed IFN gamma production upon infection. Tyk2 deficient mice are also more prone to lymphoid tumors due to reduced tumor surveillance from NK and NKT cells (Strobl et al., 2011).

1.8 Tyk2 regulation

Tyk2 is activated during cytokine binding through trans-phosphorylation by the Jak kinase associated with the other part of the receptor dimer. This activation is mediated through phosphorylation of two tyrosines within the activation loop of the kinase domain: tyrosines 1054 and 1055. Phosphorylation at these two residues has been shown to be massively upregulated upon interferon stimulation and is critical for activation of downstream STAT proteins. Phosphorylation of the activation loop is not, however, required for basal activity of these kinases, only ligand induced activation (Gauzzi et al., 1996). Jak kinases are not only regulated by phosphorylation, but also by domains within themselves. The KLD in Tyk2 is a negative regulator of kinase activation. The mechanism by which this occurs is not fully understood, but what the field has figured out is that the KLD domain binds to the hinge-side of the kinase domain in a front-to-back orientation and inhibits the activation, similar to the way Src family kinases auto-inhibit (Bradshaw, 2010; Lupardus et al., 2014b). This method of auto-inhibition appears to be critical – deleting the KLD causes increased basal Jak activity (Saharinen and Silvennoinen, 2002; Saharinen et al., 2000; Saharinen et al., 2003). Additionally, adding the KLD to the isolated kinase domain in cells or recombinant constructs is sufficient to decrease activity of the kinase domain (Lupardus et al., 2014b; Saharinen et al., 2000; Sanz et al., 2011). The method by which the KLD inhibits the kinase activity in cells is not fully understood. However – there are two competing theories. There is evidence to suggest this happens in cis – that is the KLD domain of Tyk2 inhibits its own kinase domain. The crystal structure that determined how the domains interact was of a peptide of the KLD and the kinase domain interacting in cis (Min et al., 2015). However, there also evidence that this interaction may occur in trans – that is when two Jak proteins are brought into close proximity that the KLD domain of one binds and inhibits the kinase domain of the other – and vice versa. When the KLD domain and kinase domain were expressed separately, they could be immunoprecipitated – indicating that they were binding in trans

(Brooks et al., 2014). It is possible that both of these regulatory interactions are occurring, but what is clear is that when the KLD domain is interacting with the kinase domain, the protein cannot be activated. The KLD domain appears to be locked into this inactivating conformation primarily when it is bound to ATP or an ATP- competitive inhibitor. This phenomenon has been utilized a lot recently to design more specific inhibitors to these Jak kinase family members – in particular Tyk2 (Min et al., 2015). Targeting this KLD domain allows for more specificity between the JAK kinases due to more sequence dissimilarities in the KLD domain compared to the ATP binding pocket of the actual kinase domain.

Tyk2 activation is also regulated by phosphatases, ubiquitin ligases and proteins that simply bind to the activation loop of the protein. Most of these proteins are actually upregulated by STAT proteins upon cytokine stimulation and serve as a negative feedback loop for this pathway. Tyk2 has been shown to be de-phosphorylated by SHP-1, PTP1B and CD45 (Irie-Sasaki et al., 2001; Myers et al., 2001; Yetter et al., 1995). SIAH2 is an E3 ubiquitin ligase which has been shown to mediate the degradation of Tyk2 (Muller et al., 2014). Another well characterized negative regulator of Jak-Stat signaling are the SOCS family of proteins. There are seven SOCS family members, and this group is responsible for binding to and recruiting ubiquitin ligases to mediate the degradation of many members of the Jak-STAT pathway including Jaks, STAT proteins and cytokine receptors (Yoshimura et al., 2007). Tyk2 specifically has not been shown to be ubiquitinated upon SOCS binding, unlike other Jak kinases, but SOCS proteins can still inhibit Tyk2. SOCS proteins have a kinase inhibitory region which binds to the edge of the substrate binding groove in the kinase domain of Tyk2, and this inhibits activity. SOCS1 and SOCS3 have both been shown to bind and inhibit Tyk2 activity (Babon et al., 2012). Intriguingly, there is also evidence that suggests SOCS1 binding also de-stabilizes Tyk2 expression by inhibiting a stabilizing lysine-63 polyubiquitination (Piganis et al., 2011).

1.9 Tyk2 and disease

Tyk2, though it was identified about 30 years ago, has only recently been studied in reference to cancer – particularly in comparison to the large amount of research on the other Jak kinases in cancer. Tyk2 has been primarily studied in autoimmune and auto-inflammatory diseases and has only recently been studied with respect to cancer (O'Shea et al., 2013; Schwartz et al., 2017; Woss et al., 2019). Tyk2 deficiency due to frameshift mutations has been found in patients with primary immunodeficiency and hyper IgE syndrome, although only 2 patients and 1 patient, respectively – indicating that this is fairly rare (Kilic et al., 2012; Minegishi et al., 2006). Various polymorphisms in Tyk2 have been linked to autoimmune diseases (Chu et al., 2011; Franke et al., 2010). Targeting Tyk2 specifically in autoimmune disorders has been the main focus for Tyk2 therapies, purely because Tyk2 does appear to be more causative in a lot of them and because targeting Tyk2 and not the other Jak kinases will lead to far fewer side effects since they are more widespread in their function.

Tyk2 alterations with respect to cancer have only just recently begun to be discovered. It was initially seen that Tyk2 deficient mice were much more susceptible to lymphoid tumor development due to deficiencies in tumor surveillance by NK cells (Prchal-Murphy et al., 2015; Ubel et al., 2013). This appeared to be independent of the effect of Tyk2 loss in the tumor cells themselves. Work regarding the role of Tyk2 within tumors has expanded in the past couple of years, but has been contradictory. Within cancers of the hematopoietic system, it seems clear that Tyk2 activation is oncogenic. Several oncogenic Tyk2 fusion proteins have been identified in leukemia and lymphoma (Crescenzo et al., 2015; Leitner et al., 2017; Velusamy et al., 2014), and the first and only GOF mutations in Tyk2 were identified in T-ALL patients (Sanda et al., 2013). The results in solid tumors have

been much more unclear, however. In support of Tyk2 being oncogenic – several labs have found Tyk2 to be overexpressed in prostate, ovarian, cervical and breast cancer cells (Christy and Priyadharshini, 2018; Ide et al., 2008; Santos et al., 2015; Silver et al., 2004; Song et al., 2008; Woss et al., 2019; Zhu et al., 2009). Some labs additionally found that Tyk2 increased invasiveness in prostate cancer (Ide et al., 2008). On the other hand, there is a general trend that decreased Tyk2 expression appears to be favorable in cancer patients (Leitner et al., 2017; Sang et al., 2012; Uhlen et al., 2010). This has been supported by several unbiased screening experiments. One group found in a meta-analysis of Jak-STAT pathway expression in hepatocellular carcinoma patients that normal or higher Tyk2 levels actually correlated with longer survival (Wang et al., 2019b; Woss et al., 2019). Another group found several splice mutants that render Tyk2 inactive in a screen for protein tyrosine kinase variants in cancer cell lines (Ruhe et al., 2007). More specifically, a missense LOF Tyk2 mutation was identified in breast and GI cancers as well as AML, interestingly. This mutation is within the substrate binding groove of the kinase domain and renders it functionally less active upon IFNα stimulation (Kaminker et al., 2007; Tomasson et al., 2008). In breast cancer, one group identified Tyk2 from a proteomics screen as having less expression in breast cancer samples as opposed to normal breast tissue. This group went on to show that expression level dropped in more in tumor samples of a higher grade, seeming to correlate with samples that began to spread to regional lymph nodes (Sang et al., 2012).

These data really support the fact that Tyk2 loss of function seems to play a role in tumor progression, and that role is almost completely been unexplored. This is probably due to the fact that the other Jak kinases, Jak2 in particular, play a clearly oncogenic role in all types of cancer. Downstream effectors of the Jak-STAT pathway also have been shown to be largely oncogenic. Stat3 in particular is upregulated in cancer, plays a role in a whole host of tumor promoting signaling, and has even been shown to be able to induce EMT (Gong et

al., 2018; Jin, 2020; Sullivan et al., 2009; Xiong et al., 2012; Yadav et al., 2011). Importantly, the tumor promoting role of STAT3 is primarily mediated by Jak2, or through alternative signaling that activates the protein outside of the Jak-Stat pathway. There is very limited data indicating that Tyk2 is involved in this activation. Regardless, the Jak-Stat pathway is so highly implicated in cancer that is has been termed one of the 12 core cancer pathways (Vogelstein et al., 2013). Given this bias towards defining this pathway as oncogenic, it's unsurprising that the clear tumor suppressive role Tyk2 is having in some cancers has been overlooked and even ignored in favor of the limited data suggesting Tyk2 is oncogenic in solid tumors.

1.10 Acknowledgements

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Chapter 2

Tyk2 regulates Twist1 translocation in response to rigidity

2.1 Introduction

The role of matrix stiffness in cancer development can perhaps be best understood when referencing breast cancer. Breast cancers are frequently identified through manual palpitation since the tumors feel more rigid than the normal tissue. As breast tumors develop, there are many changes in tumor microenvironment, and in particular the mechanical microenvironment as seen by an increase in the rigidity of the extracellular matrix (ECM). This increase in ECM rigidity is due largely to increased deposition, organization and crosslinking of ECM proteins, in particular collagen (Provenzano et al., 2006; Provenzano et al., 2008). This increase in rigidity is not just a consequence of tumor development, but has been correlated with tumor progression and poor recurrence free survival in patients (Levental et al., 2009; Paszek et al., 2005; Wei et al., 2015). The rigidity of the ECM or of any tissue is defined in Pascals (Pa), which is a measurement of the elastic modulus of a material. The elastic modulus describes how much a material deforms when a force is applied to it. The rigidity of the different tissues throughout the body varies significantly. Soft tissues like the brain or breast tissue are between 100 and 1000Pa, stiffer tissue such as muscle is around 30-50kPa (Butcher et al., 2009; Paszek et al., 2005). To understand how change in matrix stiffness can affect breast cell phenotype and breast cancer development, a different type of culture system had to be utilized as plastic and glass are all significantly stiffer than the normal breast tissue environment. We utilized a collagen coated polyacrylamide hydrogel system wherein we can alter the rigidity of the hydrogel by varying the concentration of the cross-linker bis-acrylamide (Engler et al., 2004; Johnson et al., 2007; Paszek, 2005). We then seeded mammary epithelial cells in a three dimensional (3D)

environment using reconstituted basement membrane (rBM) also known commercially as Matrigel (Bissell et al., 2002; Debnath et al., 2003; Lee et al., 2007; Orkin et al., 1977). It has been previously demonstrated that mammary epithelial cells form a structure called acini when seeded in 3D on polyacrylamide hydrogels that are approximately the rigidity of the normal breast environment (Levental et al., 2009; Paszek et al., 2005). When these same mammary epithelial cells were seeded on hydrogels that mimicked the rigidity of the average tumor (~5000Pa) they exhibited a very different morphology. The cells lost apical-basal polarity, they formed weaker junctions and invaded through basement membrane at higher rigidity (Levental et al., 2009; Paszek et al., 2005). This phenotype change in these cells resembles a developmental program called epithelial-mesenchymal transition (EMT) which is also utilized frequently by cancer cells to invade and metastasize (Thiery et al., 2009; Yang and Weinberg, 2008). Culturing mammary epithelial cells in an environment which is mechanically similar to that which they would experience *in vivo* allows these cells to form functionally normal 3D structures. This provides a great system in which to test how altering the rigidity can affect the formation of these functional ductal structures, while keeping all other aspects of the culture such as ECM ligand density and growth factor concentration the same.

Utilizing this culture system as described, our lab previously described a novel mechano-sensing pathway involving the activation of epithelial mesenchymal transition (EMT) through promoting nuclear translocation of the EMT-inducing transcription factor Twist1. Our lab further characterized some of the molecular changes that mammary epithelial cells undergo when cultured at a higher rigidity as opposed to a lower rigidity. In short, we found that these cells exhibit characteristic EMT molecular changes such as loss of E-cadherin expression (an epithelial junctional protein) and gain of mesenchymal markers such as fibronectin further supporting the idea that increases in rigidity were inducing EMT in

these cells (Wei et al., 2015). Work from our lab primarily utilizes two mammary epithelial cell lines Mcf10a and Eph4Ras. Mcf10a cells are non-transformed, immortalized mammary epithelial cells and Eph4Ras cells are tumorigenic mouse mammary epithelial cells (Reichmann et al., 1989; Soule et al., 1990). Importantly, both cell lines expressed the EMT inducing transcription factor Twist1 (Blick et al., 2008; Eckert et al., 2011). Our lab focused on Twist1 based on published studies showing that Twist mRNA could be mechanically induced during Drosophila development (Desprat et al., 2008). Through stable knockdown of Twist1, our lab determined that Twist1 was required for cells to undergo EMT in response to high rigidities (Wei et al., 2015). We furthermore found that nuclear translocation of Twist1 was regulated by rigidity, while its expression was not. At low rigidities, Twist1 is in the cytoplasm and at high stiffness it translocates into the nucleus where it can induce EMT as a transcription factor (Wei et al., 2015). This translocation is regulated by Twist1 being retained in the cytoplasm at low rigidities through its interaction with a cytoplasmic protein called G3BP2. This binding is regulated through a phosphorylation on Twist1 in its G3BP2 binding domain at tyrosine 107 (Y107). At high rigidities Y107 is phosphorylated, thus preventing Twist1 from binding to G3BP2 and allowing Twist1 import into the nucleus to induce EMT (Wei et al., 2015). This interaction between Twist1 and G3BP2 was also relevant for invasion and metastasis in *in vivo* models. When tumor cells with G3BP2 stably knocked down were injected into the mammary fat pad of immunocompromised mice the resulting tumors were more locally invasive than the control tumors and also metastasized more readily to the lungs (Wei et al., 2015). This indicated that not only is the regulation of Twist1 translocation relevant in *in vitro* settings, but is also relevant for models of human disease. In human patient data, low expression of G3BP2 correlated with poorer survival in breast cancer. This decrease in survival was further stratified when assaying patient samples for both G3BP2 expression and collagen fiber organization – which is an indicator of matrix stiffness. That is,

patients with both low G3BP2 expression and organized collagen (stiffer tissue) had the poorest survival outcomes. Notably, patients with organized collagen (stiff tissue) and normal G3BP2 expression stratified in the middle of this patient data set, with those patients with disorganized collagen and either normal or low G3BP2 expression having the best outcome (Wei et al., 2015). These data indicate that in addition to being relevant for increasing EMT and invasive phenotype *in vitro* and in *in vivo* mouse models, alteration in this mechano-sensing pathway or the mechanical environment appears to be relevant in human disease as well.

Our lab has since set out to understand the upstream regulators of this Twist1-mchanosensing pathway. One such upstream regulator is a protein called tyrosine kinase 2 (Tyk2). Tyk2 is a member of the Jak family of kinases, which is comprised of four kinases that are very structural similar and are canonically known to mediate cytokine signaling through the activation of various Stat transcription factors. The role of Tyk2 in regulating stiffness induced EMT is discussed here in chapter 2, while the regulation of Tyk2 with respect to rigidity, and the role of Tyk2 in human cancer are discussed in chapter 3 and chapter 4, respectively.

2.2 Tyk2 expression is critical for the maintenance of an epithelial phenotype at low rigidities

The next logical step to follow this work published in 2015 was to identify the upstream kinase that phosphorylates Twist1 at high rigidities. Our lab undertook several screening experiments of different tyrosine kinases to identify which kinase might be controlling the activation of this pathway. Based on primary sequence, the Y107 site is predicted to be a potential phosphorylation site by the Jak family of kinases. To screen for their potential effects in regulating this pathway we utilized Jak inhibitors with differential

specificity for members of the Jak kinase family. Treatment with the inhibitor filgotinib, which targets Jak1 and Jak2 at a lower concentration than Tyk2 did not result in any changes in cell phenotype. Treatment with an inhibitor which targets Jak1, Jak2 and Tyk2 at the same concentration induced invasion at lower rigidities, indicating that Tyk2 may be involved in regulating cellular responses to rigidity, and that Jak1 and Jak2 are not involved (Figure 2.1). To further assess the role of these Jak kinases, we stably knocked down two proteins in the Jak kinase family: Jak2 and Tyk2. Jak3 was dismissed because it is not well expressed outside of the hematopoietic system (Haan et al., 2006). The knockdown of Jak2 in Eph4ras cells had no effect on the cells at low or high rigidity (Figure 2.2). However, knockdown of Tyk2 caused a significant increase in the amount of invasion and nuclear translocation of Twist1 at compliant rigidities (150pa) (Figure 2.3 and Figure 2.4). Importantly, this was not the phenotype expected from depleting the cells of the kinase responsible for phosphorylating Twist1. The cells lacking this prospective kinase would, theoretically, be unable to phosphorylate Twist1 at high rigidities, thus increasing G3BP2 binding and preventing Twist1 from entering into the nucleus to promote EMT at high rigidities. Surprisingly, Tyk2 knockdown resulted in an opposite phenotype from what we were expecting, which is similar to the phenotype of G3BP2 knockdown. Together, these data suggest that Tyk2 likely functions as a negative regulator of this Twist1 mechanosensing pathway to prevent EMT when cells are at low matrix rigidities.

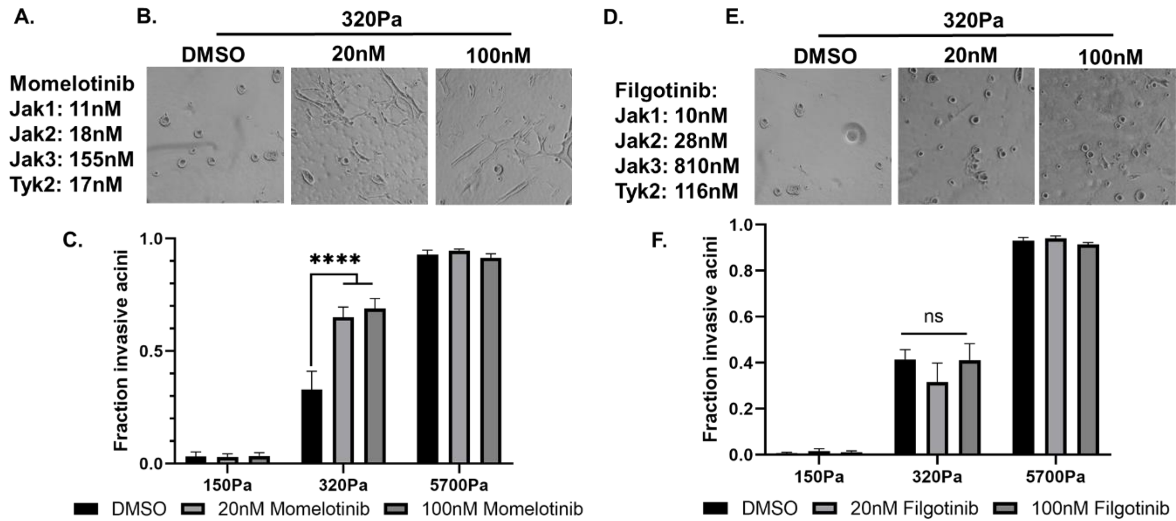
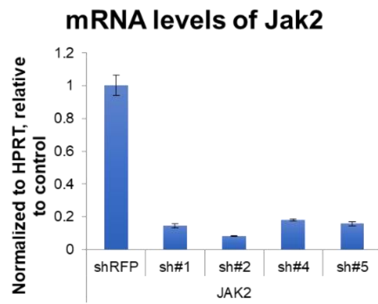


Figure 2.1: Treatment with a Jak1/2 inhibitor does not affect cell phenotype. A. IC50 values for the different Jak kinase family members for Momelotinib. B. Brighfield images of Mcf10a cells at 320Pa treated with DMSO or 20 nM or 100 nM Momelotinib. C. Quantification of the fraction of invasive acini in cells from the experiment in B **** P<0.0001. D. IC50 values for the different Jak kinase family members for Filgotinib. E. Brighfield images of Mcf10a cells at 320Pa treated with DMSO or 20 nM or 100 nM Filgotinib. Quantification of the fraction of invasive acini in cells from the experiment in E. ns: not significant.



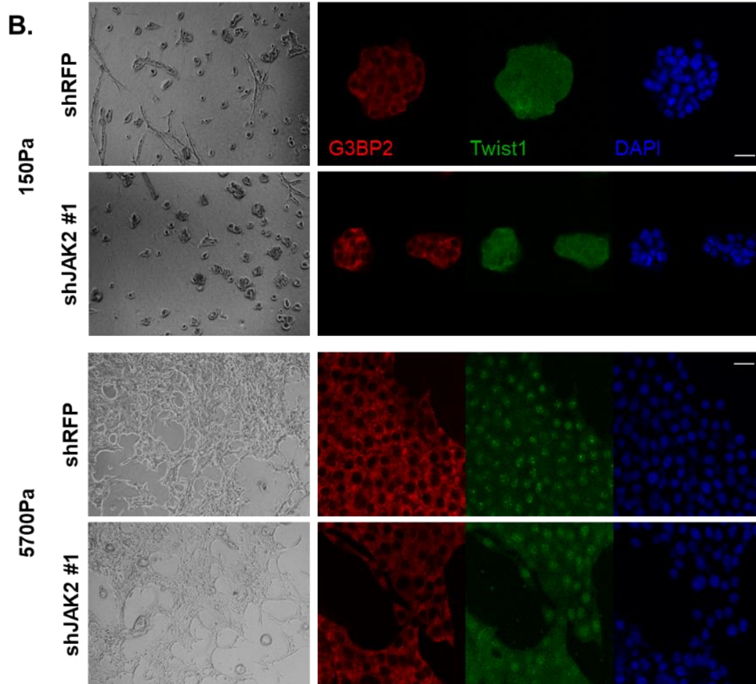


Figure 2.2: Knockdown of Jak2 in Eph4Ras cells has no effect. A. Jak2 knockdown levels measured by qPCR and normalized to HPRT. B. Control and shJak2 #1 grown for 5 days at 150Pa and 5700Pa in 3D culture. Brightfield images showing overall morphology and confocal immunofluorescence images of G3BP2 (red) Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20 μM.

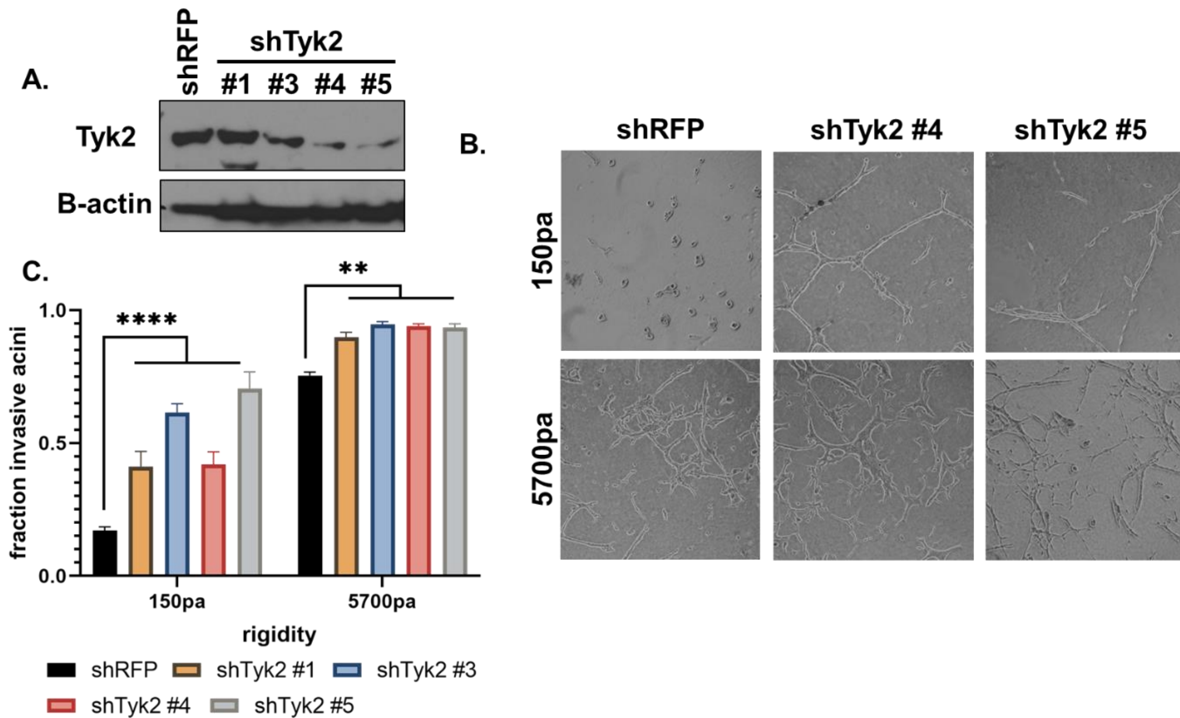


Figure 2.3: Knockdown of Tyk2 induces an invasive phenotype at low rigidities in Eph4ras cells. A. Western blot of lysates from Eph4ras cells with indicated Tyk2 shRNAs showing level of Tyk2 knockdown with beta-actin as a loading control. B. Brightfield images at 150Pa and 5700Pa for two shTyk2 lines compared to control shRFP cells. C. Quantification of brightfield images shown as the fraction of invasive acini. **** P<0.0001 ** P<0.01.

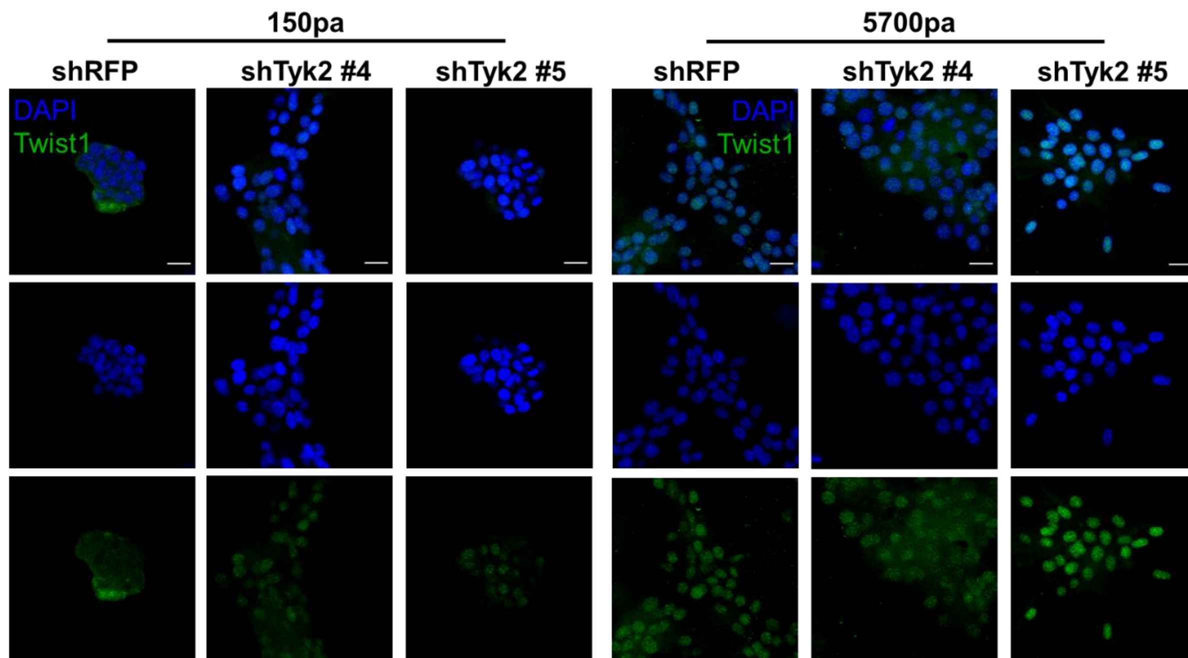


Figure 2.4: Knockdown of Tyk2 induces nuclear translocation of Twist1 at low rigidities in Eph4ras cells. Confocal immunofluorescence images of control shRFP and shTyk2 cell lines at 150Pa and 5700Pa. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20µM.

To further characterize the effect of Tyk2 knockdown, we assayed various cell types that respond to changes in rigidity including the human breast epithelial cell line mentioned previously Mcf10a, and variants of that cell line including the Mcf10DCIS and Mcf10AT. Mcf10a cells are used for the majority of experiments below. Mcf10DCIS cells are utilized later for *in vivo* experiments, and are a Mcf10a variant that forms tumors in mice. Mcf10AT are also a more tumorigenic variant of Mcf10a cells, and are transformed with Ras (Dawson et al., 1996). Stable knockdown of Tyk2 in Mcf10a cells caused an increase in invasive acini and nuclear Twist1 translocation at lower rigidities compared to the control cells, similar to the phenotype of Tyk2 knockdown in Eph4ras cells (Figure 2.5 and Figure 2.6). Knockdown of Tyk2 in Mcf10AT and MCF10DCIS caused nuclear translocation of Twist1 at low rigidities as well (Figure 2.7). It is important to note that the rigidity at which these human cells began

to invade was slightly higher than the rigidity at which the Eph4ras cells began to invade with Tyk2 knockdown (320Pa vs 150Pa). In all human cells tested, the control cells formed acini at a rate of almost 100 percent at 150Pa but presented about 10-20% of invasion at 320Pa. (Eph4ras, however, were about 20% invasive at 150pa, indicating that they are basally slightly more invasive at a lower rigidity (Figure 2.3 and Figure 2.5). In Eph4ras cells, Tyk2 knockdown caused approximately a 20-40% increase in invasion at 150pa compared to the control cells In Mcf10a cells Tyk2 knockdown caused a similar percentage increase of invasion at 320pa where the control cells had a baseline low level of invasion. Additionally, knockdown of Tyk2 caused an induction of EMT as shown by the gain of the mesenchymal marker fibronectin and a reduction in the epithelial marker E-cadherin (Figure 2.8). This indicates that Tyk2 is required at lower rigidities to maintain a noninvasive epithelial state by maintaining Twist1 cytoplasmic localization.

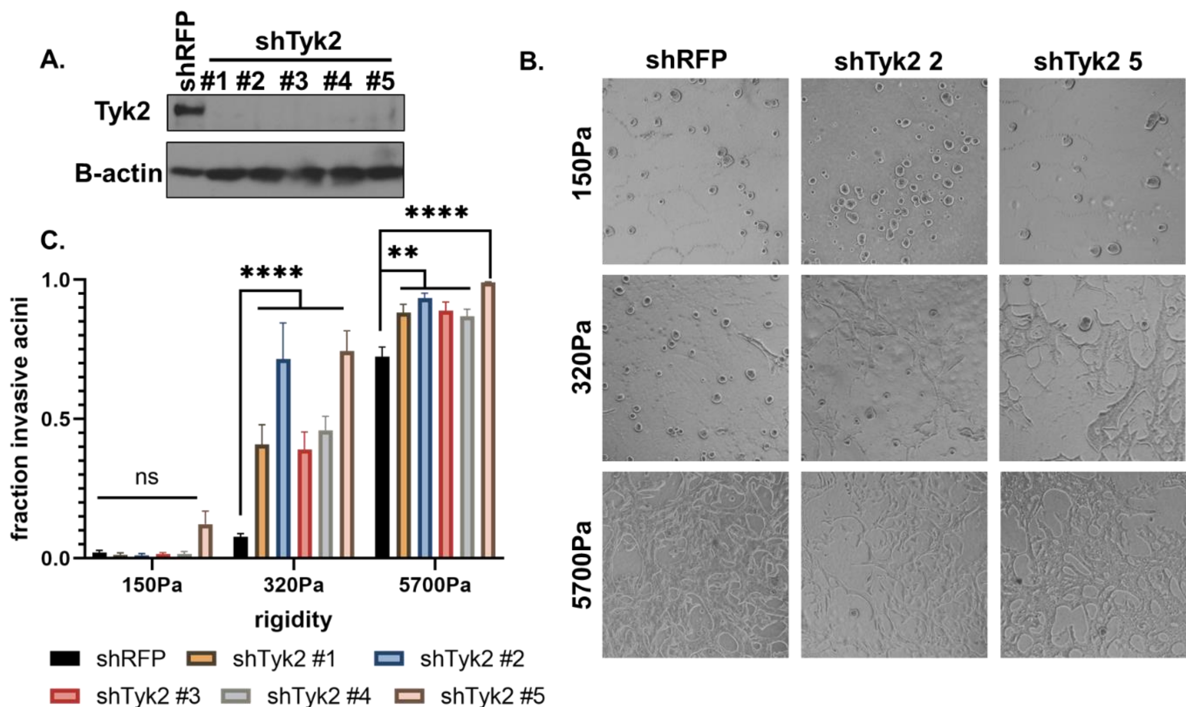


Figure 2.5: Knockdown of Tyk2 induces an invasive phenotype at low rigidities in Mcf10a cells. A. Western blot of lysates from Mcf10a cells with indicated Tyk2 shRNAs showing level of Tyk2 knockdown with beta-actin as a loading control. B. Brightfield images at 150Pa, 320Pa and 5700Pa indicating phenotype changes in two shTyk2 lines compared to control shRFP cells. C. Quantification of brightfield images shown as the fraction of invasive acini. **** P<0.0001 ** P<0.01 ns: not significant.

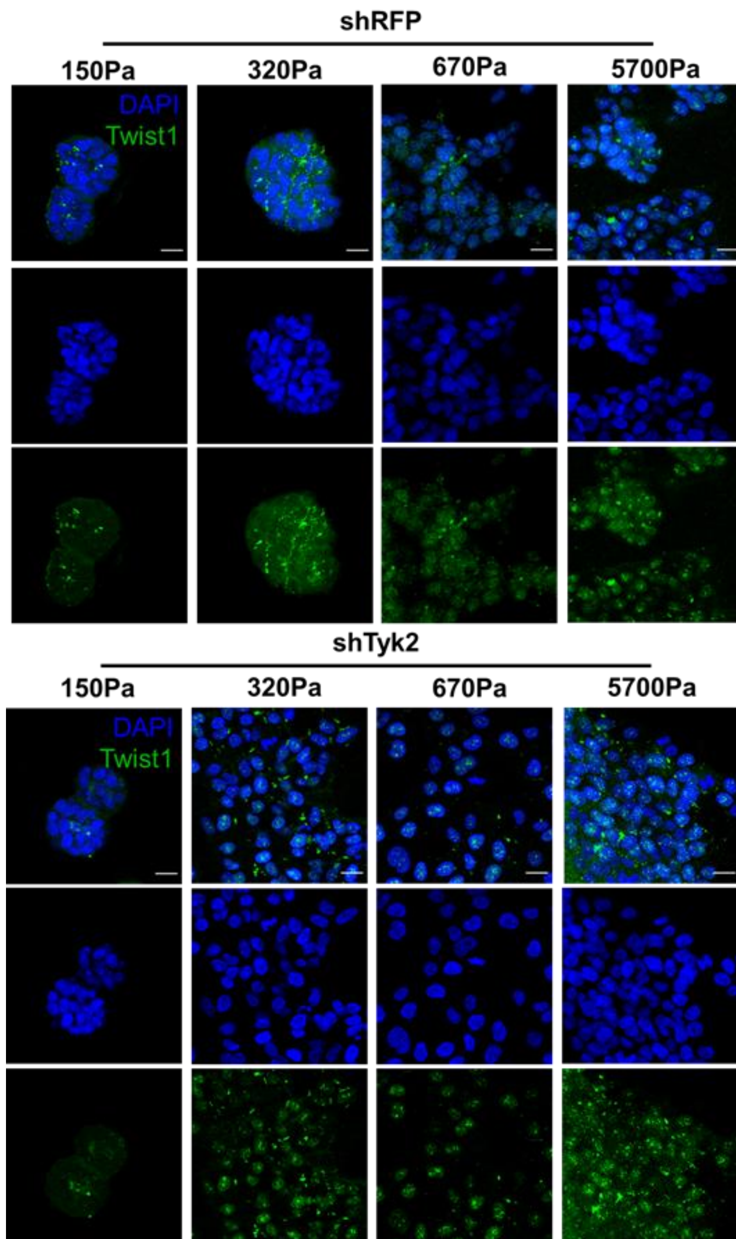


Figure 2.6: Knockdown of Tyk2 induces nuclear translocation of Twist1 at low rigidities in Mcf10a cells. Confocal immunofluorescence images of control shRFP and shTyk2 cell lines at 150Pa 320Pa, 670Pa and 5700Pa. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20 μM.

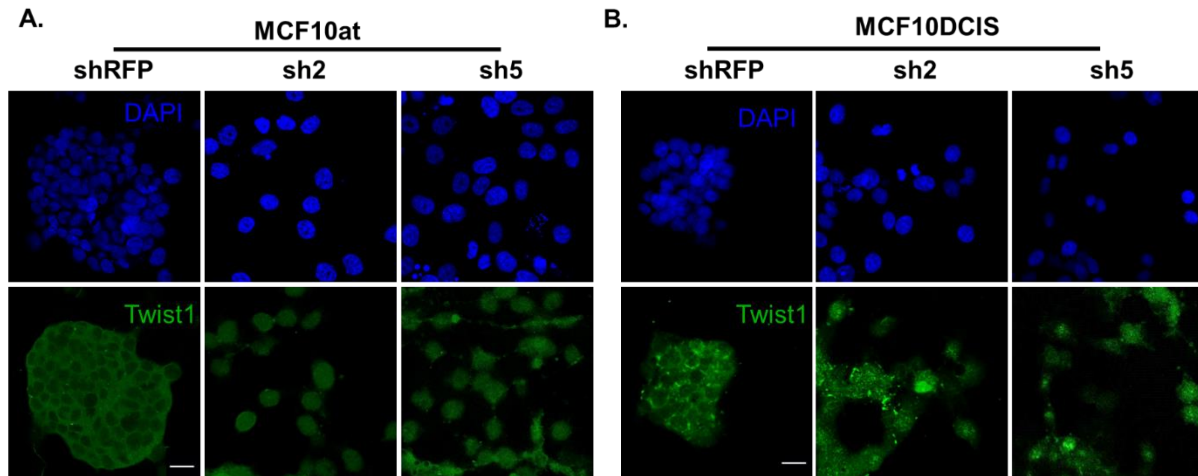


Figure 2.7: Knockdown of Tyk2 induces nuclear translocation of Twist1 at low rigidities in Mcf10AT and Mcf10DCIS cells. Confocal immunofluorescence images of control shRFP and shTyk2 (A.) Mcf10AT and (B.) Mcf10DCIS cell lines at 320Pa. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20 μM.

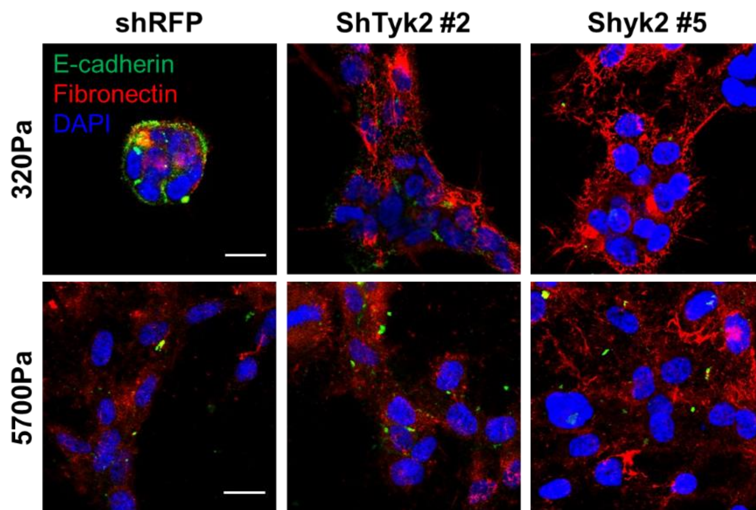


Figure 2.8: Tyk2 knockdown induces EMT at low rigidities in Mcf10a cells. Confocal immunofluorescence images of control shRFP and shTyk2 cell lines at 320Pa and 5700Pa. Stained for E-cadherin (green), Fibronectin (red) and DAPI for the nuclei (blue). Scale bar 20 μM.

2.3 Loss of Tyk2 expression induces EMT through stiffness-induced Twist1 translocation

Results fromTyk2 knockdown experiments imply that the loss of Tyk2 is inducing EMT by allowing the mechanically induced translocation of Twist1 to occur. This does not, however, rule out the hypothesis that loss of Tyk2 induces EMT through a Twist1-independent mechanism. No significant morphological changes in Tyk2 knockdown cells were observed in two dimensional cell culture on plastic dishes (Figure 2.9), supporting the notion that the EMT induced in Tyk2 knockdown cells is rigidity dependent, but not a loss of general epithelial characteristics. To address this question, we knocked down both Tyk2 and Twist1 in MCF10A cells. Briefly, I first generated stable Tyk2 knockdown Mcf10a cells using shRNAs and then used siRNAs for Twist1 to transiently knockdown the transcription factor and assess whether these cells could still undergo EMT at high matrix stiffness. These cells were plated at low (320Pa) and high (5700Pa) rigidities and assayed for the level of invasive acini and for EMT maker expression. Tyk2 knockdown alone was able to induce a higher level of invasion at low rigidities compared to control cells and displayed a more mesenchymal phenotype with the expression of fibronectin and the loss of E-cadherin. However, when Twist1 was additionally knocked down, the amount of invasive acini was reduced back to control levels at low stiffness (320Pa). In both control and Tyk2 knockdown cells, loss of Twist1 expression was sufficient to block invasion at high rigidities, (Figure 2.10). Importantly, loss of Twist1 was also sufficient to block EMT in the Tyk2 knockdown cells at low stiffness and in both control and Tyk2 knockdown cells at high stiffness (Figure 2.11). Taken together, these results indicate that Twist1 is required to induce EMT in response to high matrix stiffness, and that Tyk2 inhibits EMT at lower rigidities through maintaining Twist1 in the cytoplasm.

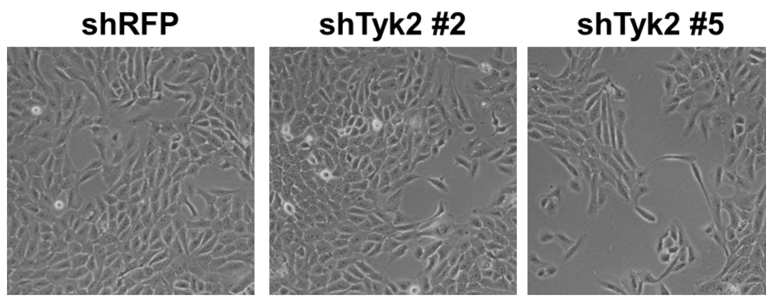


Figure 2.9: Tyk2 knockdown does not change the phenotype of Mcf10a cells in 2D culture on plastic. Control shRFP and Tyk2 knockdown cell lines grown to near confluency on 2D regular plastic culture dishes.

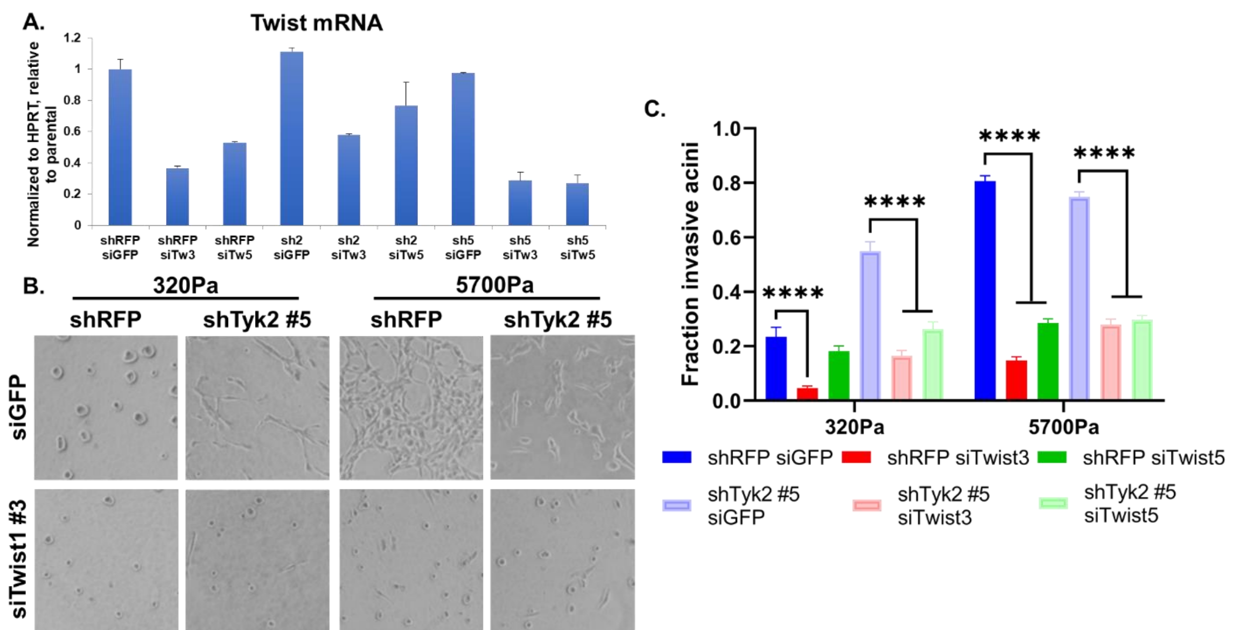


Figure 2.10: Additional knockdown of Twist1 in shTyk2 cells blocks invasion at low and high rigidities in Mcf10a cells. A. Twist1 knockdown levels measured by qPCR and normalized to HPRT. B. Brightfield images of control shRFP and shTyk2 #5 cells at 320pa and 5700Pa with either control siGFP or siTwist1 #3 siRNA treatment. C. Quantification of brightfield images shown as fraction invasive acini. **** P<0.0001.

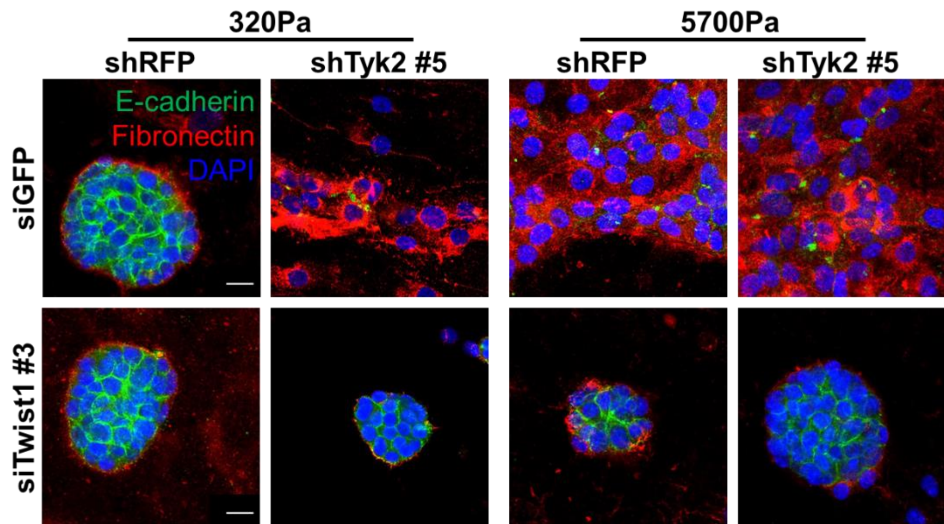


Figure 2.11: Additional knockdown of Twist1 in shTyk2 cells blocks EMT at low and high rigidities in Mcf10a cells. Confocal immunofluorescence images of control shRFP and shTyk2 cell lines treated with either control siGFP or siTwist1 #3 siRNAs at 320Pa and 5700Pa. Stained for E-cadherin (green), Fibronectin (red) and DAPI for the nuclei (blue). Scale bar 20 μM.

My data suggest that Tyk2 exerts inhibitory effects on the Twist1 mechanosensing pathway. The next question is where exactly in the mechanosensing pathway is Tyk2 exerting its effects? To examine the crux of the signaling pathway – the dissociation between Twist1 and G3BP2 – we utilized an assay called a proximity ligation assay (PLA), which assesses the interaction between two endogenous proteins and allows one to visualize where within the cell this interaction is occurring. We performed the PLA assay for Twist1 /G3BP2 interaction on control and Tyk2 knockdown Mcf10a cells at low (320Pa) and high (5700Pa) rigidities to investigate whether loss of Tyk2 affected the interaction between these two proteins. Our lab had already shown that PLA intensity, and thus Twist1-G3BP2 interaction, is high at low rigidities and occurs in the cytoplasm where G3BP2 resides. This interaction is lost at high rigidities when Twist1 enters the nucleus (Wei et al., 2015). We hypothesized that since loss of Tyk2 causes an increase in nuclear Twist1 at low rigidities, it will also lead to a decrease in PLA signals at low rigidities compared to control cells. The

alternative hypothesis is that loss of Tyk2 doesn't change the interaction between the two proteins but instead causes a translocation of the complex. We found that knockdown of Tyk2 led to a significant loss of PLA intensity at low rigidities indicating a loss of Twist1-G3BP2 interaction (Figure 2.12). This suggests that Tyk2 regulates Twist1 localization through maintaining its interaction with G3BP2 directly or indirectly. That is not to say Tyk2 is acting directly on either G3BP2 or Twist1, but that the effect Tyk2 is having on inhibiting the release of Twist1 from G3BP2 is upstream in the pathway from the dissociation event.

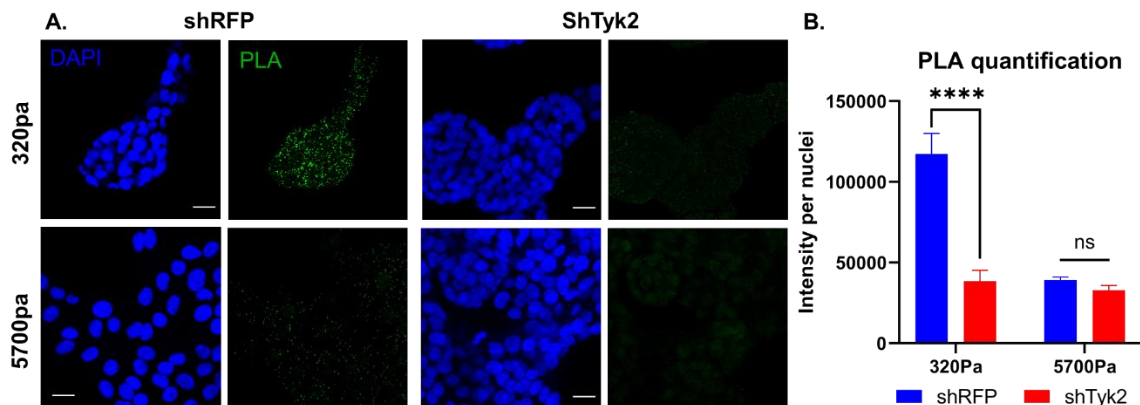


Figure 2.12: Knockdown of Tyk2 reduced Twist1-G3BP2 interaction at low rigidities. A. Confocal images of control shRFP and shTyk2 cells at 320Pa and 5700Pa. Nuclei are stained with DAPI (blue) and PLA signal is in green. Scale bar 20 μM. B. Quantification of the PLA intensity per nuclei in control and shTYk2 cells at 320Pa and 5700Pa. **** P<0.0001. ns: not significant.

I next wanted to understand at which point Tyk2 was affecting Twist1 translocation in the mechanotransduction signaling cascade. The PLA data showed that Tyk2 was acting upstream of the Twist1-G3BP2 dissociation event, so we next examined the effect of Tyk2 suppression on cell phenotype when Twist1 phosphorylation was inhibited. Work done in the lab has shown that the kinase responsible for phosphorylating Twist1 is a Src family kinase called Lyn (Fattet, 2019). Knockdown or inhibition of this kinase by a kinase inhibitor bafetinib inhibited invasion and Twist1 nuclear translocation at high rigidities (Fattet, 2019) (Figure 2.13). Treatment of Tyk2 depleted Mcf10a cells with bafetinib reduced the number of

invasive acini down to baseline levels at low rigidities (Figure 2.13). However at high rigidities, the effect of the inhibitor was not as robust. At high rigidities, bafetinib treatment blocked invasion and Twist1 nuclear localization, as shown previously by our lab (Fattet, 2019) (Figure 2.13). In the Tyk2 knockdown cells, treatment with the Lyn inhibitor did reduce the amount of non-invasive acini by about 50%, but did not completely block invasion or Twist1 translocation at high rigidity (Figure 2.13). Consistent with the morphology changes, at low rigidities, loss of Tyk2 could not induce invasion or Twist1 translocation when Lyn is inhibited and Twist1 phosphorylation is blocked. This indicates that Tyk2's effects on this mechanosensing pathway are occurring upstream of Lyn. The results at higher rigidities, however, show that loss of Tyk2 is able to drive some invasion and Twist1 translocation without Lyn kinase activity. This suggests that at high rigidities, the signaling might be more complex, or potentially that we are just seeing the limits of effectiveness for the Lyn inhibitor. Taken together these results suggest that Tyk2 could inhibit Lyn activation, or is at least acting upstream of Lyn to inhibit the activation of this mechanosensing pathway at high stiffness.

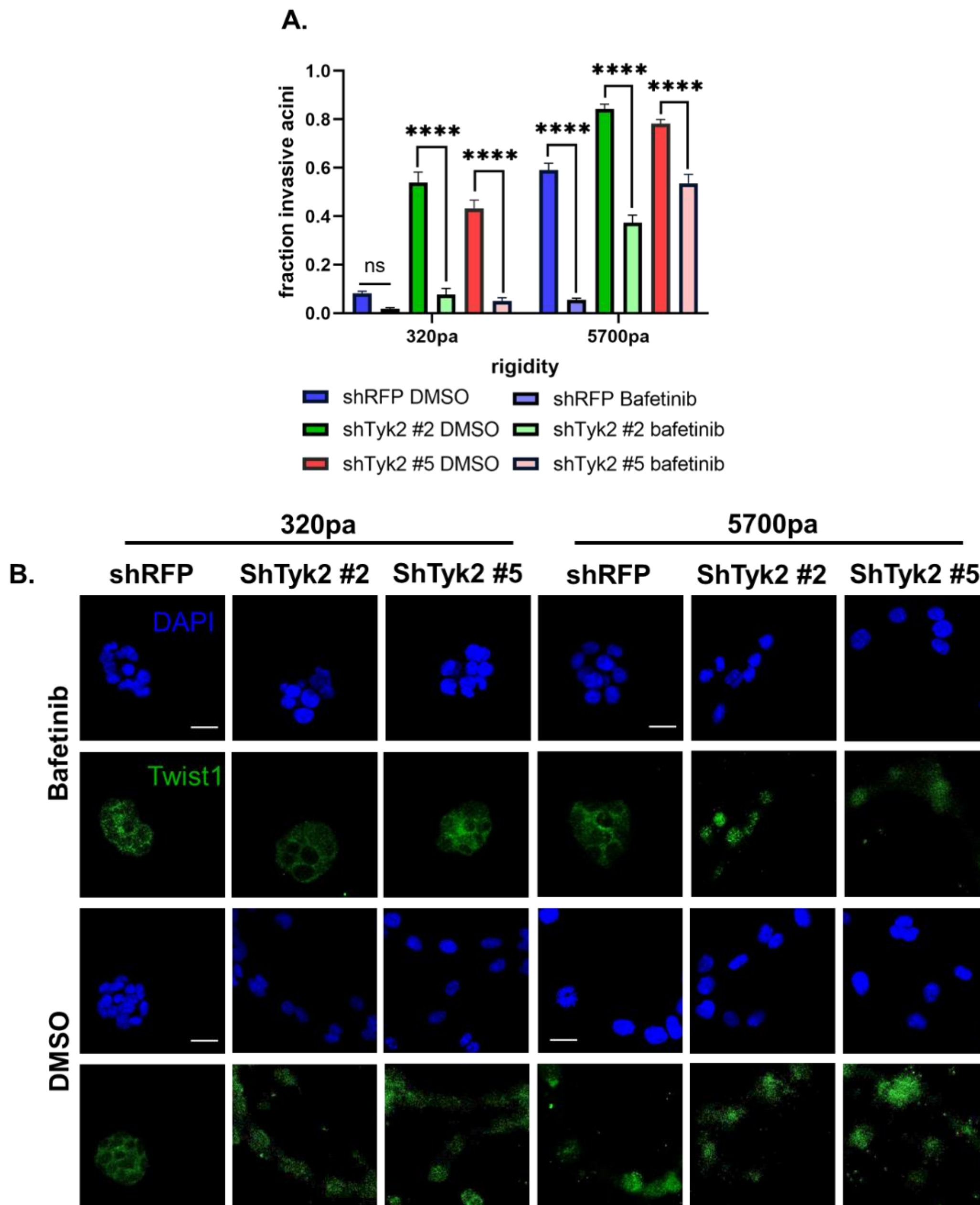


Figure 2.13: Treatment of Tyk2 knockdown cells with the Lyn inhibitor Bafetinib reduces invasion and Twist1 translocation. A. Quantification of the fraction invasive acini in control and shTYk2 cells treated with DMSO or Bafetinib at 320Pa and 5700Pa. **** P<0.0001. ns: not significant. B. Confocal immunofluorescence images of control shRFP and shTyk2 cell lines treated with DMSO or Bafetinib at 320Pa and 5700Pa. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20 μM.

2.4 Loss of Tyk2 increases metastasis *in vivo*

The results from the chapter so far show that Tyk2 is important for maintaining epithelial identity at low rigidities through inhibiting the dissociation of Twist1 from G3BP2. Tyk2 depletion allows Twist1 to dissociate from G3BP2, enter the nucleus and induce EMT. Previous work in the lab has shown that G3BP2 expression and its ability to retain Twist1 in the cytoplasm is critical for inhibiting not only EMT induction and invasive phenotypes *in vitro* but also for inhibiting invasion and metastasis *in vivo*. When Mcf10DCIS cells with G3BP2 knockdown were injected orthotopically into the mammary fat pad of immunocompromised mice, the resulting breast tumors were more locally invasive into the peritoneum and generated more metastatic nodules in the lungs (Wei et al., 2015). This shows that proper regulation of this mechanotransduction pathway is critical for inhibiting invasion and metastasis *in vivo*. Therefore, I asked whether Tyk2 was critical for maintaining a noninvasive, non-metastatic tumor in a similar mouse model for human disease.

To answer this question, we first generated green fluorescent protein (GFP) labeled Mcf10DCIS cells. This cell line is a variant of Mcf10a cells that form tumors when injected into the mammary fat pad of NOD-SCID mice. The resulting tumors from this cell line recapitulate a non-invasive disease state in humans called ductal carcinoma in situ (DCIS). These DCIS lesions are tumors that reside within the mammary ductal tissue, and are noninvasive and non-metastatic. The tumors that form in mice are initially non-invasive and very similar to DCIS in humans. After longer growth periods in the mouse, the tumors eventually become invasive (Miller et al., 2000). For our purposes, we wanted to determine whether alterations in signaling pathways can enhance metastasis in a system that otherwise does not metastasize frequently. Therefore we analyzed our tumors at about 5-6 weeks after injection. After generating the GFP labeled Mcf10DCIS cell, we depleted Tyk2 using shRNAs, expanded the cells and injected them bilaterally into the abdominal mammary fat pads of NOD-SCID mice in a 50/50 mix of cells and Matrigel (Figure 2.14). The mice were

sacrificed after five to six weeks, and the tumors were weighed, and lungs were imaged for GFP+ metastatic nodules. There was not a significant difference in the weight of the primary tumors generated by control or Tyk2 knockdown cells, except in the second experiment where we saw a slight decrease in tumor weight in tumors generated from one of the Tyk2 knockdown lines (Figure 2.15). The mice that were injected with control Mcf10DCIS cells presented very few metastases in the lung (Figure 2.16). The mice with tumors generated from Tyk2 knockdown cells, however, had significantly more metastases in the two independent lines tested (Figure 2.16). This result shows that Tyk2 is required for Mcf10DCIS tumors to remain in a non-invasive state, and that loss of Tyk2 promotes invasion and metastasis.

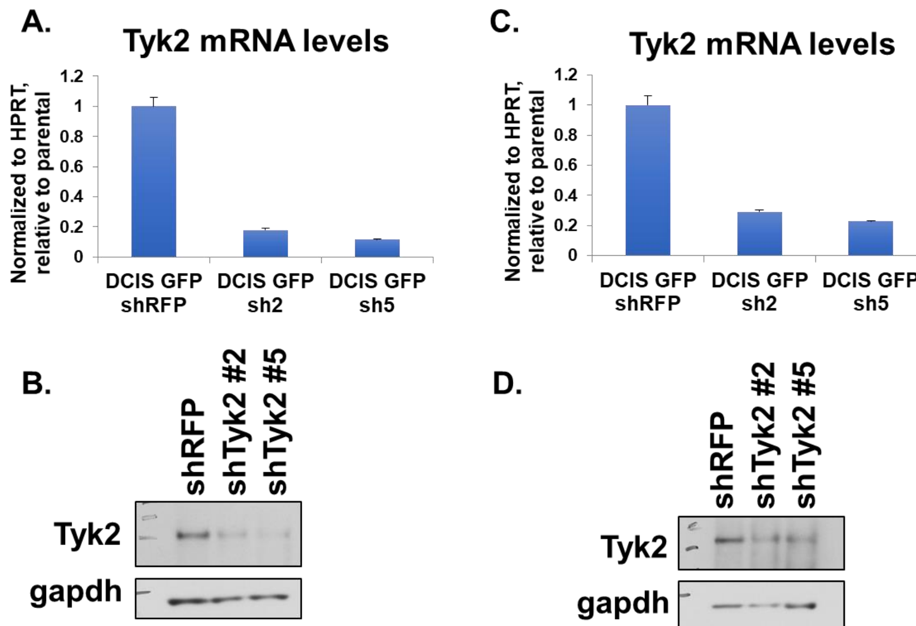


Figure 2.14: Tyk2 knockdown level in Mcf10DCIS-GFP for each *in vivo* experiment. Tyk2 knockdown levels measured by qPCR and normalized to HPRT for experiment one (A.) and experiment two (C.). Western blot of lysates from Mcf10DCIS-GFP cells with indicated Tyk2 shRNAs showing level of Tyk2 knockdown with GAPDH as a loading control for experiment one (B.) and experiment two (D.).

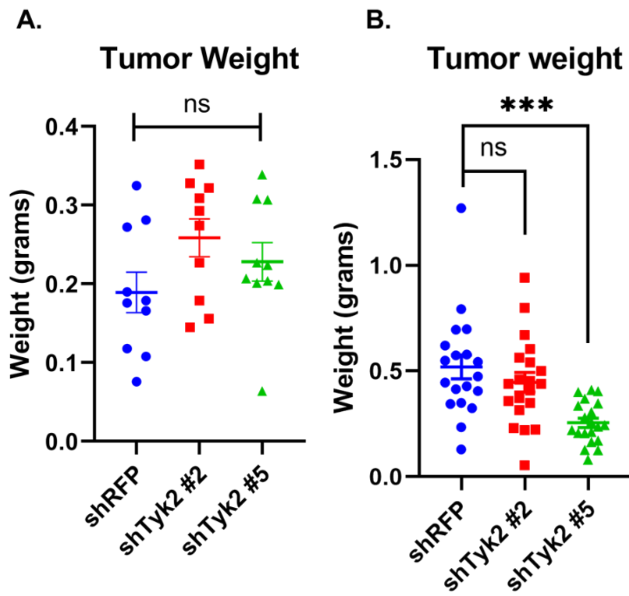


Figure 2.15: Tyk2 knockdown had no effect on primary tumor growth. Quantification of primary tumor weight in grams for each tumor, with two tumors per mouse. A. experiment one. B. experiment two. *** P<0.001. ns: not significant.

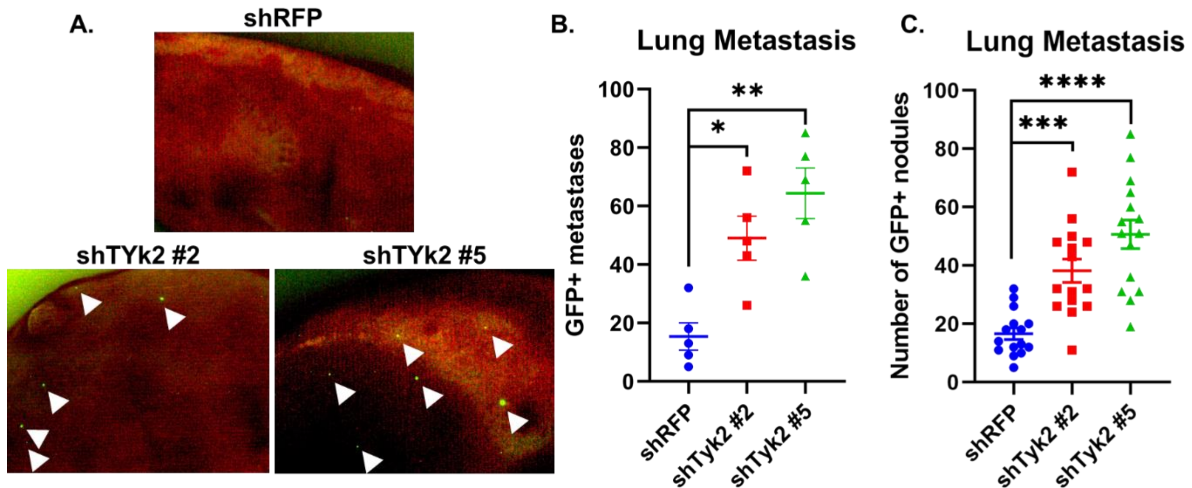


Figure 2.16: Tyk2 knockdown reduced metastasis to the lungs. A. Images of lungs from mice with tumors generated from control shRFP Mcf10DCIS-GFP cells or shTyk2 MCF10DCIS-GFP cells. White triangles indicate GFP+ lung metastases. Quantification of the number of lungs metastases generated in each mouse from experiment one (B.) and experiment two (C.). * P<0.05 ** P<0.01 *** P<0.001 **** P<0.0001.

2.5 Conclusion

In this chapter, we showed that Tyk2 is a novel regulator of Twist1 translocation in response to rigidity. We found that loss of Tyk2 induced an increase in invasive acini, Twist1 nuclear translocation and EMT marker expression at a lower rigidity than control cells. This conclusion is supported by data from a variety of human and mouse mammary epithelial cell lines tested. We found that loss of Tyk2 specifically induces EMT through Twist1, and that additionally depleting the cells of Twist1 was able to block the induction of EMT. Tyk2 appears to be controlling the translocation of Twist1 to the nucleus through maintaining the interaction between Twist1 and G3BP2 – when Tyk2 is lost, this interaction is lost at lower rigidities. Blockade of the Twist1-directed kinase Lyn largely prevents the translocation of Twist in control cells and in cells with Tyk2 depleted, further indicating that Tyk2 likely exerts its inhibitory effects on this pathway further upstream. Last, our tumor xenograft studies in vivo indicated that loss of Tyk2 also induces metastasis in vivo. Taken together, these results indicated that Tyk2 inhibits Lyn and Twist1 to prevent EMT at lower rigidities, and that loss of Tyk2 is sufficient to drive invasion and EMT through Twist1 translocation both *in vitro* and *in vivo*.

While loss of Tyk2 had an effect on the phenotype of all mammary epithelial cell lines tested, we noted that the effect occurred at different rigidities in Eph4Ras cells as compared to the human lines tested. The Eph4ras cells had a higher level of basal invasion at the lowest rigidity we test – 150pa, whereas Mcf10a cells (and the variants of this line tested) did not. Instead Mcf10a cells had a level of basal invasion at 320pa, which is where the effect of Tyk2 knockdown could be seen. This could indicate that the ‘threshold’ for activation of this pathway is different in these different cell lines, and that additional proteins need to be activated for the cells to invade besides the loss of Tyk2. This difference in threshold for invasion could be due to a variety of factors. Eph4ras cells are the only mouse line tested, so this could be a species difference. Mouse and human mammary glands could naturally be at

a different level of rigidity, and therefore have different baselines for what is perceived as 'normal' by the cells. The Eph4Ras cells are also more prone to tumorigenesis than the Mcf10a cells, as they are transformed with oncogenic Ras. However, the Mcf10AT variant is also transformed with Ras, and we saw no enhanced invasion at 150pa as we observed in Eph4ras cells, so Ras transformation may not account for these differences. More specific answers on the 'threshold' for these different cell types are more likely to come from experiments analyzing specifically at what rigidity different components of the mechanosensing pathway get activated instead of simply examining these components at 'low' and 'high.'

In general, the results from the PLA assay and the Lyn inhibitor treatment of Tyk2 knockdown cells suggest that Tyk2 functions upstream to known regulators of this mechanosensing pathway. Loss of Tyk2 led to an aberrant decrease in Twist1-G3BP2 interaction at lower rigidities, which indicates that Tyk2 is acting to inhibit this dissociation event. That data does not differentiate whether Tyk2 acts directly on either G3BP2 or Twist1, or indirectly upstream in the pathway from the dissociation event. The results at low rigidities show that blockade of Lyn activity additionally blocked the ability of Tyk2 knockdown to induce invasion – fully supporting the idea that Tyk2 is acting upstream of Lyn. The results at higher rigidities, however, show that loss of Tyk2 is able to drive some invasion and Twist1 translocation even in the presence of the Lyn inhibitor. This suggests that at high rigidities, the Tyk2 signaling might be more complex and that the loss of Tyk2 could drive Twist1 translocation through an alternative mechanism to Lyn activation. We could also simply be at the limits of the effectiveness of our inhibitor and that the inhibition of Lyn by Bafetinib is not complete at high rigidities. The overall percent reduction in invasion at high rigidities (about 40-50%) is the same between control and Tyk2 depleted cells, the control cells just have a lower basal level of invasion than the Tyk2 knockdown cells (about 60% vs 80%) (Figure

2.13). This means that the inhibitor is reducing invasion at high rigidities to the same extent in both control and Tyk2 knockdown cells. We need to closely examine Lyn activation under these settings by examining Lyn phosphorylation and Twist1 Y107 phosphorylation to further understand these differences.

Our *in vivo* experiments show that loss of Tyk2 leads to more metastasis. This indicates that within epithelial tumors, Tyk2 has a metastasis suppressive effect considering there was no increase of Tyk2 knockdown on primary tumor growth. This is in sharp contrast to the canonical role of Tyk2 in cancers of the hematopoietic system where Tyk2 is activated or upregulated, and is thought to be an oncogene. This idea that Tyk2 is an oncogene is not solidified within the field and, as discussed in the introduction chapter, there are several papers that indicate that higher Tyk2 expression is actually correlated with better prognosis in patients with various solid tumors. Our results in mice are consistent with this notion as Tyk2 loss alone was able to drive more metastatic disease.

2.6 Acknowledgements

Chapter 2, in full, is currently being prepared for submission for publication of the material. Majeski, H.E., Hu, Z., Fattet, L., Yang J. Tyk2 regulates matrix stiffness-driven EMT and metastasis. *In preparation.* The dissertation author was the primary investigator and first author of this material.

Chapter 3

Tyk2 activity is regulated by rigidity and is critical for regulating Twist1 translocation in response to rigidity

3.1 Introduction

Tyk2 is a member of the Jak kinase family. This family of proteins has several known regulatory mechanisms, the most well-characterized being their roles in cytokine signaling. In short, Jak kinases associate with a variety of different cytokine receptors and, upon ligand binding, the receptors dimerize (usually a heterodimer) to bring two receptor-associated Jak kinases together. These kinases are then phosphorylated by either auto- or cross phosphorylation on two tandem tyrosines within the activation loop of the kinase (Darnell et al., 1994). Phosphorylation at these residues activates the kinase, which subsequently phosphorylates the receptors themselves and various downstream targets – most well-known being the Stat family of transcription factors (Darnell et al., 1994). Broadly, these Stat proteins, once phosphorylated, enter the nucleus and interact with transcription factors to stimulate the transcription of a group of cytokine response genes. The gene sets included in this cytokine response signature vary based on a number of factors including which cytokine, which receptor and which Stat proteins are activated and which additional factors are interacting with this signaling pathway. The network stimulated by different Jak proteins is vast, and depends a lot on the specific cell type. Negative regulators of Jak kinases include a number of phosphatases and proteins members of the SOCS family of proteins, which instigate the degradation of Jak family members and can also inhibit their activity through binding to the kinase domain (Babon et al., 2012; Myers et al., 2001; Piganis et al., 2011; Yetter et al., 1995). The SOCS family of proteins are actually interferon response genes and represent a negative feedback loop (Yoshimura et al., 2007). Another important negative

regulator of Jak kinase activity is the pseudo-kinase domain of the protein, which is located directly next to the kinase domain at the c-terminus of Jak family kinases. The pseudo-kinase domain (also called the kinase-like-domain or KLD) has a structurally very similar activation loop to the kinase domain, but contains some key residue substitutions that render it catalytically inactive (Lupardus et al., 2014a). The KLD plays a very important role in regulating the activity of the kinase domain, and recent structural data suggests that when the KLD is stabilized by binding either ATP or an ATP competitive inhibitor, the KLD domain binds to and inhibits the kinase domain (Min et al., 2015; Tokarski et al., 2015). This has made the KLD domain a hotspot for hyperactive mutants in disease in these Jak kinases, presumably through preventing the KLD domain from binding to the kinase domain, thus rendering the kinase constitutively active.

We wanted to understand whether Tyk2 activity was important for its ability to maintain cytoplasmic localization of Twist1 at low rigidities. We addressed this question in three ways. The first was to assess the Tyk2 activation status by assaying the phosphorylation status of the protein at different rigidities and phosphorylation of a known Tyk2 substrate Stat3. Second, we examined the effect of Tyk2 inhibition on cellular phenotype and Twist1 translocation at different rigidities to determine whether Tyk2 kinase activity is necessary for maintaining an epithelial phenotype at low rigidities. Lastly, we generated mutants of Tyk2 that either activated the kinase or rendered it catalytically dead and expressed these mutants in cells grown in our 3D culture system to determine whether changes in Tyk2 activity are sufficient to drive changes in cell phenotype.

3.2 Tyk2 activity decreases with increasing matrix rigidity

When trying to understand what role Tyk2 plays in maintaining an epithelial phenotype at low rigidities, we assayed whether Tyk2 kinase activity is regulated by rigidity

via examining the phosphorylation status of the protein at different rigidities. We found that endogenous Tyk2 immunoprecipitated from lysates of Mcf10a cells grown at low rigidities presented a high level of tyrosine phosphorylation as indicated with a pan phospho-tyrosine antibody. This level of tyrosine phosphorylation on Tyk2 was significantly reduced in Tyk2 isolated from high rigidity lysates (Figure 3.1). This indicates that the protein is being de-phosphorylated at high rigidities, but does not directly show that de-phosphorylation occurs on the activation site as other tyrosines on Tyk2 have been shown to be phosphorylated, albeit with no known functions (Prchal-Murphy et al., 2012). To examine phosphorylation specifically on the tyrosines in the activation loop, we utilized an antibody specific to phosphorylated tyrosine 1054 and 1055, referred to from here on as the pTyk2 antibody. There was no specific band at the right size for Tyk2 in the endogenous Tyk2 immunoprecipitation from either low or high rigidity lysates (Figure 3.2). This could indicate that Tyk2 is not activated in Mcf10a cells and that the high level of tyrosine phosphorylation we see on Tyk2 at low rigidities is actually phosphorylation on other tyrosine residues within the protein. This could also mean that the level of active site phosphorylation on Tyk2 is low or that the antibody for phosphorylated Tyk2 is not sensitive enough to detect endogenous Tyk2 phosphorylation. To address the second possibility, we over-expressed a C-terminally tagged wild-type Tyk2 in Mcf10a cells, and immunoprecipitated the overexpressed protein from lysates of Mcf10a cells grown at low or high rigidity. In an overexpressed system it is possible that there is too much of the protein expressed to detect regulatory changes that might be very relevant for the protein expressed at endogenous levels. When we examined over-expressed Tyk2 for phosphorylation at the active site residues using the pTyk2 antibody, the presence of a clean signal at the right molecular weight for Tyk2 in the immunoprecipitation indicates that the overexpressed Tyk2 protein is phosphorylated on these residues (Figure 3.3). This signal, though present at both low and high rigidities,

decreases significantly at high rigidities. This suggests that the Tyk2 kinase is more active at low rigidities than at high rigidities. This data supports the idea that Tyk2 activation is regulated by matrix rigidities.

Figure 3.1: There is a reduction in Tyk2 tyrosine phosphorylation at high rigidities. Endogenously expressed Tyk2 from Mcf10a cell lysates was immunoprecipitated and analyzed by SDS-PAGE, and probed for Tyk2 and a pan-phosphotyrosine antibody (pTyr).

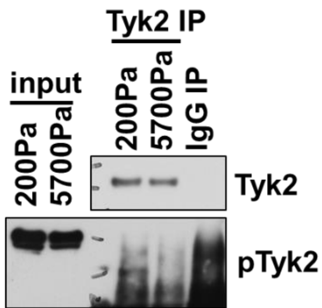


Figure 3.2: Inability to visualize activation loop phosphorylation on endogenous Tyk2. Endogenously expressed Tyk2 from Mcf10a cell lysates was immunoprecipitated and analyzed by SDS-PAGE, and probed for Tyk2 and a phospho-Tyk2 antibody specific for Tyk2 phosphorylation on tyrosine 1054 and tyrosine 1055.

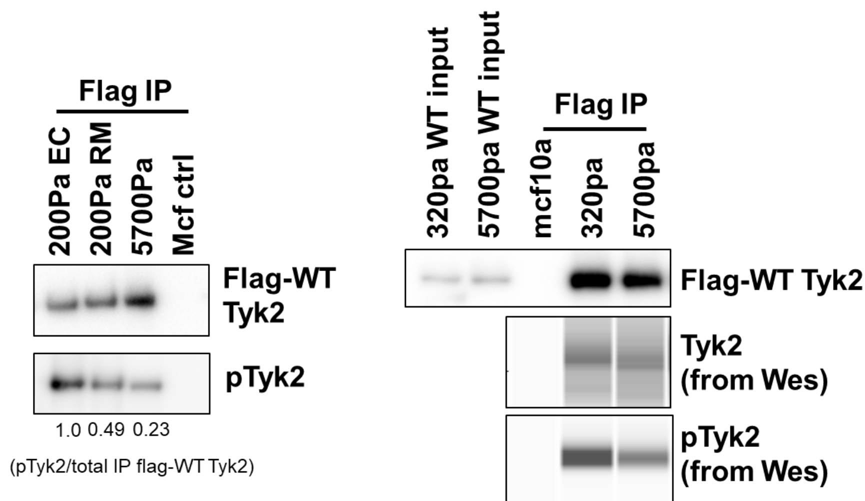


Figure 3.3: There is a reduction in the phosphorylation of the activation loop in overexpressed Tyk2 at high rigidities. Exogenously expressed wild type (WT) c-terminally Flag tagged Tyk2 from Mcf10a cell lysates was immunoprecipitated and analyzed by SDS-PAGE (left) or Simple western (right, lower), and probed for Tyk2 and a phospho-Tyk2 antibody specific for Tyk2 phosphorylation on tyrosine 1054 and tyrosine 1055. EC and RM refer to coating methods used for Matrigen hydrogels. Quantification of band intensity relative to low rigidities shown beneath images.

An alternative way of determining Tyk2 activity is to examine phosphorylation signals on potential Tyk2 substrates – most commonly Stat transcription factors. We examined phosphorylation of Stat3 in part because it is highly expressed in our system whereas other Stat proteins are not. Additionally Stat3 is well known to play a role in cancer progression and even EMT activation (Jin, 2020; Yu et al., 2009). This is predominantly through activation by Jak2, although we do not see Jak2 playing a role in Twist1 mechanoregulation. When we examined Stat3 phosphorylation in Mcf10a lysates at a range of rigidities, we found a significant decrease in Stat3 phosphorylation at high rigidities (Figure 3.4). This signal, compared to the amount of Stat3 present in the lysates is relatively low at all rigidities, but is almost completely gone at high rigidities. This supports the previous data indicating that Tyk2 kinase activity decreases at high rigidity. While we use Stat3 phosphorylation as a readout of Tyk2 kinase activity, this data does not indicate a functional role of Stat3 in Twist1

mechanoregulation. Taken together, these results indicate that the kinase Tyk2 activity is regulated by matrix rigidity to impact its function as a kinase.

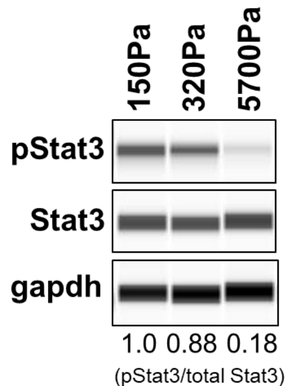


Figure 3.4: There is a reduction in Stat3 phosphorylation at high rigidities. Lysates from Mcf10a cells isolated from 150Pa, 320Ps and 5700Pa analyzed by Simple western were probed for Stat3, pStat3 and gapdh as a loading control. Quantification of band intensity relative to 150Pa shown beneath images.

3.3 Tyk2 activity is necessary for maintaining a non-invasive state at low rigidities

We next sought to determine whether Tyk2 kinase activity was necessary for its role in maintaining epithelial identity at low rigidities. We utilized two specific inhibitors for Tyk2 acquired from a company called Nimbus Therapeutics. Both of these Tyk2 inhibitors were generated to be very specific for Tyk2 over other Jak kinase family members in order to better treat psoriasis specific myeloproliferative disorders that involve the hyper-activation of Tyk2, while eliminating side effects caused by targeting Jak1, Jak2 or Jak3 inhibition. We used two specific inhibitors from Nimbus: one is a classic ATP competitive inhibitor for the catalytic domain of Tyk2 (407); The other is an allosteric inhibitor that binds to the ATP binding site of the KLD and locks it into an auto-inhibitory conformation similar to other recent inhibitors developed (Lupardus et al., 2014a) (217). Treatment with these inhibitors caused a decrease in overall phosphorylation of the endogenous protein immunoprecipitated from both low and high rigidities (Figure 3.5). We also examined phosphorylation of Stat3, a substrate

of Tyk2, as an additional readout of Tyk2 activity in these inhibitor treated cells and found that treatment with the catalytic inhibitor caused a decrease in the phosphorylation of Stat3 at all rigidities (Figure 3.6). Treatment of Mcf10a cells with either of these inhibitors led to more invasive acini at low rigidities – a 30-40% compared to control at 320Pa – very similar to the increase generated upon Tyk2 knockdown (Figure 3.7). This shows that Tyk2 kinase activity is required to establish a non-invasive state at low rigidities. We also assayed for Twist1 translocation in these cells through immunofluorescence staining and found that treatment with either the catalytic or the allosteric inhibitor caused nuclear translocation of Twist1 at low rigidities, again similar to Tyk2 knockdown (Figure 3.8). We also used these inhibitors on Eph4ras cells, and the allosteric inhibitor in particular induced invasion and nuclear translocation of Twist1 at low rigidities (Figure 3.9). Taken together, these results show that Tyk2 kinase activity is important for establishing a noninvasive epithelial state at low rigidities by keeping Twist1 in the cytoplasm, thus inhibiting EMT and invasion.

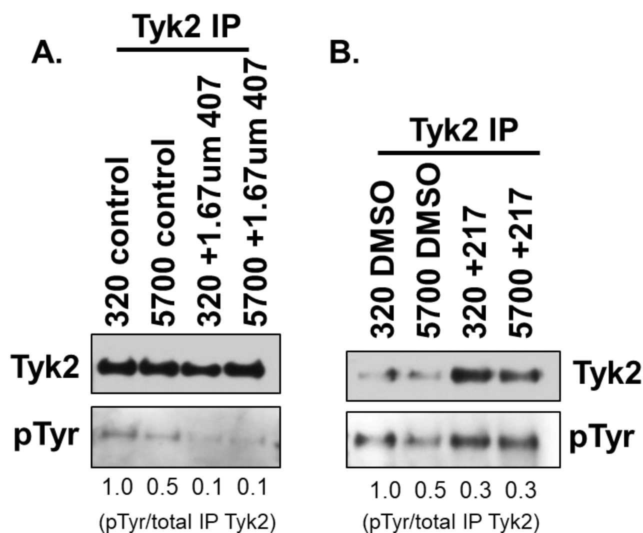


Figure 3.5: Treatment with a Tyk2 inhibitors reduces Tyk2 phosphorylation. Endogenously expressed Tyk2 from Mcf10a cell lysates treated with catalytic inhibitor (407) (A.) or allosteric inhibitor (217) (B.) was immunoprecipitated and analyzed by SDS-PAGE, and probed for Tyk2 and a pan-phosphotyrosine antibody (pTyr). Quantification of band intensity relative to 320Pa DMSO shown beneath images.

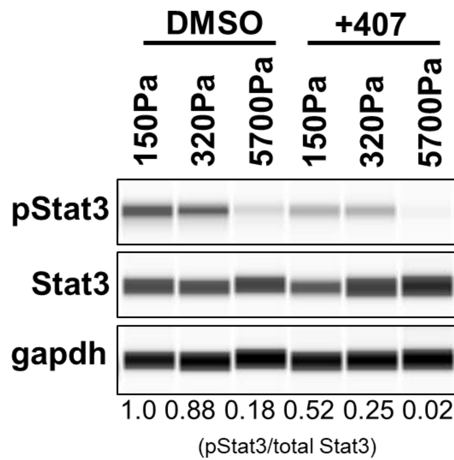


Figure 3.6: Treatment with a Tyk2 inhibitor reduces Stat3 phosphorylation. Lysates from Mcf10a cells treated with DMSO or catalytic inhibitor (407) isolated from 150Pa, 320Ps and 5700Pa analyzed by Simple western were probed for Stat3, pStat3 and gapdh as a loading control. Quantification of band intensity relative to 150Pa shown beneath images.

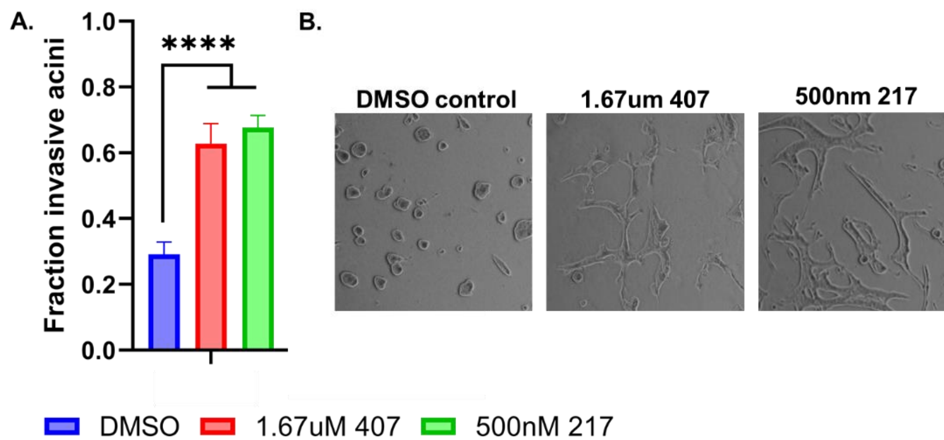


Figure 3.7: Treatment with Tyk2 inhibitors increases invasion at low rigidities. Mcf10a cells cultured for 5 days in 3D were treated with the catalytic Tyk2 inhibitor (407) or the allosteric Tyk2 inhibitor (217). A. Quantification of the fraction of invasive acini at 320Pa. **** $P<0.0001$. B. Representative brightfield images indicating phenotypic changes observed with inhibitor treatment that are quantified in A.

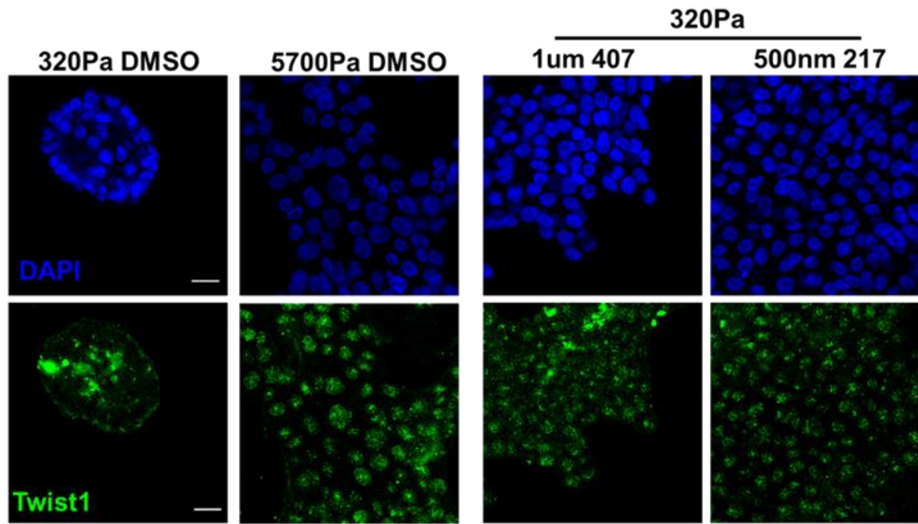


Figure 3.8: Treatment with Tyk2 inhibitors increases Twist1 nuclear translocation at low rigidities. Confocal immunofluorescence images of Mcf10a cells treated with DMSO, a catalytic Tyk2 inhibitor (407) or an allosteric Tyk2 inhibitor (217) at 320Pa and 5700Pa. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20 μM.

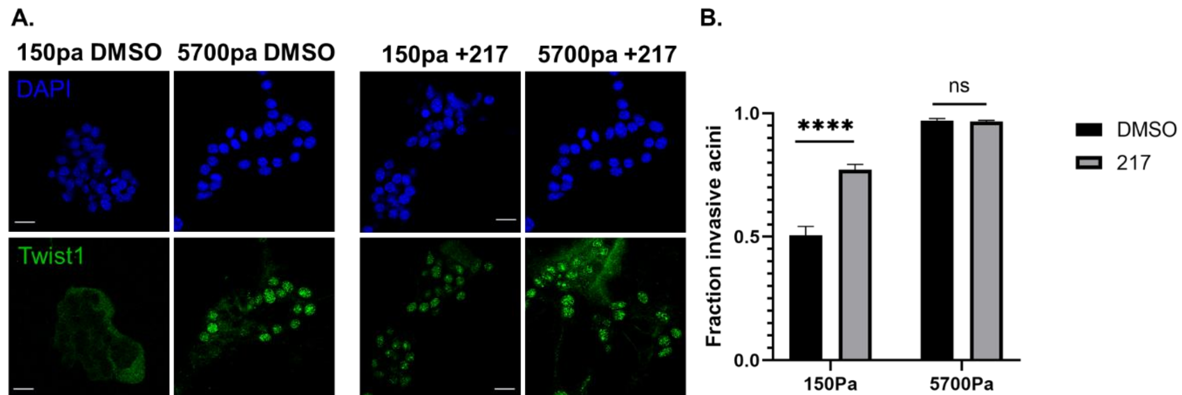


Figure 3.9: Treatment of Eph4ras cells with a Tyk2 inhibitor increases invasion and Twist1 nuclear translocation at low rigidities. A. Confocal immunofluorescence images of Eph4ras cells treated with DMSO or an allosteric Tyk2 inhibitor (217) at 150Pa and 5700Pa. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20 μM. B. Quantification of the fraction invasive acini in DMSO or inhibitor treated cells 150Pa and 5700Pa. **** $P<0.0001$.

We next wanted to understand whether Tyk2 activity is important early to establish non-invasive organoids or whether constant Tyk2 kinase activity is required to maintain a

noninvasive state. Rigidity sensing is a constant phenomenon, but the rigidity sensing that occurs early during cell spreading is particularly important for establishing cell phenotype. In the 3D cultures, cells change morphology within 24 hours upon seeding, and rigidity-dependent Twist1 dissociation from G3BP2 happens in single cells within 24 hours (Wei et al., 2015). To determine whether Tyk2 kinase activity is important for continuously maintaining an epithelial state, we treated Mcf10a cells with both of the Tyk2 inhibitors after they had already been in culture for three days and had already begun to either invade (high rigidity) or form acini (low rigidity). Treatment with the Tyk2 inhibitor at this time point was still able to induce invasion to a similar level that treatment at seeding caused (Figure 3.10). Upon two days of inhibitor treatment, cells that had already formed acini were invading. These results show that Tyk2 activity is not only important for establishing a noninvasive phenotype in these cells, but that continuous Tyk2 activity is required to maintain this noninvasive state.

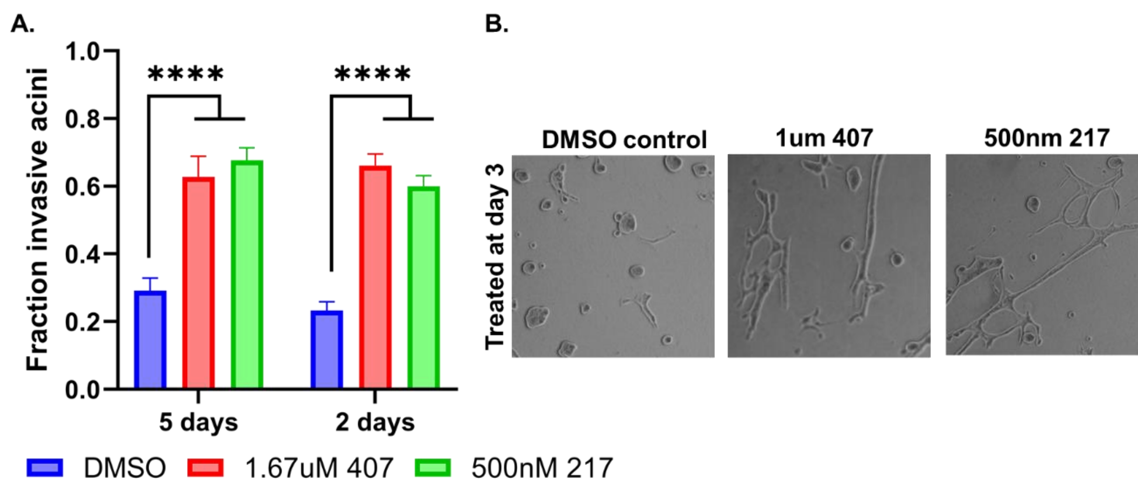


Figure 3.10: Treatment with Tyk2 inhibitors induced invasion at low rigidities. A. Quantification of the fraction of invasive acini at 320Pa for Mcf10a cells treated with DMSO, a catalytic Tyk2 inhibitor (407) or an allosteric Tyk2 inhibitor (217) for either 5 full day or for 2 days (treated at day 3 in culture). **** $P<0.0001$. B. Representative brightfield images at 320Pa from B.

Interestingly, we performed shorter time courses for inhibitor treatment and assessed how quickly Tyk2 inhibition could induce nuclear Twist1 translocation. We cultured Mcf10a cells for five days, and then we treated the cells with the catalytic Tyk2 inhibitor for either five hours or two hours. No change in cell phenotype was observed within this short timeframe of inhibitor treatment, as was expected. However, we observed robust Twist1 nuclear localization with five hours of inhibitor treatment, and nuclear translocation of Twist1 in some cells within two hours of inhibitor treatment although it was not quite as robust (Figure 3.11). Taken together, these results indicate that Tyk2 activity is necessary to both establish and maintain a noninvasive state because it is necessary to maintain cytoplasmic Twist1.

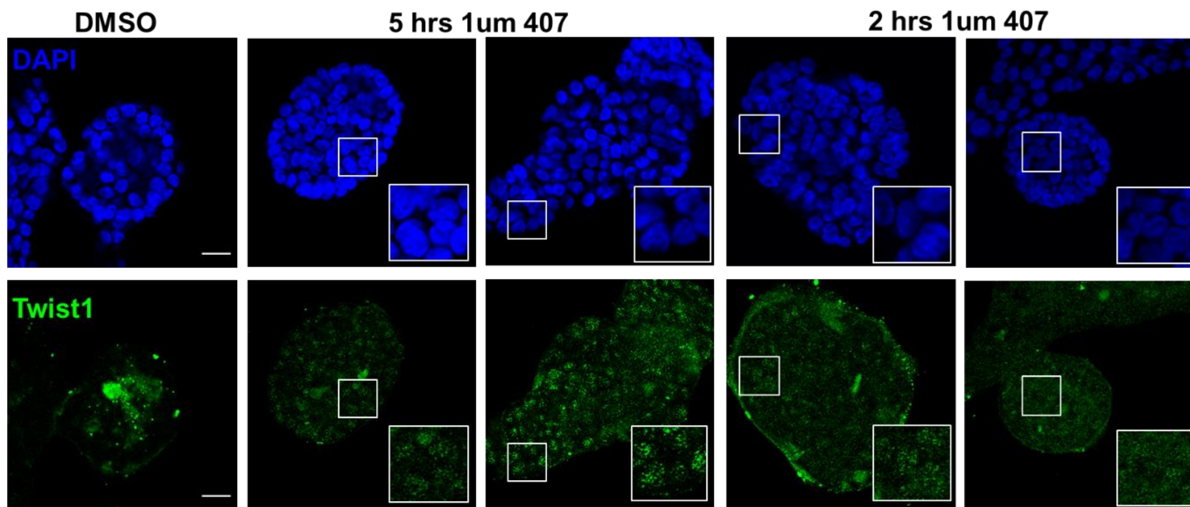


Figure 3.11: Short term treatment with a Tyk2 inhibitor increases nuclear Twist1 translocation at low rigidities. Confocal immunofluorescence images of Mcf10a cells seeded in 3D culture at 320Pa treated with DMSO or a catalytic Tyk2 inhibitor (407) at 320Pa for either 5 hours or two hours. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20μM.

3.4 Tyk2 inhibition activates upstream regulators of rigidity-induced Twist1 translocation

In chapter two, the role of Lyn kinase in regulating Twist1 translocation was discussed. Briefly, Lyn kinase is activated to phosphorylate Twist1 on tyrosine 107

specifically at high rigidities, and phosphorylation at this site causes Twist1 to be unable to bind to G3BP2 and allows it to enter the nucleus. Lyn is a Src family kinase and, like other Src family kinases, contains an activating tyrosine phosphorylation site and a inhibitory tyrosine phosphorylation site. Phosphorylation at the inhibitory site locks the kinase into an auto-inhibited state. Phosphorylation at the activating site stimulates activity of the kinase for downstream effectors. Phosphorylation at these sites is mutually exclusive. Lyn kinase is activated at high rigidities, which can be monitored through an increase in phosphorylation at the activation site (pSFK416) (Fattet, 2019) (Figure 3.12). Treatment with a Tyk2 inhibitor, in this case the catalytic inhibitor, caused an increased level of invasive acini and an increased level of Twist1 translocation into the nucleus due. Given that Lyn is the kinase responsible for instigating the dissociation of Twist1 from G3BP2, we hypothesized that Tyk2 inhibition might increase the activity of Lyn kinase aberrantly at lower rigidities. We found that when Tyk2 was inhibited, there was a small increase in active tyrosine phosphorylation of Lyn at lower rigidities than the control cells (Figure 3.12). This indicates that Tyk2 is acting to inhibit Lyn activation at low rigidities.

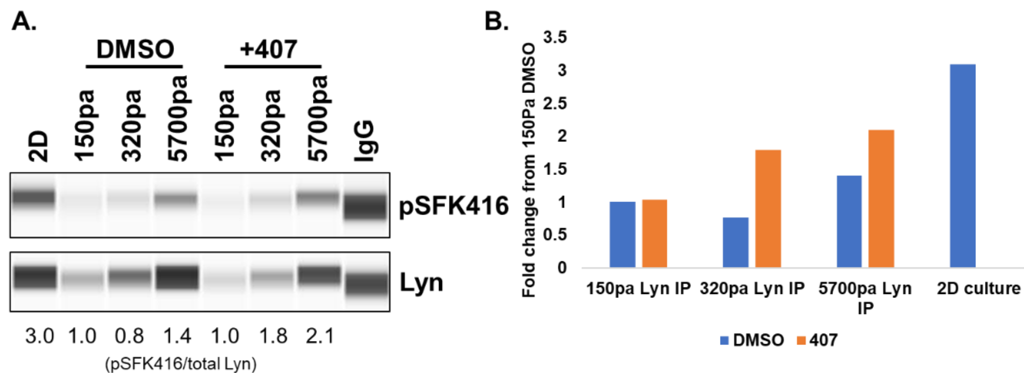


Figure 3.12: Treatment with a Tyk2 inhibitor increases Lyn phosphorylation at lower rigidities. A. Mcf10a cells from 2D or from 3D cultures at 150Pa, 320Pa or 5700Pa were treated with DMSO or a catalytic Tyk2 inhibitor (407). Endogenous Lyn from these Mcf10a cell lysates was immunoprecipitated and analyzed by Simple western and probed for Lyn and an antibody specific for the activating Src family kinase phosphorylation site on tyrosine 416 (PSFK416). B. Quantification of intensity for PSFK416 over the intensity for the total immunoprecipitated Lyn is plotted.

Several of the signaling pathways which regulate Lyn in response to matrix stiffening have been discovered in our lab recently. We recently identified a receptor tyrosine kinase called EphA2 as an upstream regulator of Lyn in this pathway. The EphA2 kinase activity is activated by binding to its cell-surface tethered ligands such as ephrinA1. This canonical pathway stimulates a variety of downstream effects and is generally thought to be tumor suppressive by suppressing Akt. However, a ligand independent function of EphA2 is mediated by phosphorylation of serine 897 by kinases such as RSK and is thought to promote invasion and metastasis (Miao et al., 2009; Pasquale, 2010). In the case of mechanically regulated EphrinA2, we found that high rigidities promote phosphorylation of S897 (Fattet, 2019) (Figure 3.13). Interestingly, Tyk2 inhibition led to an increase in the levels of S897 phosphorylation on EphA2 specifically at 320pa in the Mcf10a cells which is the rigidity at which we see an increase in invasion (Figure 3.13). This indicates that loss of Tyk2 activity was sufficient to activate this upstream regulator of Lyn to a similar level that is

seen at high rigidities, and that Tyk2 appears to be regulating Twist1 translocation through inhibiting the EphA2/Lyn/Twist1 mechanosensing pathway members.

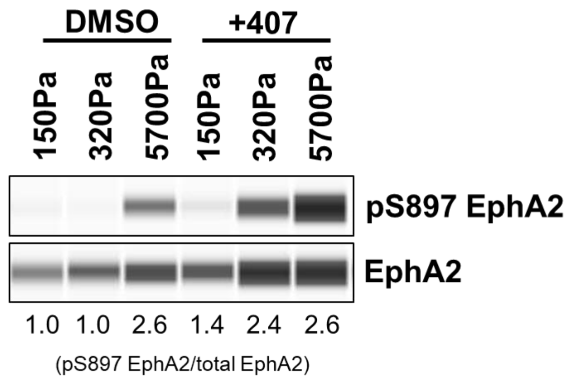


Figure 3.13: Treatment with a Tyk2 inhibitor increases EphA2 phosphorylation at lower rigidities. Lysates from Mcf10a cells treated with DMSO or catalytic inhibitor (407) isolated from 150Pa, 320Ps and 5700Pa analyzed by Simple western were probed for EphA2, and an antibody specific to phosphorylation of serine 897 of EphA2 (pS897 EphA2). Quantification of band intensity relative to 150Pa shown beneath images.

3.5 Alterations in Tyk2 kinase activity through mutation are sufficient to drive phenotypic changes

Experiments with the Tyk2 inhibitors demonstrate that Tyk2 is necessary to maintain a noninvasive epithelial state at low rigidities through maintaining Twist1 cytoplasmic localization. We wanted to determine whether altering Tyk2 activity through a constitutively active Tyk2 was sufficient to inhibit rigidity-induced invasion by inhibiting the EphA2/Lyn pathway. We also wanted to test whether a catalytically dead Tyk2 mutant could have a dominant-negative effect on the cells – that is whether overexpression of a kinase dead version of Tyk2 could compete with endogenous Tky2 to drive invasion via activation of Lyn and EphA2 at low rigidities. To do so we generated a number of Tyk2 point mutants that alter its kinase activity. The constitutively active Tyk2 is a mutation of a valine within the KLD to a phenylalanine (V678F). This mutation is a homologous mutation to a V617F mutation found

in Jak2 in human patients with the myeloproliferative disorder polycythemia vera (Baxter et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Staerk et al., 2005). This mutation causes constitutive phosphorylation of the activation loop tyrosines on Tyk2 (1054 and 1055) without stimulation (Figure 3.14) (Gakovic et al., 2008; Staerk et al., 2005). As briefly mentioned in the introduction to this chapter, the KLD has a regulatory function for the kinase domain, and mutations within this domain, such as the V678F mutation, are thought to be activating by loosening the negative regulatory effect of the KLD on the kinase domain (Gakovic et al., 2008). We additionally created a catalytically dead mutant by mutating a lysine within the kinase domain that is essential for catalytic activity to an arginine (Prchal-Murphy et al., 2012). This generated a Tyk2 that is unable to phosphorylate substrates or auto-phosphorylate (Figure 3.14) (Prchal-Murphy et al., 2012). Lastly, we created a Tyk2 that had both the activation loop tyrosines mutated to un-phosphorylatable phenylalanines (YY10541055FF). This mutant could not be phosphorylated on the activation loop, but retained some of the ability to auto-phosphorylate (Figure 3.14) (Gauzzi et al., 1996). All of these Tyk2 variants, and the wild type Tyk2, were Flag-tagged to allow for easy immunoprecipitation and identification via immunofluorescence.

V678F: constitutively active Tyk2
K930R: catalytically dead Tyk2
Y1054/1055F: un-phosphorylatable Tyk2

2D culture Flag-Tyk2 overexpressed

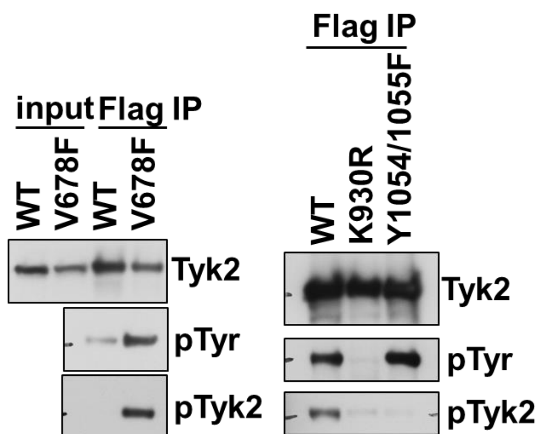


Figure 3.14: The Tyk2 constitutively active mutant has higher levels of phosphorylation and the kinase dead and activation loop phosphorylation mutants have lower levels of phosphorylation. Mcf10a cells stably expressing Flag-tagged wild type Tyk2 (WT) kinase dead Tyk2 (K930R) constitutively active Tyk2 (V678F) or activation loop mutant Tyk2 (YY1054/1055FF). Lysates from these cells in 2D culture isolated were analyzed by SDS-PAGE and were probed for Tyk2, a pan-phospho tyrosine antibody (pTyr) and an activation loop phosphorylation specific antibody (pTyk2).

To determine whether any of these mutants have a dominant negative effect on the cell phenotype, we stably overexpressed these constructs, including the tagged wild type Tyk2, in normal Mcf10a cells and assayed their effects on invasive acini formation. When analyzed at day 5, the time when we normally collect our cultures for analysis, we found that overexpression of the kinase dead Tyk2 mutant had a modest effect at low rigidities – causing about a 20% increase in invasion compared to control cells (Figure 3.15). The constitutively active mutant, however, did not appear to have any inhibitory effects on cell invasion at higher rigidities (Figure 3.15). At day five, the V678F Tyk2 expressing cells had no significant changes in invasive acini formation at any rigidity from the control cells (Figure 3.15). Interestingly, however, when we looked at the phenotype of the cells over the duration

of the five days that they were in culture, we found that there was initially a strong phenotypic difference in the V678F Tyk2 expressing cells that faded over time in culture (Figure 3.16). At day three in culture Mcf10a cells expressing the V678F Tyk2 were significantly less invasive than control cells both at 320pa and at 5700pa, but by day five, these cells appeared to be able to invade. The un-phosphorylatable mutant, however, did not have an effect on cell phenotype at any rigidity at any time-point (Figure 3.17). This suggests that the effect of the kinase dead mutant is due to its inability to phosphorylate its substrate, not due to its inability to be activated. Taken together, these results indicate that Tyk2 mutants altering its kinase activity are sufficient to change the phenotype of Mcf10a cells in 3D culture.

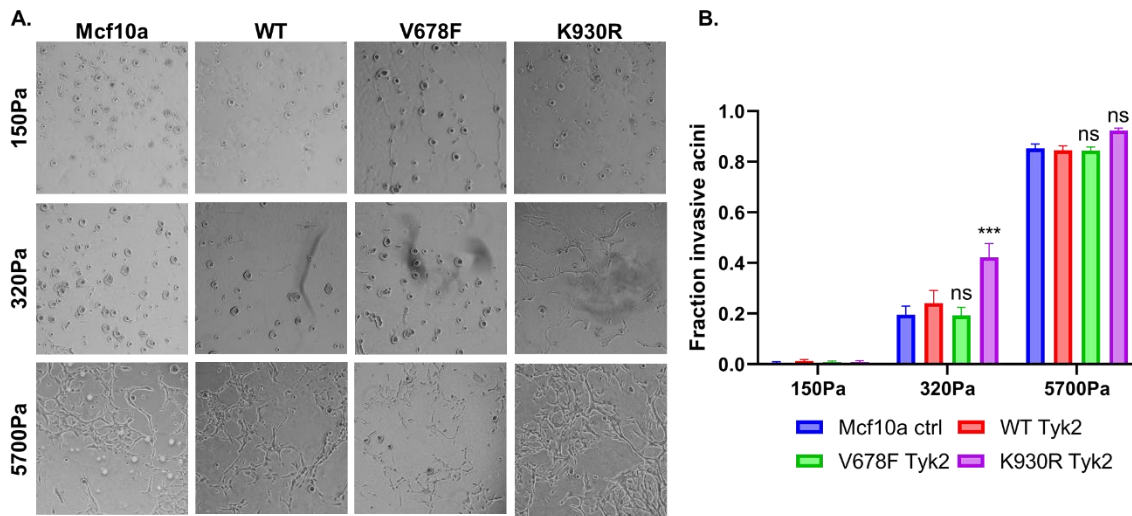


Figure 3.15: Tyk2 kinase dead mutant expression increases invasion at low rigidities. A. Brightfield images of control Mcf10a cells, cells overexpressing WT Tyk2, kinase dead Tyk2 (K930R) or constitutively active Tyk2 (V678F) cultured in 3D at 150Pa, 320Pa or 5700Pa. B. Quantification of the fraction of invasive acini for the cells shown in A. *** P<0.001. ns: not significant.

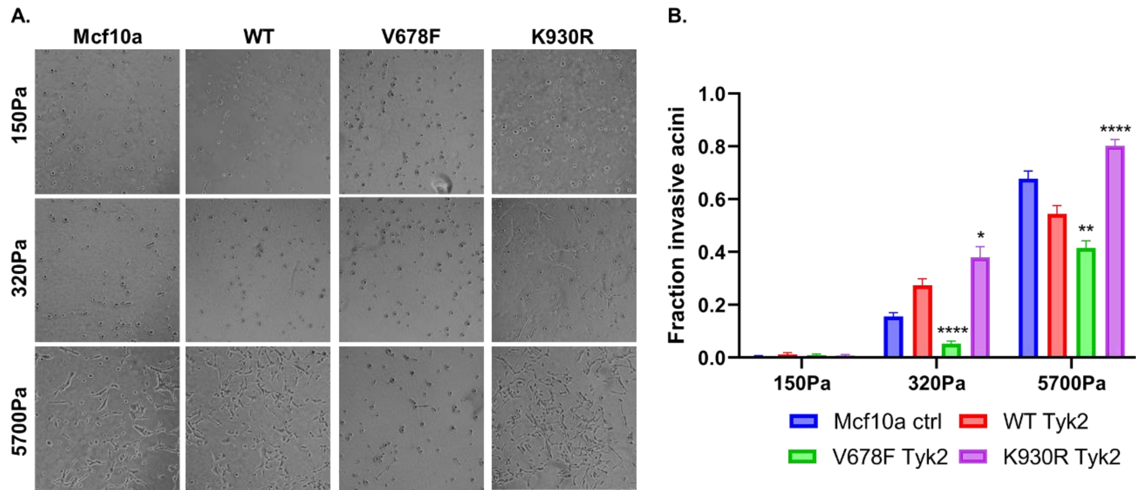


Figure 3.16: Tyk2 constitutively active mutant overexpression reduces invasion at day 3, Tyk2 kinase dead mutant overexpression enhances invasion at day 3. A. Brightfield images of control Mcf10a cells, cells overexpressing WT Tyk2, kinase dead Tyk2 (K930R) or constitutively active Tyk2 (V678F) cultured 3D at 150Pa, 320Pa or 5700Pa for 3 days B. Quantification of the fraction of invasive acini for the cells shown in A. * P<0.05 ** P<0.01 **** P<0.0001.

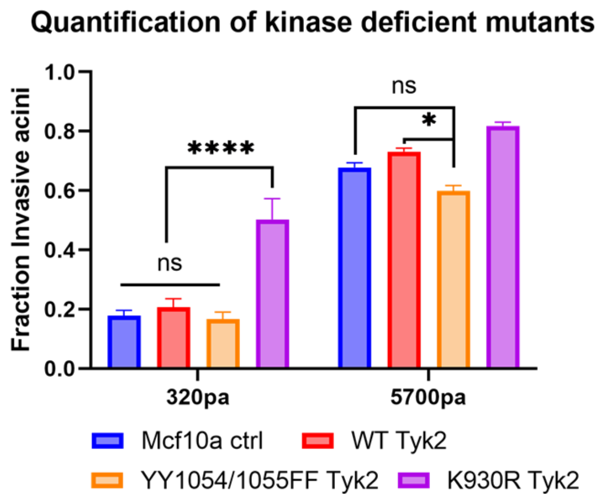


Figure 3.17: Activation loop phosphorylation mutant Tyk2 does not induce invasion. Quantification of the fraction of invasive acini after 5 days in 3D culture of Mcf10a cells expressing wild type (WT) Tyk2, activation loop phosphorylation site mutant Tyk2 (YY10541055FF) or kinase dead Tyk2 (K930R) * P<0.05 **** P<0.0001.

To begin to understand why the phenotype of the V678F mutant was decreasing over time in culture we started to look at the expression levels of the different Tyk2 mutant constructs. When measuring Tyk2 mRNA levels by qPCR we found that the V678F mutant expression level was slightly decreased at day five, both in low and high rigidities (Figure 3.18). Additionally, when examining Tyk2 by immunofluorescence we saw that at day three there was robust expression of the wild type and mutant constructs indicated by flag staining. However, immunofluorescence done at day five showed that the V678F Tyk2 intensity was decreased (Figure 3.19). The intensity of WT Tyk2 or the K930R mutant did not change with respect to how many days the cells had been in culture (Figure 3.19). This indicates that expression of the V678F Tyk2 construct is downregulated and explains the limited effect this construct has on cell phenotype after five days in culture. These data suggest that constitutive activation of Tyk2 is sufficient to block invasion in Mcf10a cells, albeit limited due to downregulation. Additionally these data indicate that overexpression of a kinase dead Tyk2 had a dominant negative effect on endogenous Tyk2 to drive invasion at low stiffness.

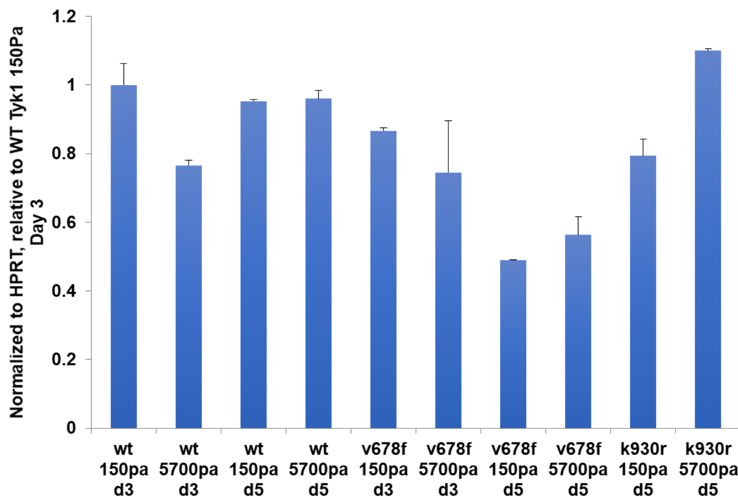


Figure 3.18: Tyk2 V678F expression is reduced at day 5 as compared to day 3 in 3D culture. Mcf10a cells expressing wild type Tyk2, constitutively active Tyk2, or kinase dead Tyk2 were cultured at 150Pa or 5700Pa for 3 or 5 days. Tyk2 mRNA expression level in these cells was measured by qPCR, normalized to HPRT and shown relative to wild type Tyk2 expression cells at 150Pa at day 3.

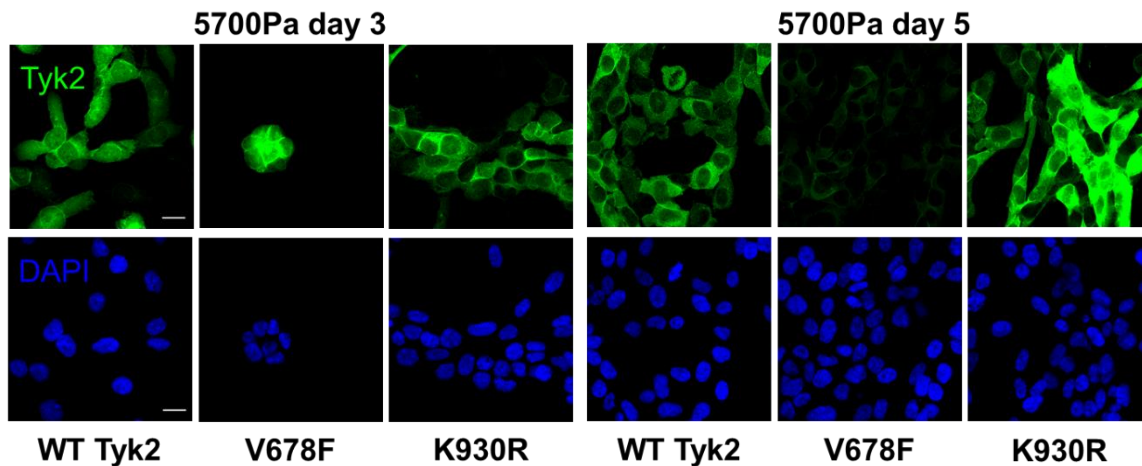


Figure 3.19: Constitutively active Tyk2 expression is reduced at high rigidities after 5 days in 3D culture. Confocal immunofluorescence of Mcf10a cells expressing wild type (WT) Tyk2, constitutively active Tyk2 (V678F) or kinase dead Tyk2 (K930R) cultured at 5700Pa for 3 days or 5 days. Stained for Twist1 (green) and DAPI for the nuclei (blue). Scale bar 20μM.

3.6 Conclusions

In this chapter we provided evidence that Tyk2 kinase activity is decreased upon increasing rigidities. We assessed Tyk2 activation status through examining phosphorylation changes in Tyk2 at different rigidities and by examining phosphorylation changes to a substrate of Tyk2, Stat3. We found that Tyk2 phosphorylation decreased with rigidity, as did Stat3 phosphorylation. We further show that Tyk2 kinase activity is required to maintain a non-invasive state and cytoplasmic Twist1 localization at low rigidities through affecting upstream regulators such as Lyn and EphA2. Treatment with Tyk2 inhibitors was able to stimulate invasion and nuclear translocation of Twist1. Lastly we showed that Tyk2 mutants which altered the activity of the kinase were sufficient to drive a change in cell phenotype. All together these data indicate that Tyk2 kinase plays a major role in this mechanosensing pathway. The phosphorylation of endogenous Tyk2 at low rigidities could only be observed

with a pan phospho-tyrosine antibody and not with an activation loop phosphorylation specific antibody. The levels of Stat3 phosphorylation additionally, were relatively low even at low rigidities compared to cytokine-induced Stat3 phosphorylation. The lack of robust signal for pTyk2 at any rigidity and the weak phosphorylation of Stat3 means that Tyk2 is probably not activated through cytokine stimulation in this system. Generally, when Tyk2 is activated in such a way, there is a sharp and transient increase in the pTyk2 signal, which we do not see in our system. This could mean that it is activated transiently very early on during rigidity sensing, and that we are simply missing the window that it is activated in since we take protein lysates from the cells five days after seeding. However, short time-course experiments with the Tyk2 inhibitor generally rule that hypothesis out. Additionally, expressing a mutant of Tyk2, which is unable to be phosphorylated on the activation loop, did not induce the same phenotype change within our system as the kinase dead Tyk2 mutant did. This indicates that while Tyk2 activity is important for regulating this Twist1 mechanosensing pathway, cytokine stimulated activation indicated by activation loop phosphorylation is not. The kinase activity of Tyk2 does appear to play a central role in its ability to regulate this pathway, however, as there is no significant difference in the amount of invasion at low rigidities with Tyk2 knockdown as compared to Tyk2 inhibition. This suggests that Tyk2 kinase activity is responsible for the entire effect Tyk2 is having on establishing epithelial identity.

Research from other labs has shown that Tyk2 can possess kinase activity, particularly auto-phosphorylation capabilities, without cytokine stimulation or phosphorylation of activation loop tyrosines (Gakovic et al., 2008). Our results suggest that basal Tyk2 activity is actually physiologically relevant and that the function of Tyk2 in this system does not require the activation of the kinase by cytokine stimulation. The results from Stat3 indicate that Tyk2 is still able to phosphorylate, albeit at a low level, a known substrate and this

presents the possibility that Tyk2 is able to phosphorylate an important downstream regulator of the mechanosensing pathway with this basal level of activity. Alternatively the ability of Tyk2 to auto-phosphorylate may be important, or perhaps there are structural changes that are important for regulating the access of Tyk2 to important downstream regulators. Further characterization of potentially relevant downstream effectors of Tyk2 will be critical for answering these questions.

The strong effect of Tyk2 inhibition on upstream mechanosensing pathway members was somewhat surprising. We discussed in chapter 2 how Tyk2 knockdown had no effect at 150pa in the Mcf10a cells compared to the Eph4ras cells, but only had an effect starting at 320pa. We reasoned that this was due to the level of basal invasion – at 150pa Ephr4as cells had a non-zero level of invasion, whereas the Mcf10a cells didn't have a non-zero level of invasion until 320pa. When we examined the effects of Tyk2 inhibition on EphA2, there was only a slight increase in serine phosphorylated EphA2 at 150pa in the inhibitor treated cells, which coincides with the lack of increase in invasion of these cells at 150pa. These data support the hypothesis that Tyk2 inhibition of this EphA2-Lyn-Twist1 signaling cascade is important for maintenance of an epithelial phenotype at low rigidities, but that removing this inhibitory pathway by inhibiting Tyk2 is not sufficient to drive the activation of this pathway at 150Pa. This implies that there must be additional activation events that occur to initiate this signaling cascade, and that these regulators are only activated at rigidities higher than 150pa. This is in line with our general hypothesis that Tyk2 activity is an inhibitory pathway and that Lyn is an activating pathway. The results obtained here indicate that these pathways are more closely related than we anticipated, but still suggests that in addition to reduced Tyk2 kinase activity, there may be additional signals that activate Lyn fully at high matrix stiffness.

The result that the constitutively active V678F Tyk2 mutant seems to be downregulated in our system is both surprising and somewhat predicable. The literature suggests that Tyk2 has many mechanisms for downregulating or de-activating Tyk2 upon cytokine stimulation. However, when other groups examined Tyk2 mutants including this V678F, and the homologous mutation in mice (K923E) to our kinase dead K930R, they did not find similar results with respect to stability. One group found that the kinase dead mutant was unstable, and was degraded through the autophagy-lysosomal pathway (Prchal-Murphy et al., 2012). Another group found that the hyper-activation mutant V678F had no effect on protein stability (Gakovic et al., 2008). This is the direct opposite of what we observed in our system – the kinase dead mutant was stable, whereas the constitutively active mutant appeared to be unstable. This could be a regulatory mechanism specific to our cells or specific to our culture method or even specific to rigidity. Importantly, the group that found decreased stability with their kinase dead Tyk2 mutant also saw a decrease in Tyk2 protein levels upon Tyk2 inhibition, which we did not see with our inhibitors (Prchal-Murphy et al., 2012). This further indicates that the mechanism by which this particular mutant is de-stabilized is probably specific to our system. There are several mechanisms by which this could be occurring – several phosphatases and ubiquitin ligases are known to affect Tyk2 stability. All of these proteins and mechanisms need to be examined as they could be relevant for decreases in endogenous Tyk2 activity at high rigidities. Although it is discussed in the next chapter in more detail, it is important to note here that we do not see changes in Tyk2 expression with respect to rigidity so phosphatase regulation or auto-inhibition or binding of proteins to the activation loop – such as the SOCS family of proteins – are much more likely that to be relevant than degradation mechanisms.

Since we saw a phenotype with the kinase dead Tyk2 mutant, we intend to use that mutant to probe for differential Tyk2 interacting proteins in the future. Due to technical

limitations of the 3D culture system, performing mass spectrometry on an endogenous Tyk2 immunoprecipitation proved to be technically challenging. As an alternative, we have decided to perform IP mass spectrometry on overexpressed wild type or kinase dead Tyk2 at low and high rigidities. Kinase dead Tyk2 is able to drive invasion at lower rigidities, so we hypothesize that it may be interacting with a separate set of proteins than the wild type Tyk2 is interacting with at low rigidities, and we are hopeful that we can parse out those differential interacting proteins. While performing this experiment on overexpressed proteins presents its own set of challenges – such as possible high background and false candidates due to the overexpression system – it is technically easier to procure enough protein using a tagged exogenous system such as this. As shown in figure 3.20, approximately 50 ng of Tyk2 can be immunoprecipitated from 5 mg of lysate from 3D culture using commercially available Flag-conjugated magnetic beads. The band at the size of Tyk2 is specific to cells overexpressing Tyk2, and was not pulled out of lysates of parental Mcf10a cells. This amount of initial lysate (5 mg) can be isolated from about five 50 mm PA gel coverslips of 3D cultured cells. These 50 mm gels are the upper limit in size of gel that we can make. Scaling up this IP of exogenous Tyk2 to about triple this test run should generate enough immunoprecipitated Tyk2 to perform and IP-mass spec, and this is technically feasible at about 15 of 50 mm coverslips per condition.

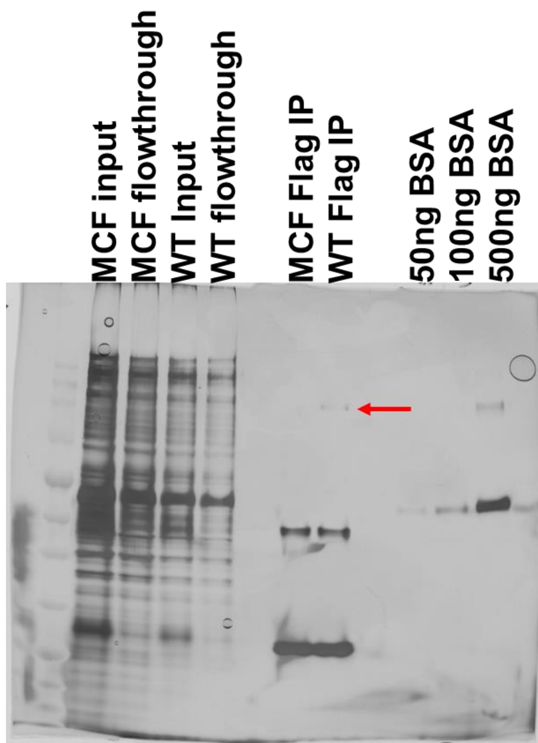


Figure 3.20: Flag-WT Tyk2 immunoprecipitated from Mcf10a cells at low rigidities for IP-mass spectrometry. Immunoprecipitation of exogenous Flag-tagged wild type Tyk2 from Mcf10a cell lysates resolved by SDS-PAGE and silver stained. BSA standards were used to assay for the amount of protein pulled down. Control Mcf10a cells were used to determine specificity of the IP. Red arrow indicates Tyk2 band.

3.7 Acknowledgements

Chapter 3, in full, is currently being prepared for submission for publication of the material. Majeski, H.E., Hu, Z., Fattet, L., Yang J. Tyk2 regulates matrix stiffness-driven EMT and metastasis. *In preparation.* The dissertation author was the primary investigator and first author of this material.

Chapter 4

Tyk2 localization change in response to rigidity

4.1 Introduction

The role of Tyk2 in cancer is only recently being explored. The other three Jak-family kinases were the primary targets for cancer therapeutics and are all generally thought to be oncogenic. Tyk2 was initially a target for auto-immune disease and has only in the past decade or so been examined in the frame of cancer (O'Shea et al., 2013; Schwartz et al., 2017). Currently, the role of Tyk2 in cancer is found to be diverse. Tyk2 plays a clear role in immunosurveillance, and in the context of the immune system regulating cancer growth – it is tumor suppressive (Leitner et al., 2017). The role of Tyk2 within solid tumors is less clear, however. Several studies have shown that there appears to be an upregulation of Tyk2 in some cancer cell lines (Christy and Priyadharshini, 2018; Ide et al., 2008; Santos et al., 2015; Silver et al., 2004; Song et al., 2008; Zhu et al., 2009). One of the downstream targets of Tyk2, Stat3 has been implicated in tumor growth, EMT induction and cancer stem cell maintenance (Yu et al., 2009). Stat3 is generally downstream of Jak2 activation in the context of breast cancer however, and the role – if any – of Tyk2 in this activation is not well described (Jin, 2020). On the other hand, there are also several studies that indicate that lower levels of Tyk2 are correlated with better prognosis in patients (Sang et al., 2012; Uhlen et al., 2010; Wang et al., 2019b). Sang et al. examined the expression of Tyk2 in different subtypes of breast cancer in more detail. Tyk2 actually came out of a proteomic screen they performed searching for proteins with differential expression in breast cancers of different hormone receptor expression patterns. Tyk2 expression, assayed by immunohistochemistry (IHC) of breast cancer patient samples, decreased with increasing tumor stage, and was particularly downregulated in patients with local metastases (Sang et al., 2012). When they

tried to explain these results with *in vitro* experiments, they found that Tyk2 knockdown actually decreased transwell migration in MCF10a cells – confounding their own results and adding to the confusion within the field about the role of Tyk2 in breast cancer.

Interestingly, there appeared to be a rather robust Tyk2 localization change in human breast cancers by IHC, although this was not discussed in the study that reported the data. Tyk2 protein appeared more membranous in normal human breast epithelial cells, while Tyk2 protein in the cancerous samples examined did not appear to be on the membrane. The localization of Jak kinases, including Tyk2, to the membrane is itself well characterized. While Tyk2, like other Jak family kinases, does not contain any transmembrane domains, it is thought to interact strongly and constitutively with its upstream receptors such as IFNAR1, which tethers it to the cell membrane (Gakovic et al., 2008). However, there is very limited literature describing Tyk2 localization in cells and there are no reports on howTyk2 comes off the membrane or if it dissociates from cognate receptors. Tyk2 degradation has been described – both via the proteasome and the lysosome (Muller et al., 2014; Prchal-Murphy et al., 2012). It is also well established that Tyk2 is required to maintain membrane localization of IFNAR1, and that the loss of Tyk2 leads to recycling of the receptor (Gauzzi et al., 1997; Ragimbeau et al., 2003). Tyk2 trafficking with any of its cognate receptors has not been described either. Although only one published study describes the potential regulation of Tyk2 expression and localization in breast cancer, the results are interesting and provided a basis for our own work in examining the regulation of Tyk2 in human breast cancers. Assaying for Tyk2 activity in human breast cancer samples did not work – the pTyk2 antibody was too weak and non-specific for IHC or IF. Upregulation of Stat3 phosphorylation by other Jak family kinases in a variety of cancers confounds the utility of using pStat3 as a readout of Tyk2 activation in human breast cancer patient samples. So, to understand how Tyk2 might be regulated in human breast cancer, we first examined Tyk2 expression and

localization in our 3D culture system over a range of rigidities. We then determined whether those changes we saw *in vitro* could be correlated with changes in human breast cancer patient samples.

4.2 Tyk2 localization and expression over a range of rigidities

In order to characterize the role of Tyk2 in human breast cancers, we first examined Tyk2 expression levels and localization in the 3D culture system and determined how rigidity may or may not affect this. Neither the mRNA level nor protein level of Tyk2 show a difference with respect to rigidity changes (Figure 4.1). This indicates that Tyk2 protein is not being degraded as a result of increased rigidity. We next examined Tyk2 localization through immunofluorescent staining of Mcf10 and Eph4Ras cells at different rigidities and found a striking difference in Tyk2 localization with respect to rigidity (Figure 4.2). At lower rigidities, the cells formed acini and presented robust Tyk2 membrane localization, which is again characteristic for Jak family kinases. At higher rigidities, however, Tyk2 signal at the membrane was completely diminished and became more diffused within the cytoplasm (Figure 4.2). It is important to note that this result is true using two different antibodies for Tyk2 – one for human Tyk2 and the other for mouse Tyk2 as each antibody only recognizes Tyk2 of the corresponding species (Figure 4.3). With staining of the mouse cells with the mouse specific Tyk2 antibody we occasionally see robust nuclear staining at high rigidities, which we do not ever see in the human cells with the human specific Tyk2 antibody. We believe that the nuclear staining is a non-specific signal for a number of reasons. When we used the mouse specific Tyk2 antibody on human Mcf10a cells, we observed this nuclear signal at high rigidities, even though western blots indicate that this antibody does not recognize human Tyk2 (Figure 4.4). Furthermore, when we processed Tyk2 knockdown cells for immunofluorescence, we still observed this nuclear staining, even when specific Tyk2

signals (such as the membrane staining) were lost (Figure 4.4).Taken together, these results indicate that Tyk2 dis-localizes from the membrane at high rigidities and adopts a more diffused cytoplasmic staining. This is similar to the staining patterns observed in normal human breast tissues compared to cancerous breast tissues, so we were very curious to examine the subcellular localization changes of Tyk2 *in vitro* and to determine whether Tyk2 localization change is correlated with changes in matrix composition in human breast tissues.

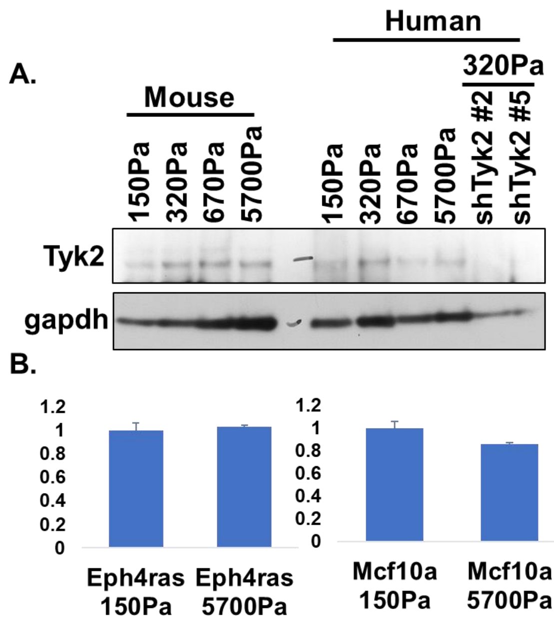


Figure 4.1: Tyk2 expression levels do not change with rigidity. A. Eph4ras cells (mouse) and Mcf10a cells (human) were lysed from a range of rigidities (150Pa, 320Pa, 670Pa and 5700Pa). Lysates were analyzed by SDS-PAGE gel and probed for Tyk2 and gapdh as a loading control. Tyk2 knockdown cells were additionally asses for specificity. B. RNA was isolated from Eph4ras and Mcf10a cells from 150Pa and 5700Pa and analyzed by qPCR. Values are normalized to control genes GAPDH (mouse) or HPRT (human) and shown relative to 150pa. No significant changes were observed.

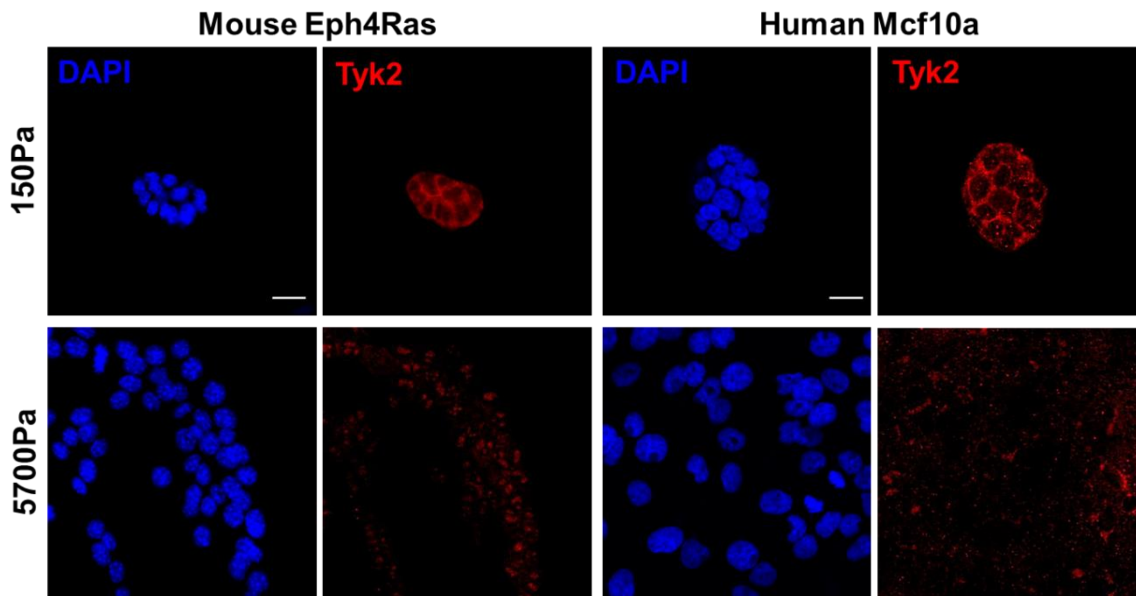


Figure 4.2: Tyk2 localization changes with rigidity. Confocal immunofluorescence images of mouse Eph4ras cells and human Mcf10a cells cultured at 150Pa and 5700Pa. Stained for Tyk2 (red) and DAPI for the nuclei (blue). Scale bar 20 μM.

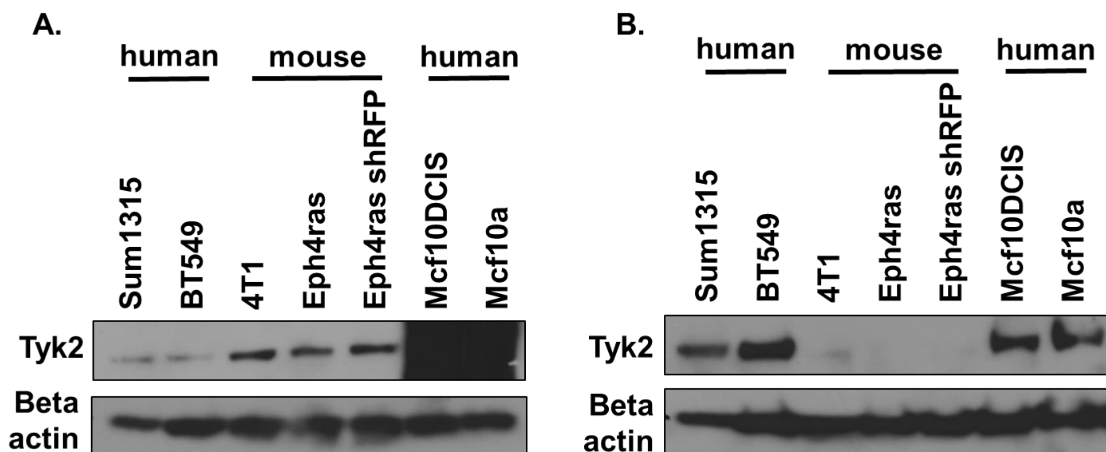


Figure 4.3: Mouse and human antibodies are specific only to mouse or human Tyk2. Lysates from a panel of human and mouse cell lines were isolated from regular 2D culture and run on an SDS-PAGE gel. The gel was probed for a (A.) mouse specific Tyk2 antibody or (B.) human specific Tyk2 antibody. Beta actin was probed as a loading control.

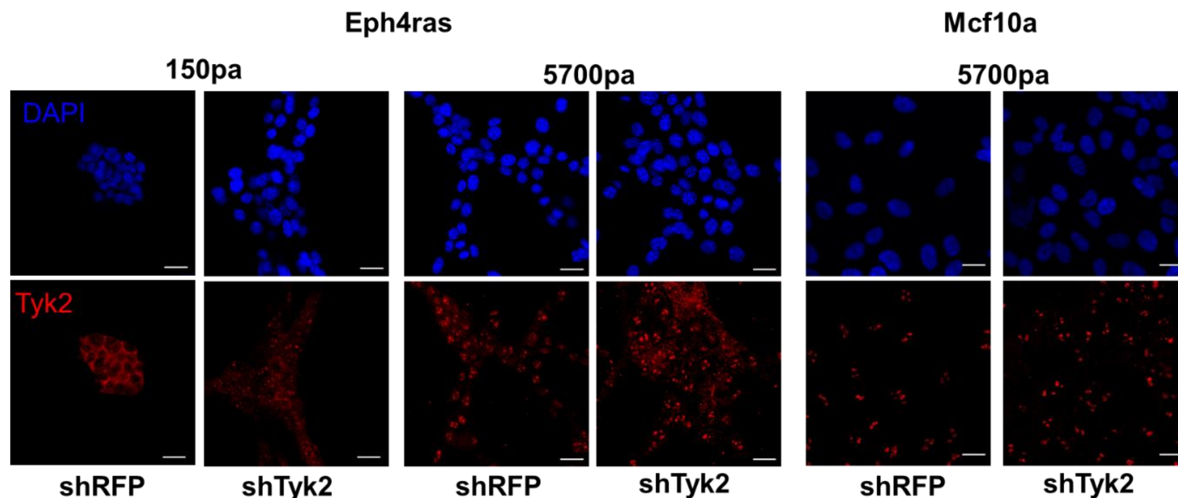


Figure 4.4: Mouse Tyk2 antibody shows non-specific staining in the nucleus. Confocal immunofluorescence images of mouse Eph4ras cells or human Mcf10a cells cultured at 150Pa or 5700Pa stained for Tyk2 (red) using the mouse specific antibody and DAPI for the nuclei (blue). Tyk2 knockdown cells were used as a control for positive Tyk2 staining. Scale bar 20 μM.

4.3 Tyk2 localization changes in response to rigidity independent of changes in cell polarity, cell-cell junctions, or alterations of the downstream mechanotransduction pathway components

Tyk2 translocation from cell membrane to the cytoplasm occurs between low and high rigidities. However, it is unclear whether this translocation event is directly regulated by the rigidity or is the consequence of the morphological changes in response to rigidity changes. In Mcf10a cells, Tyk2 localization change was assessed over a range of rigidities and it appears to dislocate from the membrane between 320pa and 670pa (Figure 4.5). This is also the rigidity where Tyk2 inhibition or Tyk2 knockdown has an effect on the cell invasion phenotype. We wondered, then, whether this localization change could be the result of Tyk2 inactivation, or even be potentially driving the loss of Tyk2 activity with respect to rigidity. The activity of other Jak kinase family members is shown to be regulated by their interactions with receptor proteins (Funakoshi-Tago et al., 2008; Zhou et al., 2001). To understand whether

changes in Tyk2 activity would affect Tyk2 localization, we examined the localization of Tyk2 by immunofluorescence in Mcf10a cells treated with Tyk2 inhibitors. These cells showed more diffuse Tyk2 staining at lower rigidities (320pa) than the control cells (Figure 4.6). This suggests that loss of Tyk2 activity may change Tyk2 localization. However, there are several changes that occur in these cells between low and high rigidities that could affect the localization of this protein, and these morphological changes also occur in inhibitor treated cells at lower rigidities. The cells go from a partially polarized acinar structure with intact cell-cell adhesion complexes to more mesenchymal cells that spread on the gel and do not form 3D structures and have different cell-cell junctional complexes and are not polarized. Any number of these changes could alter Tyk2 localization, and since these changes all also occur at lower rigidities in inhibitor treated cells, we cannot rule these factors out as being causative for the localization change of Tyk2.

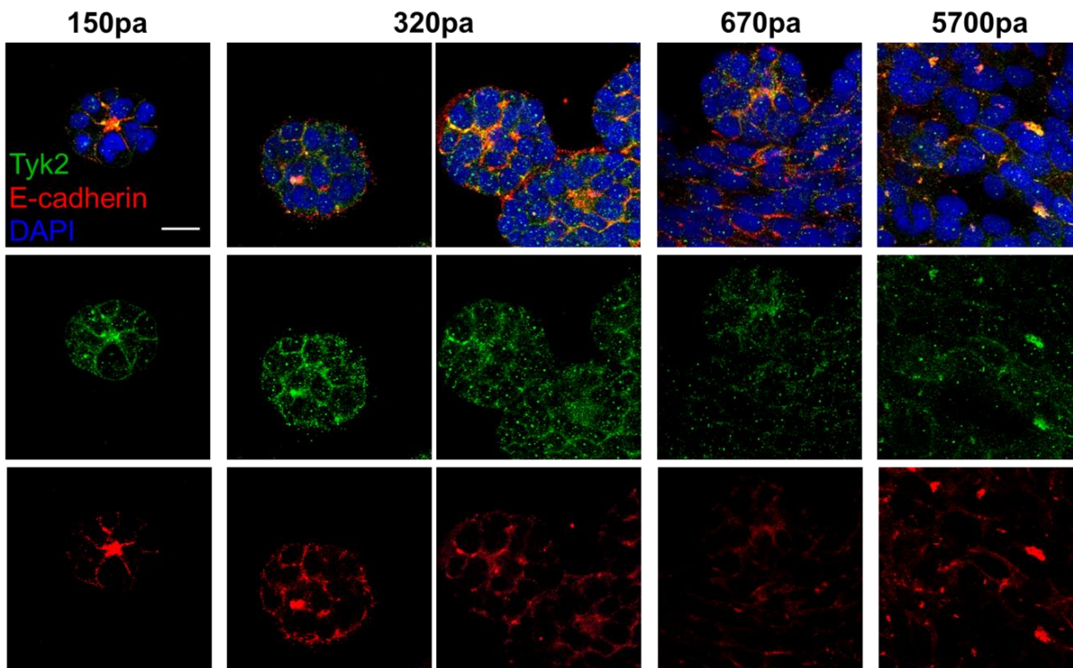


Figure 4.5: Tyk2 localization changes between 320pa and 670pa. Confocal immunofluorescence images of Mcf10a cells cultured at 150Pa, 320Pa, 670Pa and 5700Pa stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Scale bar 20 μM.

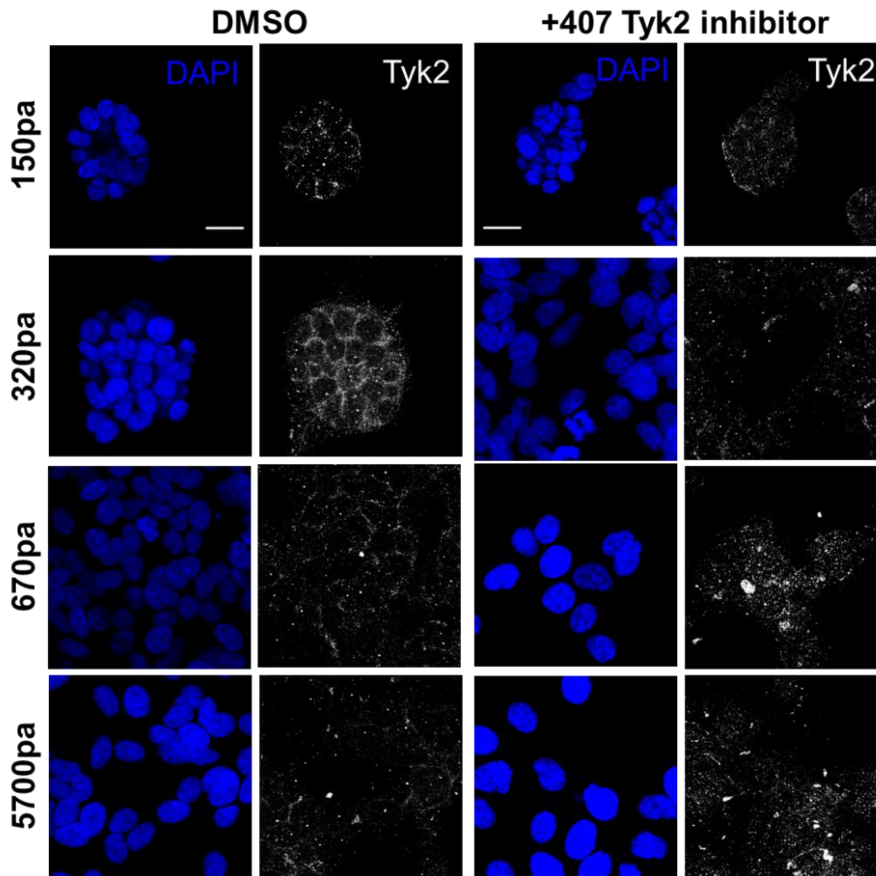


Figure 4.6: Tyk2 localization changes with Tyk2 inhibitor treatment. Confocal immunofluorescence images of Mcf10a cells treated with DMSO or the catalytic Tyk2 inhibitor (407) cultured at 150Pa, 320Pa, 670Pa and 5700Pa stained for Tyk2 (white) using the human specific antibody and DAPI for the nuclei (blue). Scale bar 20 μM.

In order to address whether epithelial polarity regulates Tyk2 localization, we switched to a colon carcinoma cell line Caco2. Caco2 cells form fully polarized acini when grown in 3D culture. Mcf10a cells will form partially polarized structures, especially if kept in 3D culture for time periods around two weeks. Mcf10a cells cannot fully polarize, however, because they lack wild type crumbs3 gene, a crucial polarity protein (Fogg et al., 2005). To determine whether epithelial polarization affects Tyk2 localization we utilized an intestinal epithelial cell line called Caco2, which form fully polarized acini with a hollow lumen

surrounded by a single layer of cells (Jaffe et al., 2008; Zhang et al., 2003). Examining Tyk2 expression in this cell line would allow us to visualize whether fully intact polarity was important for Tyk2 localization, if Tyk2 co-localized with any polarity markers, and if disturbing polarity caused a change in Tyk2 localization. What we found, however, was that Tyk2 localization on the membrane appeared to be unaffected by polarity, at least in these cells. Tyk2 localized to the membrane only in a small portion of the acini we examined, but most of the acini where Tyk2 was on the membrane were not completely polarized (i.e. had more than a single layer of cells surrounding the lumen) (Figure 4.7). In the fully polarized acini, Tyk2 did not localize to the membrane at all (Figure 4.7). This provides evidence that Tyk2 localization is not regulated by polarity, but does not indicate that polarity plays no role in Tyk2 localization within breast cells. Intestinal epithelial do have robust Tyk2 expression, and IHC on these tissues shows that Tyk2 is membranous in intestinal epithelial cells, but it is unclear whether there is any localization change in intestinal cancers (Uhlen et al., 2010). The Caco2 cell line utilized here is also a cancer cell line, so the localization may have already become dysregulated – although this hypothesis itself does indicate that the localization dysregulation is not due to polarity changes.

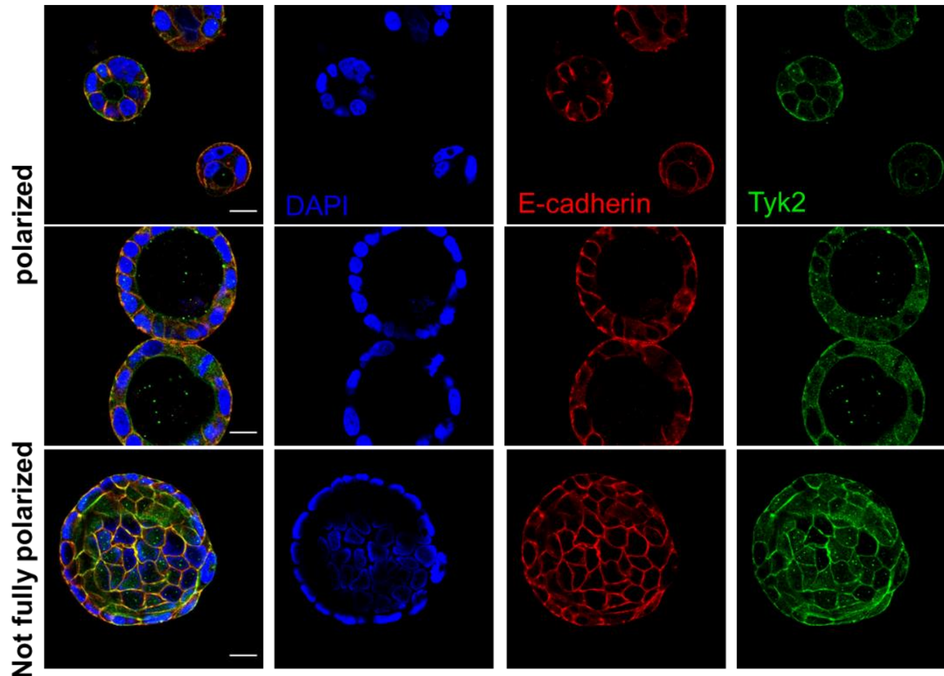


Figure 4.7: Tyk2 doesn't localize to the membrane in fully polarized Caco2 cells. Confocal immunofluorescence images of Caco2 cells cultured in 3D and stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Scale bar 20 μM.

Another way of examining the role of polarity in regulating the localization of Tyk2 is to disrupt the polarity in the breast 3D culture system. One way to do this is to examine single cells at low and high rigidities. Examining Tyk2 localization in single cells additionally removes any effects of cell-cell junctions on Tyk2 localization. Single cell culture in our 3D system is achieved by simply processing our cultures for immunofluorescence about 24 hours after seeding before cells form organoids. Unpublished data from our lab has shown that within 24 hours, Twist1 translocation into the nucleus at high rigidities can be observed in single cells. This indicates that the mechanosensing pathway that triggers the nuclear translocation of Twist1 has two important properties. One: mechanosensing occurs within 24 hours, and two: Twist1 mechanically regulated translocation does not require cell-cell interactions. If Tyk2 location change is purely a rigidity-sensing effect, Tyk2 should localize to

the membrane in single cells at low rigidities and to the cytoplasm in single cells at high rigidities.

Single cell immunofluorescence of endogenous Tyk2 proved to be technically very difficult due to the sensitivity of the antibody. In order to assess Tyk2 localization in single cells, we overexpressed Flag-tagged Tyk2. To evaluate whether overexpressed Tyk2 localization was still regulated in response to rigidity, we performed immunofluorescence on Mcf10a cells overexpressing wild type Tyk2 at low and high rigidities. Overexpressed Tyk2 protein localized correctly, even though there was a significant increase in the amount of protein. Wild type flag Tyk2 localized to the membrane at low rigidities, and became diffuse throughout the cell at high rigidities (Figure 4.8). This enabled us to use the Tyk2 overexpression system for single cell experiments. Single cell immunofluorescence revealed that Tyk2 was diffuse throughout the cytoplasm in single cells at high rigidities, and appeared to be membrane localized at low rigidities (Figure 4.9). Low rigidity localization was more difficult to determine, however, because the cells are both smaller and more rounded as single cells at low rigidities and this makes differentiating between membrane and the cytoplasmic space difficult. We utilized a cell membrane dye in these cultures to differentiate the membrane and the cytoplasm in these rounder cells. Tyk2 appeared to co-localize with the membrane dye and not in the cytoplasmic space at low rigidities (Figure 4.10). These data demonstrate that Tyk2 localization to the membrane at low rigidities is independent of epithelial polarity and cell-cell junctions and suggests that Tyk2 localization is specifically regulated by rigidity.

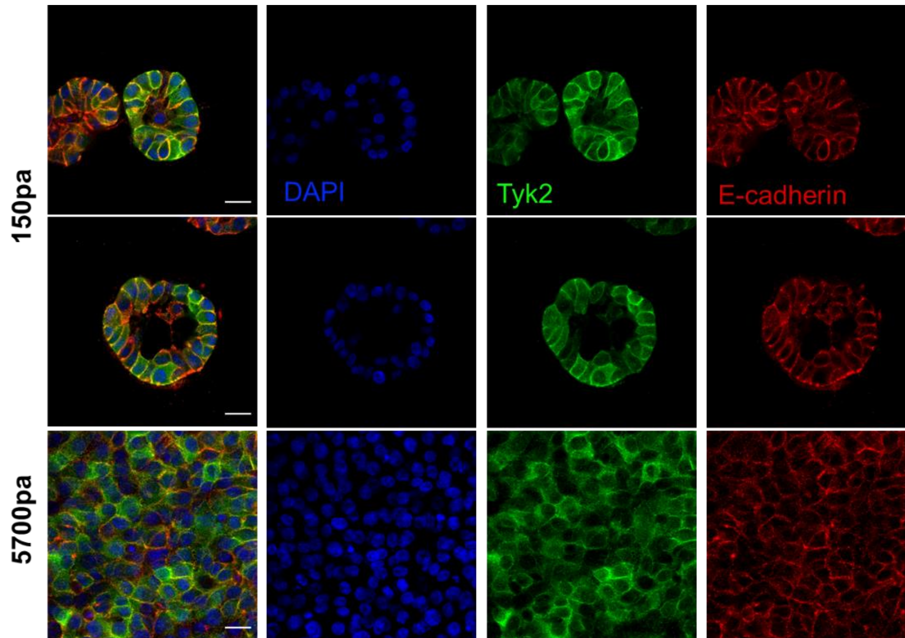


Figure 4.8: Overexpressed wild type Tyk2 localized to the membrane at low rigidity and cytoplasm at high rigidity. Confocal immunofluorescence images of Mcf10a cells expressing a Flag-WT Tyk2 cultured at 150Pa and 5700Pa and stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Scale bar 20 μM.

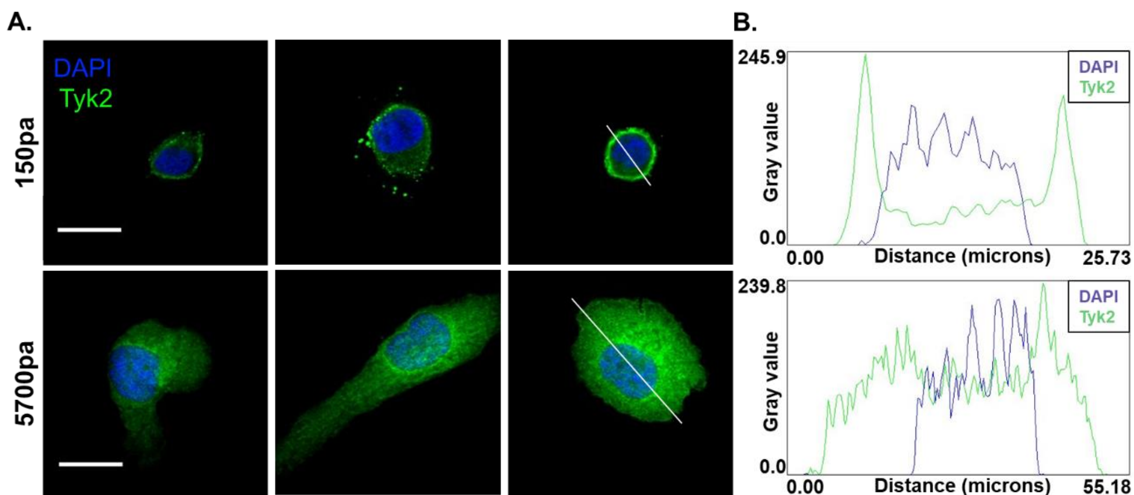


Figure 4.9: Wild type tyk2 localizes to the membrane at low rigidity and the cytoplasm at high rigidity in single cells. (A.) Confocal immunofluorescence images of Mcf10a single cells expressing a Flag-WT Tyk2 cultured at 150Pa and 5700Pa for 24 hours and stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Scale bar 20 μM. White lines on far-right images indicate lines used for B. (B). Line traces of fluorescence intensity over distance for the lines indicated in A for DAPI (blue) or Tyk2 (green).

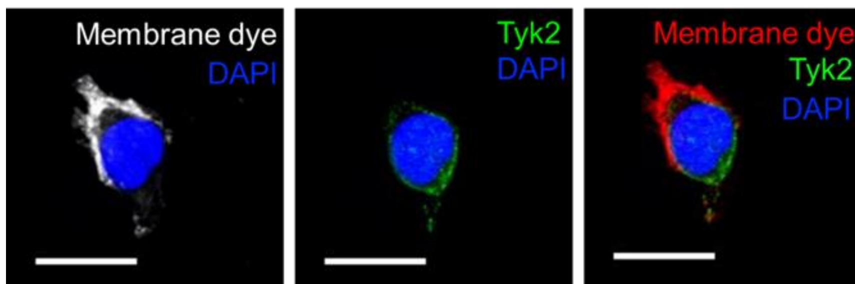


Figure 4.10: Tyk2 localizes with membrane dye at low rigidity. Confocal immunofluorescence images of Mcf10a single cells expressing a Flag-WT Tyk2 cultured at 150Pa and 5700Pa for 24 hours. Incorporated membrane dye is in red, stained for Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Scale bar 20 μM.

To demonstrate that the localization is due to rigidity sensing upstream of Tyk2 and not an downstream effect of cell morphological changes due to activation of the mechanosensing pathway, we assayed for Tyk2 localization at low and high rigidities in cells treated with the Lyn inhibitor Bafetinib. Cells treated with Bafetinib form acini at high rigidities and are not able to invade because Lyn is inactive and cannot phosphorylate Twist1. This is an interesting situation in which to probe Tyk2 because while mechanosensing upstream of Lyn should still be intact, the cells do not change their morphology. Therefore if Tyk2 is truly upstream of Lyn as we expect, then Tyk2 localization change should still occur at high rigidities even though the cells still form acini. Interestingly, we found that in Mcf10a cells Flag-WT Tyk2 was localized to the cytoplasm at high rigidities in both the control and Bafetinib treat cells (Figure 4.11). This indicates that even though the cells were forming acini due to lack of Lyn activity, Tyk2 localization was still changing with rigidity, independent of all of the structural, junctional and polarity changes that occur when invasion is induced.

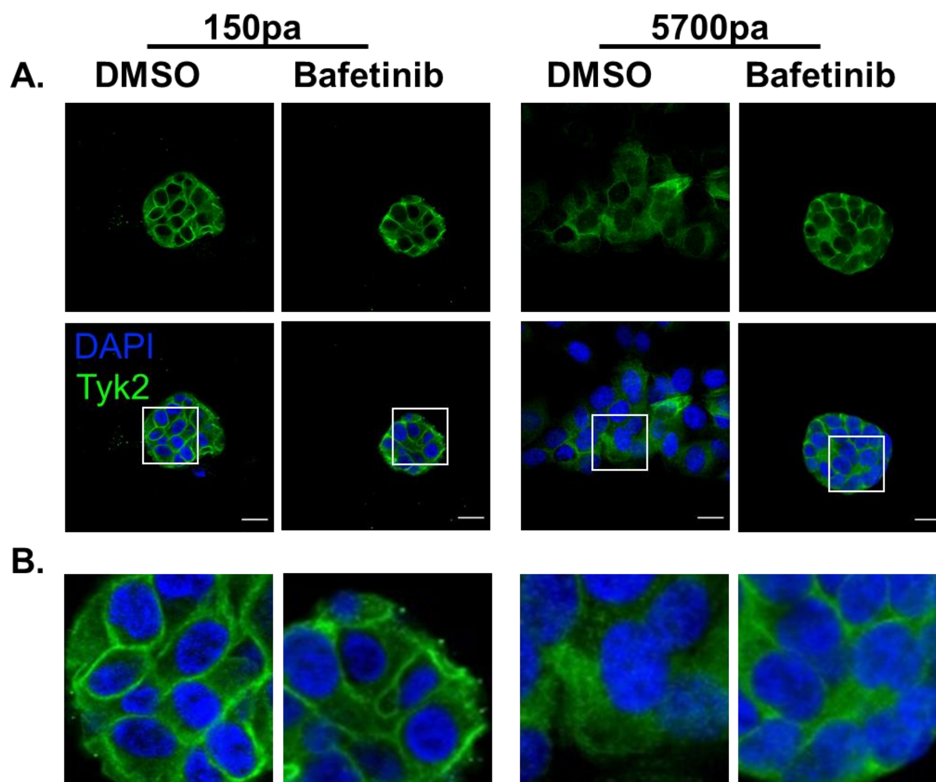


Figure 4.11: Tyk2 localizes to the cytoplasm at high rigidities in bafetinib treated cells. (A.) Confocal immunofluorescence images of Mcf10a single cells expressing a Flag-WT Tyk2 cultured at 150Pa and 5700Pa treated with DMSO or the Lyn inhibitor bafetinib. Stained for Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Areas in white represent increased magnification shown in B. Scale bar 20μM. (B.) Increased magnification for areas traced in a white box in the image directly above in figure A.

4.4 Tyk2 localizes to the membrane in normal human breast tissues and is cytoplasmic in breast tumor tissues

The reason why we initially wanted to examine Tyk2 expression and localization in our *in vitro* system was to eventually determine whether the expression and/or localization of Tyk2 at low and high rigidities was at all correlated with its expression and/or localization in normal and cancerous human breast tissue. The literature mentioned in the introduction of this chapter found that Tyk2 expression appeared to be inversely correlated with disease progression in breast cancer, and since Tyk2 localization appeared to be correlated in our *in*

vitro system, we wanted to determine whether Tyk2 localization is also appreciably altered in human tumor samples.

To do this, we first assessed Tyk2 expression via immunohistochemistry in normal human breast tissue. Tyk2 showed robust signals in the epithelial ductal tissue in all the normal breast samples we examined (Figure 4.12). This staining was relatively specific for the ductal structures. Interestingly, the Tyk2 signal appeared to be strongly membranous in these ductal tissues, very similar to the staining observed by immunofluorescence in our acini cultures (Figure 4.12). We then examined a tissue microarray (TMA) of human breast tumor samples. The TMA contains tumor cores from a variety of tumor stages, but was primarily from stage II tumors, with a few stage I and a few stage III/IV. Strikingly, in all breast tumor samples examined Tyk2 did not present any membrane staining (Figure 4.13). Instead, the Tyk2 signal was diffused throughout the cytoplasm in the breast tumor cells with some signals outside of the bulk tumor (Figure 4.13). Furthermore, the Tyk2 signal appeared to be much weaker in the tumor samples – potentially indicating a reduction in Tyk2 expression. However, the intensity difference observed could also be due to dispersion of the signal throughout the cell instead of concentrated on the membrane. This diffuse signal was observed in all tumor samples assayed, regardless of the stage of the tumor. We also did not find any tumors with a heterogeneous population where part of the tumor had cytoplasmic Tyk2 and part of the tumor had membranous Tyk2. To further determine Tyk2 localization, we performed immunofluorescence on these same normal and cancerous breast tissue samples and co-stained with E-cadherin, which localizes at epithelial cell membranes. The result was very similar to what we found in the IHC experiments. Tyk2 localized to the membrane in normal epithelial ducts and co-localized with E-cadherin well (Figure 4.14). All the breast tumor samples analyzed, regardless of stage, did not present Tyk2 membrane localization, and there was little co-localization with E-cadherin (Figure 4.14). Interestingly

there did seem to be some heterogeneity in Tyk2 intensity in the breast tumor samples that we were not able to pick up by IHC. These differences in intensity of the Tyk2 signal did not appear to correlate with tumor stage (Figure 4.14). Using both IHC and immunofluorescence we were able to observe Tyk2 localization change from membranous in normal tissue to cytoplasmic in cancerous tissue with no samples that had any heterogeneity in Tyk2 localization. The lack of samples with membrane Tyk2 staining suggests that mislocalization of Tyk2 likely occurs during early stage of tumor development.

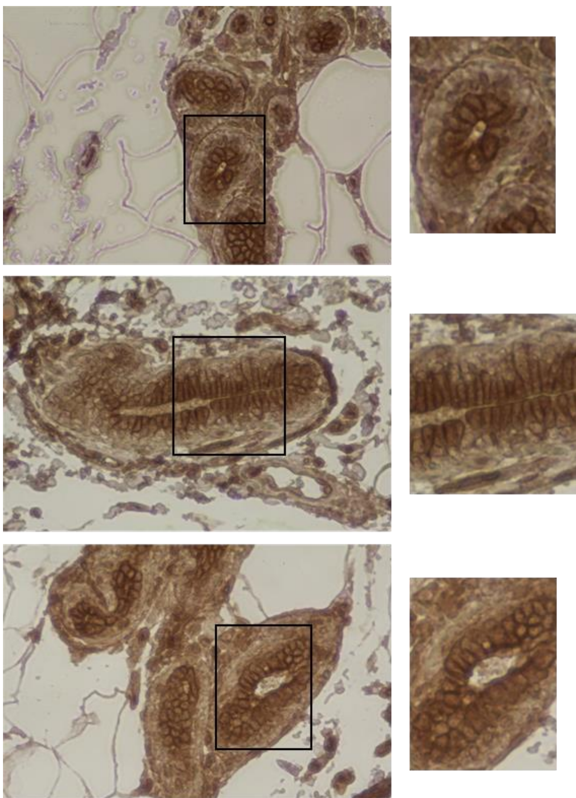
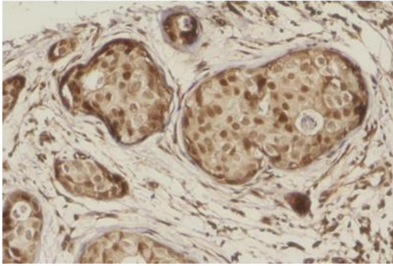
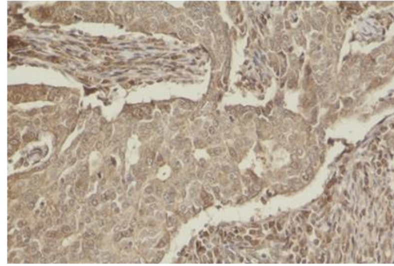
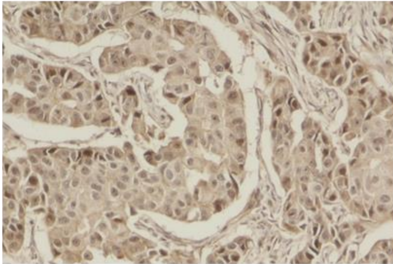


Figure 4.12: Tyk2 localizes to the membrane in mammary ductal epithelial cells in normal human tissue. Immunohistochemistry using the human specific Tyk2 antibody of normal human breast tissue, focused on the ductal structures. Black boxes indicate the location of the higher magnification on the right.







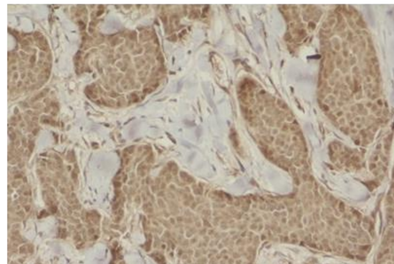


Figure 4.13: Tyk2 is cytoplasmic in all human breast cancer samples tested. Immunohistochemistry using the human specific Tyk2 on an array of human breast tumor samples. Example images of each tumor stage included in the array shown here.

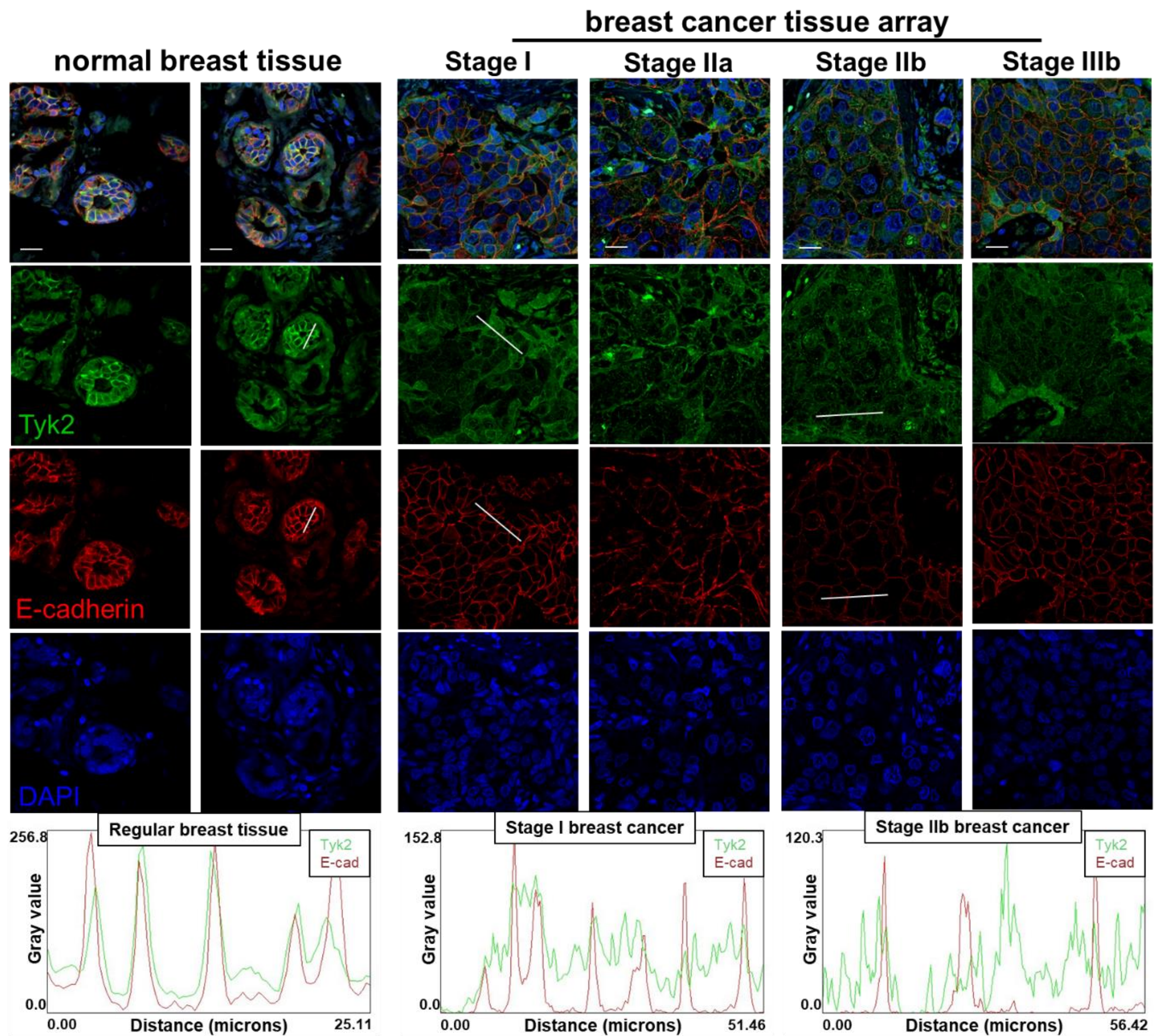


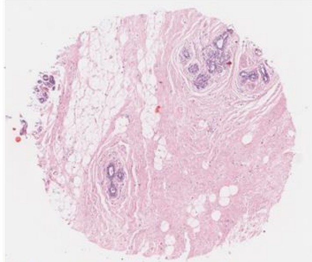
Figure 4.14: Tyk2 co-localizes on the membrane with E-cadherin in normal breast ductal epithelial tissues and is in the cytoplasm in all breast cancer samples. Immunofluorescence of normal human breast tissue and an array of human breast tumor samples. Stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). Example images of each tumor stage included in the array shown here. Scale bar 20 μM. Line traces of fluorescence intensity over distance for the lines indicated in the images above. E-cadherin intensity trace shown in red, Tyk2 intensity trace shown in green.

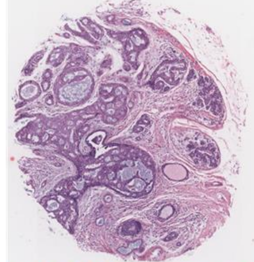
4.5 Tyk2 localization changes early in breast lesion development, and is correlated with changes in collagen organization

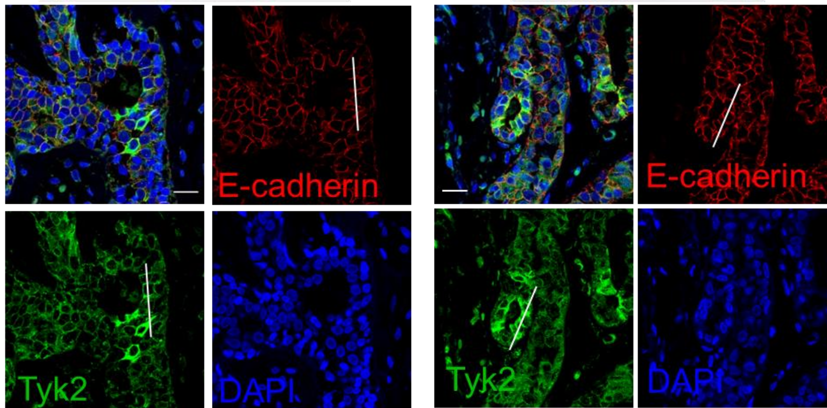
To understand the specific stages of tumor development where mislocalization of Tyk2 occurs, we examined another breast tissue TMA array that contains very early-stage breast lesions – named ductal intraepithelial neoplasias (DIN). The earliest stage of these lesions is called DIN1a or flat epithelial atypia. These samples largely resemble normal breast tissue – they have normal looking ductal architecture and are defined by hyperproliferation and cytological abnormalities (Said et al., 2015). The next stage is called DIN1b, or atypical ductal hyperplasia. These samples no longer look like normal breast tissue and are defined by hyperproliferation, cytological abnormalities and alterations in the architecture of the ducts (Tavassoli and Norris, 1990). The final two stages on this DIN array are DIN2 and DIN3 which are grade 2 and grade 3 ductal carcinoma *in situ* (DCIS), respectively. DCIS is an early stage tumor which develops within the ducts of the breast tissue but has not invaded into other areas of the breast or spread to any lymph nodes (Bendifallah et al., 2012). When we assayed for Tyk2 expression by immunofluorescence in these samples, we found that Tyk2 localized to the membrane in the majority of the DIN1a samples (Figure 4.15) and the staining appeared very similar to what is observed in normal human breast tissue. In DIN1b samples, about half of them presented membranous Tyk2 that co-localized with E-cadherin, while the other half showed more mixed staining or complete cytoplasmic Tyk2 signals (Figure 4.15). This DIN1b set of samples did appear to be the stage at which Tyk2 was coming off of the membrane because in all the low grade DCIS samples analyzed (grade 2 or grade 3 DCIS) Tyk2 was cytoplasmic and did not co-localize with E-cadherin (Figure 4.16), which is very similar to the tumor samples analyzed with respect to Tyk2 localization. Taken together, these data show that Tyk2 miclocalization to the cytoplasm from the membrane occurs very early on around the hyperplasia stage during breast tumor development (Figure 4.17).

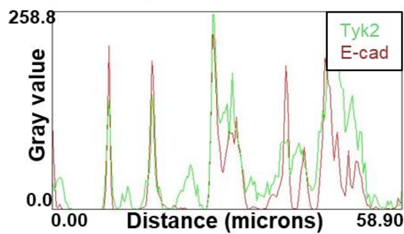
A. DIN1a
Flat epithelia atypia
8/12 membranous Tyk2

B. DIN1b
Atypical ductal hyperplasia
5/10 membranous Tyk2









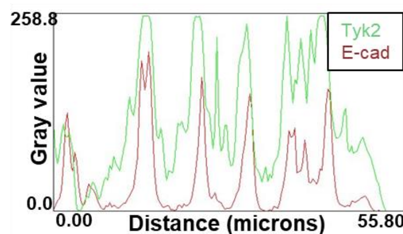


Figure 4.15: Tyk2 co-localizes on the membrane with E-cadherin in most DIN1a samples. Example immunofluorescence of a (A.) DIN1a and a (B.) DIN1b sample from an array of human breast ductal intraepithelial neoplasia. Stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). H&E image of the given sample provided with the array and shown above. Number of samples showing membranous Tyk2 staining out of total DIN1a samples in array shown above. Scale bar 20 μM. Line traces of fluorescence intensity over distance for the lines indicated in the images above. E-cadherin intensity trace shown in red, Tyk2 intensity trace shown in green.

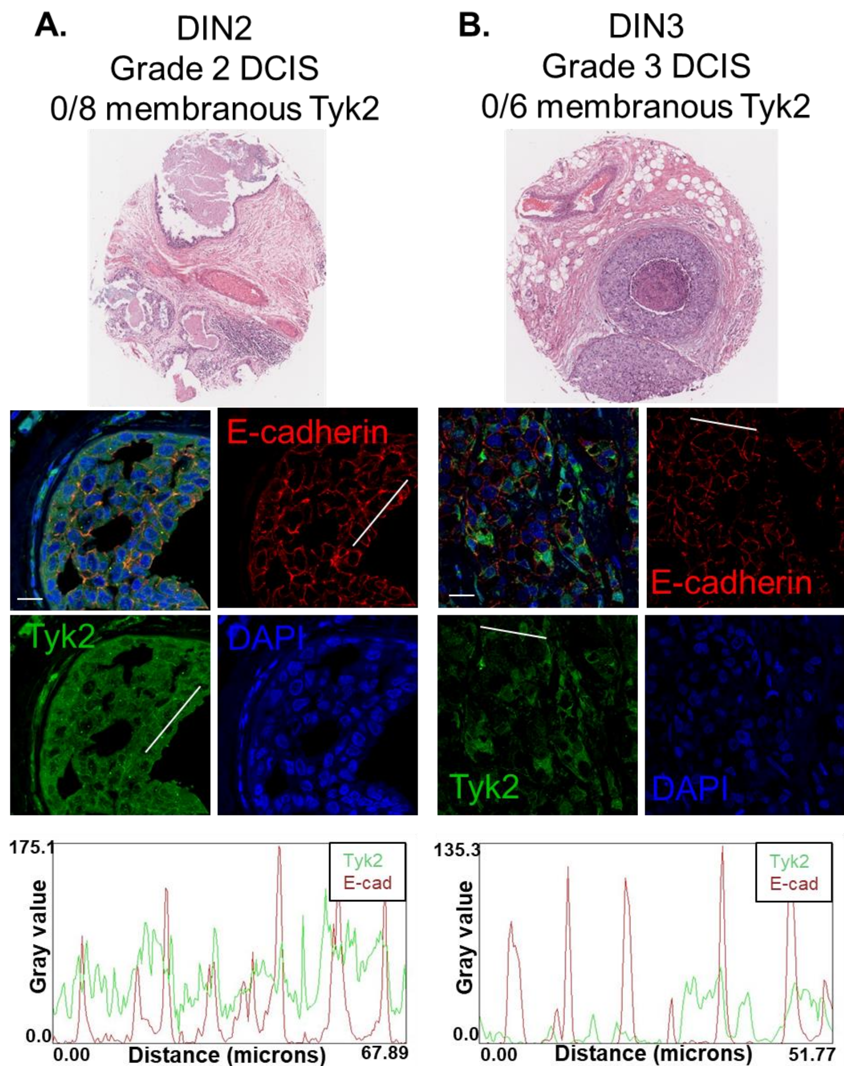


Figure 4.16: Tyk2 is localized in the cytoplasm in all low grade DCIS samples. Example immunofluorescence of a DIN2 and a DIN3 (or grade 2 and grade 3 DCIS) sample from an array of human breast ductal intraepithelial neoplasia. Stained for E-cadherin (red), Tyk2 (green) using the human specific antibody and DAPI for the nuclei (blue). H&E image of the given sample provided with the array and shown above. Number of DIN2 or DIN3 samples showing membranous Tyk2 staining out of total DIN2 or DIN3 samples in array shown above. Scale bar 20 μM. Line traces of fluorescence intensity over distance for the lines indicated in the images above. E-cadherin intensity trace shown in red, Tyk2 intensity trace shown in green.

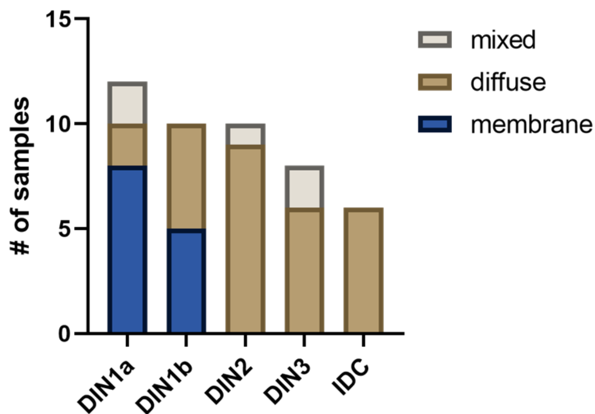


Figure 4.17: Tyk2 localization is correlated with staging in ductal intraepithelial neoplasia samples. The number of samples in each stage included in the array of human breast ductal intraepithelial neoplasia which contained membranous, cytoplasmic or a mixed Tyk2 immunofluorescence staining is indicated in each row by a separate color.

In our *in vitro* system we found that Tyk2 localization changed between low and high rigidities, with the 'mixed population' occurring between 320pa and 670pa. We wondered whether matrix stiffening also contributes to Tyk2 mislocalization in human patient samples. Our *in vitro* data indicated that the localization change of Tyk2 was likely due to the changes in rigidity, and not changes in polarity or 3D structure formation. To determine whether changes in rigidity in these human samples are correlated with changes in Tyk2 localization, we needed to determine rigidity in these early-stage breast lesions. The increase in rigidity seen in breast tumors as they develop and become more aggressive is well characterized (Calvo et al., 2013; Leight et al., 2012; Levental et al., 2009; Paszek et al., 2005; Wei et al., 2015). For early lesions, however, there is much less data. Valerie Weaver's group has examined DCIS samples by second harmonic generation (SHG) microscopy and found that compared to normal tissue, they had longer and more aligned collagen fibers, an indicator of a more rigid matrix. Invasive tumors present a much stiffer collagen signature than DCIS, however (Acerbi et al., 2015). The same group also examined tumor rigidity in mice as tumors progressed from benign lesions to more invasive tumors and found a similar increase

in rigidity as the tumors developed (Lopez et al., 2011). The only data on the rigidity of early breast cancer lesions was performed by groups using various techniques, like shear-wave elastography, on patients as a diagnostic and screening tool. One group found that ADH lesions were more rigid than normal tissue, but less rigid than DCIS, which was in turn less rigid than IDC (Berg et al., 2015). The number of ADH patients assessed was too small to yield statistically significant correlations on, however. Considering the human patient samples on TMA were already fixed, we decided to measure collagen fiber organization via SHG using a two-photon microscope. Collagen is one of the most abundant extracellular matrix (ECM) components of breast tissue. Increased deposition and crosslinking of collagen is the key mechanisms by which ECM stiffening occurs during breast tumor development (Provenzano et al., 2006; Provenzano et al., 2008). Visualization of collagen fibers by SHG could reveal aspects of collagen fiber organization, such as fiber length, curvature, and alignment of fibers with each other. Collagen signatures with short, curved fibers with little alignment are what we term 'disorganized' collagen and are a direct indicator of a tissue with lower rigidity. In stiffer tissues the collagen fibers visualized by SHG are much longer, much straighter and much more aligned with each other. This signature is called 'organized' collagen and is indicative of a more rigid tissue. A more 'organized' collagen signature in tumor samples is correlated with poorer survival in patients (Colpaert et al., 2001; Conklin et al., 2011).

We performed SHG on our DIN array to determine whether collagen signatures were appreciably different within these early stage lesions and if any differences could be correlated with stage or, more importantly, with Tyk2 localization. We found a wide range of collagen signatures in the samples that we examined. The amount of collagen in the samples varied a lot – although this was not correlated with staging of the lesion or with Tyk2 localization. The variation in collagen amount seemed to be correlated more with the portion

of tumor vs. stroma in a sample. Some tumor samples analyzed showed very little collagen purely because the entire sample was from the tumor core where there was no stroma (Figure 4.18). We next looked at collagen organization and found that tumors categorized as 'disorganized' were correlated with membranous Tyk2 (Figure 4.19), while those with organized collagen were correlated with cytoplasmic Tyk2 (Figure 4.20). We found a statistically significant association between mislocalization of Tyk2 and organized collagen (Figure 4.21). While we were not able to directly measure rigidity, using the collagen signature as an indicator of matrix rigidity, we conclude that Tyk2 translocation to the cytoplasm does appear to correlate with increasing rigidity in early-stage human breast lesions.

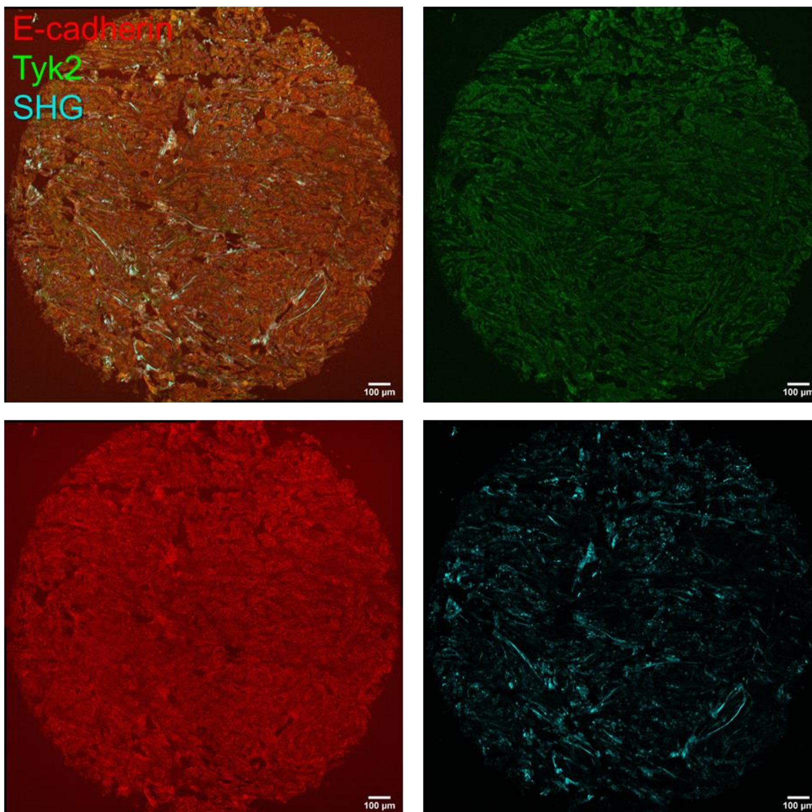


Figure 4.18: An examples of a low SHG signature in a breast tumor sample. Immunofluorescence images of a breast tumor sample stained for E-cadherin (red) and Tyk2 (green). Second harmonic generation (SHG) signal for collagen fibers was obtained in tandem and shown in cyan. Scale bar 100 µM.

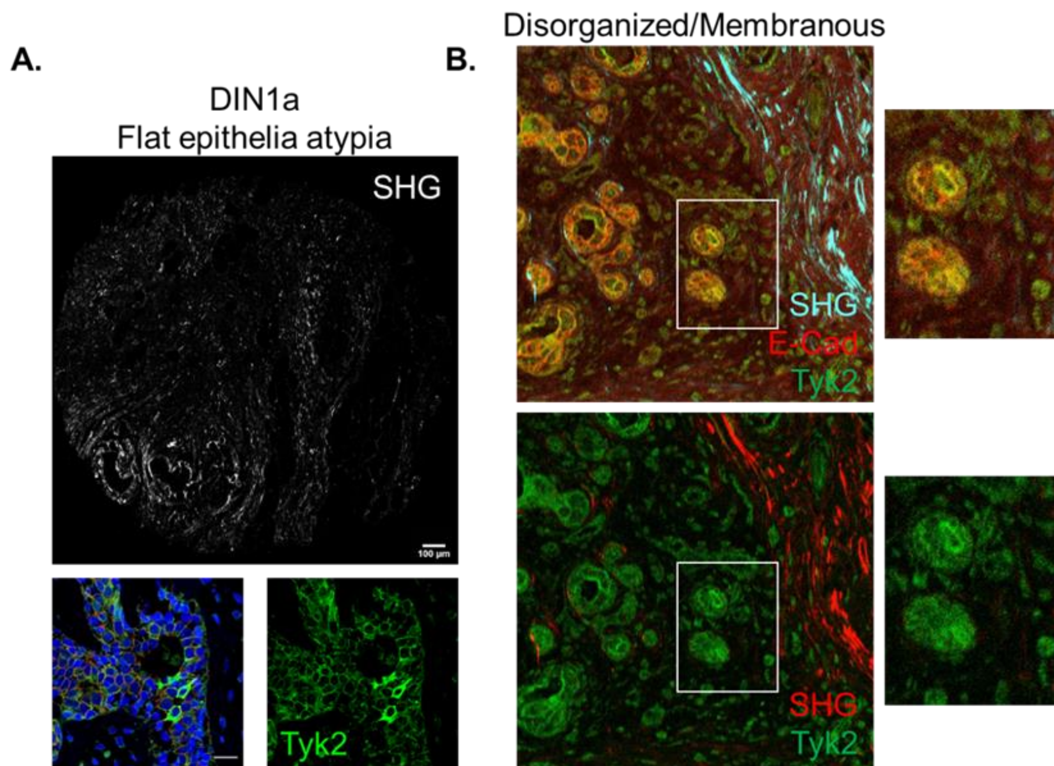


Figure 4.19: Membranous Tyk2 is correlated with a more disorganized collagen signature. A. Second harmonic generation (SHG) signal from a DIN1a sample. Scale bar 100μM. Corresponding immunofluorescence for the same DIN1a sample shown below. Left stained for E-cadherin (red) Tyk2 (green) and DAPI for the nuclei (blue). Right is Tyk2 stain alone. Scale bar 20 μM. B. Top image is a section of a DIN1b sample with disorganized collagen and membranous Tyk2 stained for E-cadherin (red) Tyk2 (green) with the SHG signal obtained in tandem (cyan). Bottom image is the same section, but with Tyk2 stained in green and the SHG signal in red. White section indicates portion of image shown at an increased magnification on the right.

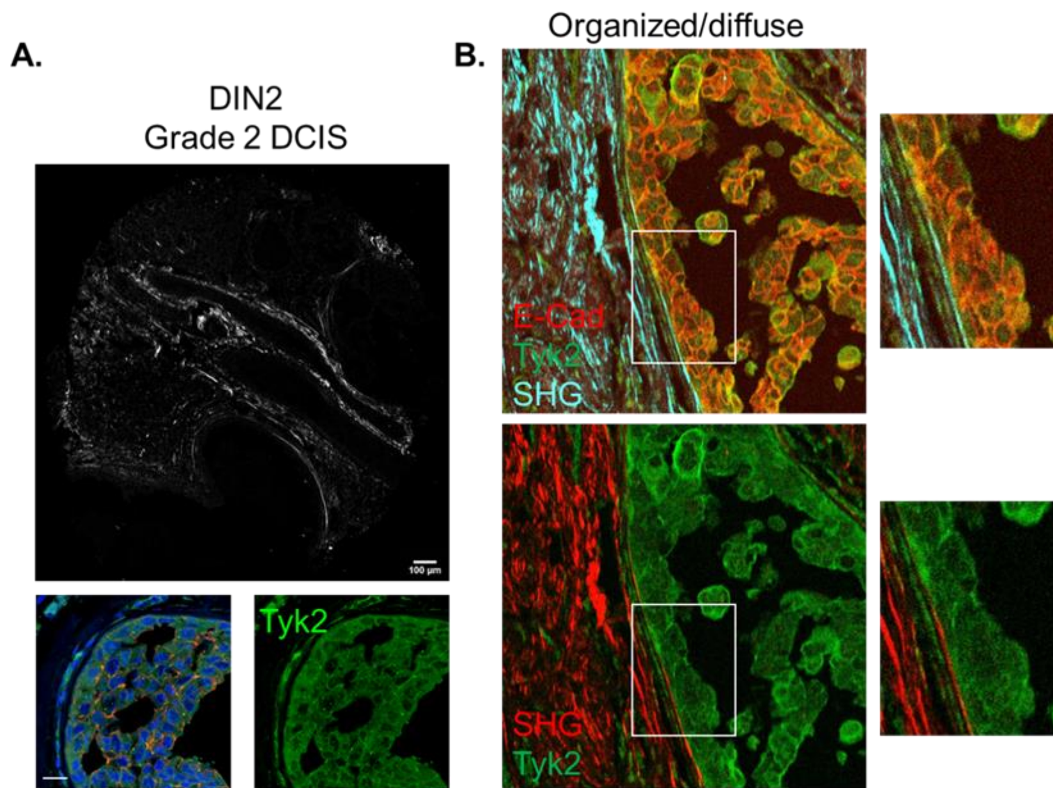
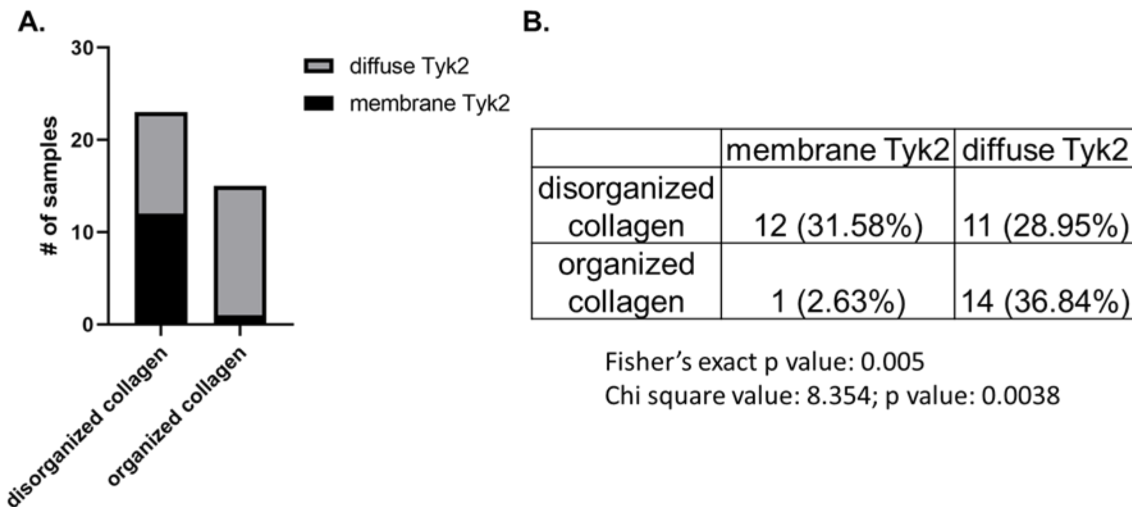


Figure 4.20: Cytoplasmic Tyk2 is correlated with a more organized collagen signature. A. Second harmonic generation (SHG) signal from a DIN2 sample. Scale bar 100μM. Corresponding immunofluorescence for the same DIN2 sample shown below. Left stained for E-cadherin (red) Tyk2 (green) and DAPI for the nuclei (blue). Right is Tyk2 stain alone. Scale bar 20 μM. B. Top image is a section of a DIN1b sample with organized collagen and diffuse Tyk2 stained for E-cadherin (red) Tyk2 (green) with the SHG signal obtained in tandem (cyan). Bottom image is the same section, but with Tyk2 stained in green and the SHG signal in red. White section indicates portion of image shown at an increased magnification on the right.



	membrane Tyk2	diffuse Tyk2
disorganized collagen	12 (31.58%)	11 (28.95%)
organized collagen	1 (2.63%)	14 (36.84%)

Figure 4.21: Tyk2 localization is correlated with collagen organization in ductal intraepithelial neoplasia samples. (A.) The number of samples in the array of human breast ductal intraepithelial neoplasia with disorganized or organized collagen which contained membranous, or diffuse cytoplasmic Tyk2 immunofluorescence staining is indicated in each row by a separate shade of grey. (B.) The total number of samples with organized or disorganized collagen and membranous or diffuse cytoplasmic Tyk2 is indicated, with the percent of the total number of samples in the matrix shown in parentheses. P values for a Fisher's exact test and a Chi- square test are indicated below.

4.6 Conclusions

In this chapter we found that in our 3D culture system Tyk2 expression levels do not change with rigidity, but Tyk2 localization does. At lower rigidities, Tyk2 localizes to the membrane, and becomes more diffuse and cytoplasmic at higher rigidities. This localization change occurs between 320pa and 670pa. Not only is the localization change correlated with increases in rigidity, but also decreases in Tyk2 activity – treatment with a Tyk2 inhibitor caused cytoplasmic localization of Tyk2 to happen at earlier rigidities. This localization change appears to be independent of changes in 3D organoid structure, polarity, and cell-cell junctions. This indicates that the alterations in localization are likely due either directly to changes in rigidity, or due to changes in Tyk2 activity that occur at different rigidities. Whether rigidity is changing activity and that changes the localization of Tyk2 or whether

rigidity is changing the localization of Tyk2 and that changes Tyk2 activity is still unknown. Importantly, localization of both the kinase dead and constitutively active Tyk2 phenocopy endogenous Tyk2 and overexpressed WT tyk2 (data not shown). This would indicate that changes in activity cannot drive a localization event. However, the localization change with the Tyk2 inhibitor supports the opposite conclusion. We hypothesize that these events are concurrent, but one may not necessarily be the cause the other, and they may just both be events that occur due to the same upstream signal. In order to really discover what is regulating this localization change, we need to know how Tyk2 is associated with the membrane. Screening of various known interacting proteins while concurrently performing the IP mass spectrometry experiments discussed in chapter 3 will hopefully lead us to this receptor. Once identified, mutating Tyk2 to perturb its interaction with this receptor will be possible, and will be incredibly useful for understanding the importance of Tyk2 localization.

We also examined human samples in this chapter, and found that Tyk2 was strongly expressed in normal human breast ductal epithelial cells, and that this membrane localization was lost in breast cancer samples. In the breast cancer samples examined, we found that Tyk2 expression level was variable, but that Tyk2 was localized to the cytoplasm in all samples tested regardless of stage. When we examined a panel of ductal intraepithelial neoplasia, we found that Tyk2 localized to the membrane in most of the earliest stage lesions (classified as DIN1a), but began to localize to the cytoplasm in atypical ductal hyperplasia (DIN1b) samples and all early stage DCIS samples had cytoplasmic Tyk2. Our *in vitro* data indicated that the localization change of Tyk2 is likely is not associated with changes in polarity, adherens junctions, or 3D structure formation, which is actually rather surprising after examining the human patient samples. The hematoxylin and eosin (H&E) staining that was provided for serial sections of the samples of this array indicated that one of the most striking differences between DIN1a and DIN1b samples is the change in polarity and ductal

architecture. It is possible that these changes in polarity and ductal structure that we are seeing in the human samples are in part due to changes in rigidity, and that the effects on Tyk2 localization and ductal structure are correlated but that one is not causing the other. This is why we also decided to examine the collagen signatures of these samples by using SHG microscopy. We saw that there was a wide variety of collagen architecture within these samples. We found a correlation between those samples that had membranous Tyk2 and those that had ‘disorganized’ collagen signatures characterized by shorter, more curved fibers. Collagen organization is an indirect marker of matrix rigidity, but there are many previous studies that show that a disorganized collagen signature is an indicator of a softer matrix. This indicates that there is a correlation in these samples between a softer matrix and more membranous Tyk2, just like what we observed in 3D organoid cultures.

This is the first examination of the collagen signatures in these early stage lesions, and the fact that changes in collagen organization seems to occur so early is actually rather surprising. This provides evidence that the stiffening of the ECM that occurs during tumor development is happening very early. It is important to point out that while these changes in fiber alignment are likely correlated with change in ECM rigidity, such change in rigidity is relatively small compared to invasive tumors. Collagen signatures that are associated with very aggressive tumors not only have aligned and elongated fibers, but these collagen fibers are also oriented perpendicular to the tumor boundary. This is thought to provide a sort of path for tumor cell invasion to occur (Han et al., 2016). We did not see any collagen fibers orienting in such a way, and in fact all of the elongated fibers we found that were around the lesion borders were parallel, and most even curved along with the lesion boundary. So while these signatures imply an increase in ECM rigidity, we did not see any of the ECM composition changes that are associated with very aggressive tumor development. This

suggests that there is still a lot of ECM modulations – including further increases in rigidity – which happen later during tumor development and progression.

These data provide early evidence that Tyk2 localization could potentially be a marker for staging these early lesions. These DIN1a and DIN1b lesions are currently characterized by pathologists based primarily on structural features. The difference between a pathologist characterizing a lesion as DCIS instead of atypical ductal hyperplasia is often simply the size of the lesion (Bendifallah et al., 2012). The addition of Tyk2 localization could be useful for correctly assigning patients to one of these stages. More importantly, there is not currently a good way to predict which atypical ductal hyperplasia lesions will progress to DCIS, although patients with ADH do have an increased risk of developing DCIS and invasive breast cancer (Cichon et al., 2010; Degnim et al., 2007; Hartmann et al., 2014). Some patients with atypical ductal hyperplasia will carry them for years and even develop an increasing number of them without ever progressing to DCIS or invasive ductal carcinoma, whereas other progress rapidly (Hartmann et al., 2014). Since the atypical ductal hyperplasia samples we analyzed were the ones that had this split population with respect to Tyk2 localization, it provides the intriguing possibility that Tyk2 localization could be a useful prognostic marker with Tyk2 cytoplasmic localization being an indicator that those patients might progress. We cannot directly test this hypothesis because the TMA set we analyzed does not include information about how these patients progressed over time. Acquiring samples with that information and correlating Tyk2 localization in their early lesions with their disease progression could provide much needed biomarkers to predict breast tumor risk in these patients.

4.7 Acknowledgements

Chapter 4, in full, is currently being prepared for submission for publication of the material. Majeski, H.E., Hu, Z., Fattet, L., Yang J. Tyk2 regulates matrix stiffness-driven EMT and metastasis. *In preparation.* The dissertation author was the primary investigator and first author of this material.

Chapter 5

Materials and Methods

5.1 Cell Culture

MCF10A, MCF10DCIS and MCF10AT cells were grown in DMEM/F12 media supplemented with 5% horse serum, 20 ng/ml human EGF (hEGF), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 1% penicillin and 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 hEGF, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 1% penicillin and streptomycin14. 293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. All cell lines were tested negative for mycoplasma contamination.

5.2 Generation of Stable Cell Lines

Stable gene knockdown cell lines were generated using lentiviral or retroviral plasmid vectors. Briefly, 293T cells were transfected with pCMVΔ8.2R (for lentivirus) or pUMVC3 (for retrovirus), VSVG and the pLKO.1 construct containing the shRNA of interest or the pWB retroviral construct expressing Tyk2 (ratio 2:1:10). Viral supernatants were then concentrated using Lenti-XTM concentrator (Takara). Concentrated viral supernatants were applied to target cells with 6 µg/ml protamine sulfate. Infection was repeated the next day. Infected cells were then selected with puromycin (2 µg/ml) or Blasticidin (5 µg/ml).

5.3 shRNA sequences

pSP108 lentiviral target sequences: Twist1 #3, AAGCTGAGCAAGATTCAGACC. Twist1 #5, AGGTACATCGACTTCCTGTAC. siControl (siGFP) does not target any

endogenous coding DNA. pLKO.1 (Sigma-Aldrich) lentiviral target sequences are listed in Table 5.1.

Table 5.1: List of shRNAs. This table lists the sRNAs used within the thesis for mouse and human cells, and the sequences of these shRNAs.

Target	TRC number	Sequence
shControl (shRFP)		CACCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTGAATTC
Mouse		
shTyk2#1	TRCN0000236001	CCGGCCCAAGACATGAACCTCTATTCTCGAGAATAGAGGTTCATGTCTTGGGTTTTTG
shTyk2#3	TRCN0000025886	CCGGCCCATCTTCATTAGCTGGGAACTCGAGTTCCCAGCTAATGAAGATGGGTTTTT
shTyk2#4	TRCN0000025942	CCGGCCCTTCATCAAGCTAAGTGATCTCGAGATCACTTAGCTTGATGAAGGGTTTTT
shTyk2#5	TRCN0000025964	CCGGCCACTTTAAGAATGAGAGCTTCTCGAGAAGCTCTCATTCTTAAAGTGGTTTTT
shJak2#1	TRCN0000023650	CCGGCCGTGATCTTAACAGCCTGTTCTCGAGAACAGGCTGTTAAGATCACGGTTTTT
shJak2#2	TRCN0000023649	CCGGCCTGGCAACAAGGAACATATTCTCGAGAATATGTTCCTTGTTGCCAGGTTTTT
shJak2#4	TRCN0000278124	CCGGCCAACATTACAGAGGCATAATCTCGAGATTATGCCTCTGTAATGTTGGTTTTTG
shJak2#5	TRCN0000023651	CCGGCGGCCCAATATCAATGGATTTCTCGAGAAATCCATTGATATTGGGCCGTTTTT
Human		
shTyk2#1	TRCN0000003120	CCGGGAGATCCACCACTTTAAGAATCTCGAGATTCTTAAAGTGGTGGATCTCTTTTT
shTyk2#2	TRCN0000003123	CCGGCGTGAGCCTAACCATGATCTTCTCGAGAAGATCATGGTTAGGCTCACGTTTTT
shTyk2#3	TRCN0000003124	CCGGCGAGCACATCATCAAGTACAACTCGAGTTGTACTTGATGATGTGCTCGTTTTT
shTyk2#4	TRCN0000320550	CCGGCGAGCACATCATCAAGTACAACTCGAGTTGTACTTGATGATGTGCTCGTTTTTG
shTyk2#5	TRCN0000320618	CCGGGAGATCCACCACTTTAAGAATCTCGAGATTCTTAAAGTGGTGGATCTCTTTTTG

5.4 DNA constructs

The Tyk2 cDNA was subcloned into the retroviral pWB backbone. The kinase dead (KD) Tyk2 was obtained by a mutation of K930R, the constitutively active Tyk2 was obtained by a mutation of V678F and the kinase loop mutant was obtained by a double mutation of

Y1054 and Y1055 to F1054 and F1055. Tagged constructs and mutants were obtained by site-directed mutagenesis using the QuikChange II kit (Agilent) or by overlapping PCR.

5.5 Polyacrylamide Hydrogel Preparation

Hydrogels were prepared as previously described on 12 mm and 50 mm coverslips (Chaudhuri et al., 2010). Briefly, No. 1 glass coverslips were etched using UV/Ozone Procleaner Plus, functionalized using 3-aminopropyltriethoxysilane, rinsed with dH_2O, incubated in 0.5% glutaraldehyde in PBS, dried, and then acrylamide/bis-acrylamide mixtures were polymerized between the functionalized coverslip and a glass slide coated with dichlorodimethylsiloxane. Polyacrylamide coated coverslips were then washed twice with dH_2O, 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 PBS, and sterilized. For some IP experiments, custom-made easy-coat 100mm Petrisoft dishes were purchased from Matrigen (La Brea, CA, USA).

5.6 3D Cell Culture

MCF10A and Eph4Ras cells were 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 and MCF10A cells were seeded similarly in DMEM/F12 medium supplemented with 2% horse serum, 5 ng/ml human EGF, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, penicillin, streptomycin, and 100 ng/ml cholera toxin.

5.7 Invasive Acini Quantification

Invasive acini were quantified using brightfield images with a minimum of 5 random low-magnification fields being analyzed per condition per experiment. Acini were scored as either normally developed acini or acini that adopted a spread and invasive phenotype.

5.8 3D confocal microscopy

Our protocol was adapted from the method described by Debnath *et al* (Debnath et al., 2003). Briefly, cells were fixed with 2% paraformaldehyde (PFA) for 20 minutes at room temperature, permeabilized with PBS-0.5% Triton X-100 for 10 minutes, quenched with 3 washes of 100mM PBS-glycine, and then blocked with 20% goat serum in 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 (1:200 dilution) for 1 hour at room temperature, washed 3 times with IF buffer, and then mounted in DAPI-containing mounting medium (Vector Laboratories). Confocal images were acquired using an Olympus FV1000 with 405, 488, 555, and 647 laser lines. Images were linearly analysed and pseudo-coloured using ImageJ analysis software.

5.9 Proximity Ligation Assay

Cells were cultured on 3D-PA gels for 5 days and fixed and processed as described for 3D confocal immunofluorescence before performing Duolink PLA (Sigma-Aldrich) as per manufacturer's protocol. Briefly, mouse anti-TWIST1 and rabbit anti-G3BP2 primary antibodies were used to detect endogenous proteins and subsequently recognized using species specific plus and minus PLA oligonucleotide conjugated probes at 37°C for 60 minutes. Interacting probes were then ligated at 37°C for 30 minutes and detected by polymerase mediated amplification at 37°C for 100 minutes and subsequently analyzed by fluorescent confocal microscopy. For analysis of formed 5-day acini, a minimum 50 cells from 5 random fields were quantified per condition. To quantify the PLA signal, images were

converted to 8-bit images and thresholded, the area of PLA signal was then quantified and normalized to cell number using ImageJ.

5.10 Second Harmonic Generation Microscopy

An SP8 resonant laser-scanning confocal system mounted on a DM 6000 upright microscope (Leica Microsystems) with a 10X, 0.4 NA was used for second-harmonic generation microscopy. A Ti-Sapphire femtosecond pulsed Chameleon Ultra II (Coherent Inc.) laser was tuned to 855 nm and the emitted fluorescent signal was recorded with a non-descanned hybrid detector fitted with a 425/26 nm bandpass filter. To increase the signal to noise ratio, 1248x1248 pixel 8-bit images (0.6 μm/pixel) were acquired with 16x line averaging and 8 frames image accumulation. Images were exported as TIFF files and analysed and pseudo-coloured using ImageJ analysis software. The scoring rubric (which was defined prior to blinded scoring) for SHG analysis was defined as “organized collagen” in tumors having prominent linearized collagen fibres or as “disorganized collagen” in tumors having collagen fibers with high degree of circularity (i.e. curved).

5.11 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 orthovanadate, 2.5 mM beta-glycerophosphate, 10% glycerol, pH 7.5), scraped off the culture dish, sonicated, supplemented to 400 mM NaCl, sonicated, and diluted to 200 mM NaCl. Lysates were precleared with protein G (for Lyn) or protein A (for Tyk2) beads for 1 hour at 4°C. Antibodies were conjugated to protein G beads (Lyn) or protein A beads (Tyk2) (Invitrogen), crosslinked using disuccinimidyl suberate (Pierce) as per manufacturer’s protocol, incubated with lysates overnight at 4°C, washed eight times with IP lysis buffer supplemented with 200 mM NaCl,

and eluted by incubation in LDS sample buffer with 50 mM DTT at 70°C for 10 minutes. For IP samples run on Wes (Simple Western), samples were eluted by incubation in 1% SDS and 1mM DTT at 95°C for 5 minutes. For Tyk2 constructs containing a Flag-tag, Flag pre-conjugated magnetic or agarose beads (Sigma) were utilized, with the same overall protocol.

5.12 Antibodies

Antibodies are listed in Table 5.2.

Table 5.2: List of Antibodies. This table lists the antibodies used within the thesis. It also lists the concentrations of the antibody used for different applications, the source of the antibody, and an identifier for the antibody.

Reagent	Source	Identifier
TYK2 (WB 1:1000, IF 1:80, Wes 1:50, IHC 1:100)	Sigma-Aldrich	HPA005157
Tyk2 (WB 1:1000, IF 1:100)	Genetex	GTX61449
pTYK2 (WB 1:500, Wes 1:25)	Sigma-Aldrich	T0442
TWIST1 (WB 1:200, IF 1:25)	Santa Cruz Biotechnology	sc-81417
E-CADHERIN (IF 1:100)	BD Biosciences	610181
FIBRONECTIN (IF 1:200)	Sigma-Aldrich	F3548
EPHA2 (WB 1:500, IP, Wes 1:25)	Cell Signaling Technology	#6997
LYN (WB 1:500, IP, Wes 1:50)	Cell Signaling Technology	#2796
pY416 SFK (WB 1:500, Wes 1:25)	Cell Signaling Technology	#2101
pY527 SFK (WB 1:500)	Cell Signaling Technology	#2105
GAPDH (WB 1:5000, Wes 1:400)	Genetex	GTX100118
BETA-ACTIN (WB 1:1000)	Genetex	GTX629630
Flag (WB 1:1000, IF 1:500)	Sigma Aldrich	F3165
Rabbit IgG control (IP)	Cell Signaling Technology	#2729
pS897 EPHA2 (WB 1:500, Wes 1:25)	Cell Signaling Technology	#6347
pY588 EPHA2 (WB 1:500)	Cell Signaling Technology	#12677
Goat anti-Mouse-HPR (1:10000)	Jackson Immunoresearch	115-035-003
Goat anti-Rabbit-HPR (1:10000)	Jackson Immunoresearch	111-035-144
Bovine anti-Goat-HPR (1:10000)	Jackson Immunoresearch	805-035-180
Goat anti-Mouse Alexa Fluor 488 (1:200)	Invitrogen	A-11001
Goat anti-Rabbit Alexa Fluor 546 (1:200)	Invitrogen	A-11010

5.13 Tumor Tissue Microarrays

Invasive breast cancer and ductal intraepithelial neoplasia tumor tissue microarrays (TMAs) (Biomax) were stained for Tyk2 by immunohistochemistry and for E-cadherin and Tyk2 by immunofluorescence. The ductal intraepithelial neoplasia array was concurrently imaged by confocal microscopy and SHG. Cores were scored blindly for ‘membranous Tyk2’ staining (i.e. Tyk2 staining which co-localized with E-cadherin). Tyk2 membranous localized was scored either as membranous, cytoplasmic (diffuse) or mixed. Mixed samples were omitted from correlation analysis with SHG signal because the number of samples exhibiting that signature was too small to be statistically correlated.

5.14 Xenograft Tumor Assay

1.0 x 10^6 GFP-labelled MCF10DCIS cells suspended in 15 µl Matrigel (50%, diluted in DMEM/F12) were injected bilaterally into the inguinal mammary fat pads of 6 to 8-week-old female SCID-beige mice. Mouse weight and tumor sizes were monitored for 6 weeks post tumor cell implantation, until mice were sacrificed and tumor burden analyzed. Mice were dissected and tumor invasion assessed in situ using a fluorescent dissection scope (Leica Microsystems). All work with animals was performed in accordance with UC San Diego IACUC and AAALAC guidelines.

5.15 Real-time PCR

RNA was extracted from cells using Tri Reagent (SIGMA). cDNA was generated using random hexamer primers and cDNA Reverse Transcription Kit (Applied Biosystems). Expression values were generated using ddCt values normalized to murine Gapdh or human

HPRT. Experiments were performed in biological and technical triplicate. RT-PCR primer sequences are listed in Table 5.3

Table 5.3: List of Primers. This table lists the RT-PCR primers used within the thesis. It lists the sequences for the forward and reverse primers and categorizes them relative to the species they are specific for (mouse or human).

Target	Forward	Reverse
Mouse		
Tyk2	CTGCCTGAGGTCACACAGAA	TAGCACCATCAAGCATCCTG
Jak2	TCTGTGGGAGATCTGCAGTG	GAAAGCAGGCCTGAAATCTG
Gapdh	GACCCCTTCATTGACCTCAAC	CTTCTCCATGGTGGTGAAGA
Human		
Tyk2	CCAGGAGAAACCTCCAATCT	CTGGGTGATCTCCTTCTGGT
Twist1	AAGAGGTCGTGCCAATCAG	GGCCAGTTTGATCCCAGTAT
HPRT	GCTATAAATTCTTTGCTGACCTGCTG	AATTACTTTTATGTCCCCTGTTGACTGG

5.16 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Student t-test was performed to determine statistical significant between two test groups. One-way ANOVA test was carried out to determine statistical significance of three or more test groups. The correlation between collagen organization and membranous Tyk2 signal in human breast cancer patients' samples (TMA analysis) was analyzed using Fisher's exact analysis and a χ^2 test. Statistical significance was defined as *, **, ***, or **** for $p < 0.05$, $p < 0.01$, $p < 0.001$, or $P<0.0001$, respectively, with regard to the null hypothesis.

Chapter 6

Conclusions and Future Perspectives

6.1 Tyk2 and epithelial cell mechanotransduction

Our lab identified a novel Twist1 mechanosensing pathway in the work published by Spencer Wei in 2015, but at the time the specific molecular regulation of this pathway was not well elucidated. Since that publication, our lab has focused on identifying proteins involved in the regulation of Twist1 translocation in response to rigidity. Through that work other lab members identified the kinase responsible for phosphorylating Twist1 at high rigidities (Lyn) and upstream regulators of this kinase (EphA2). Through my thesis work, we also defined a novel negative regulator of this pathway – Tyk2. This work, described in chapters 2-4, clearly indicates that Tyk2 expression and kinase activity is required to maintain epithelial identity through maintaining cytoplasmic localization of Twist1 at low rigidities. We additionally determined that both Tyk2 kinase activity and membrane localization were regulated by rigidity, and that membrane dis-localization was correlated with disease progression and changes in the ECM architecture in human breast lesions.

6.1.1 Tyk2 regulation of the EphA2-Lyn-Twist1 mechanosensing pathway

We have begun determining how Tyk2 and other known mechanoregulators of the Twist1 mechanotransduction pathway interact. Work described in chapter 3 showed that inhibition of Tyk2 leads to an increase in EphA2 S897 phosphorylation and Lyn activation. This work indicated that Tyk2 acts upstream of Lyn activation and EphA2 phosphorylation, but does not explain how Tyk2 affects the phosphorylation of these proteins. Immunoprecipitation experiments did not reveal direct interaction between Tyk2 and Lyn or EphA2 at any rigidity, making it unlikely that Tyk2 directly phosphorylates or modulates either

of these proteins. Given this, more work needs to be done to completely understand how Tyk2 is interacting with this mechanoregulatory pathway, and what other proteins are involved downstream of Tyk2 to mediate the inhibitory effects that Tyk2 has on this pathway at low rigidities. The IP-mass spectrometry experiment explained in chapter 3 is one approach we are employing to identify novel, rigidity or activity specific interactors of Tyk2. We hope that some of these novel interacting proteins of Tyk2 could connect these pathways together.

We are also screening known downstream substrates of Tyk2 such as Stat proteins and certain phosphatases. Our focus was not originally placed on canonical members of the Jak-Stat pathway primarily because the basal level of activity for Tyk2 at low stiffness does not resemble the activity activated by cytokine stimulation. Upon ligand stimulation, Tyk2 is rapidly and robustly phosphorylated on the activation loop. We instead showed that there is a basal level of Tyk2 phosphorylation at low rigidities that is lost at high rigidities. This constant basal level of Tyk2 activity was critical to the maintenance of the epithelial phenotype at low rigidities, not a transient increase in Tyk2 activity. We also screened a subset of interferon response genes by qPCR and found no rigidity specific activation or suppression. A significant number of downstream and upstream effectors of Tyk2 are mostly expressed in the hematopoietic cells and not well expressed in mammary epithelial cells. Further examination of these canonical proteins does, however, seem critical. We found that there is a low level of Stat3 phosphorylation at low rigidities that is lost at high rigidities. Inhibition of Tyk2 kinase activity leads to Twist1 nuclear localization within two hours, suggesting that the effect of Tyk2 on Twist1 likely does not require transcription. Given that the amount of Stat3 phosphorylation is low and that no induction of interferon response genes were observed, we believe that the Stat3 phosphorylation is likely to be a readout of the basal Tyk2 activity and not responsible for mediating the effects of Tyk2 activity. Stat3 has a very well characterized

role in promoting EMT and invasion, which is also not in line with Stat3 dephosphorylation observed in cells that are more invasive. Regardless, to determine whether Stat3 is critical for downstream mechanosignaling, functional assays for Stat3 – both depleting the cells of Stat3 and inducing Stat3 phosphorylation through cytokine stimulation need to be performed.

In addition to Stat protein phosphorylation and activation, Tyk2 has also been shown to phosphorylate and regulate a dual specificity phosphatase called DUSP3 or VHR (Hoyt et al., 2007). The phosphorylation of DUSP3 by Tyk2 has only been described in a single paper. However, phosphorylation of DUSP3 by Tyk2 was shown to be critical for substrate specificity of the phosphatase. For the paper, Tyk2-directed phosphorylation of DUSP3 targets the phosphatase to Stat5 as a negative feedback loop response (Hoyt et al., 2007). However, phosphorylation of DUSP3 at the same residue has been shown by other labs to be critical for targeting the phosphatase to other substrates – which includes members of the MAP kinase family (Alonso et al., 2003; Kang and Kim, 2006; Todd et al., 1999). This series of papers on this phosphatase is intriguing to follow up for two reasons. The first is that phosphorylation of DUSP3 is mediated by Tyk2 specifically, not the other Jak kinases. This is important because the effect we observed in our system is Tyk2 specific, while the vast majority of downstream phosphorylation events in the Jak-stat pathway are mediated by a combination of Jak family kinases, not Tyk2 specifically. Second, DUSP3 is an interesting target because one of its well-characterized substrates is ERK. Work done by others in the lab has shown that ERK is part of a signaling cascade that is critical for phosphorylation of EphA2 at serine 897 at high rigidities. Together, these studies indicate that DUSP3 could serve as the connection between Tyk2 and the phosphorylation of EphA2 – that is when you deplete Tyk2 activity or Tyk2 activity is lost at high rigidities, DUSP3 is no longer phosphorylated by Tyk2, and subsequently can no longer de-phosphorylate ERK, and this leads to an increase in ERK activity, and subsequent activation of signaling pathways that

lead to the phosphorylation of EPhA2. Phosphatases tend to be constitutively active and have a number of substrates making them poor specific regulators of pathway activation. However, the mechanisms of DUSP3 targeting make it more promising as a specific regulator. Additionally, we are starting to examine phosphatase regulation on other portions of this pathway. More specifically, we are determining whether phosphatases are involved in the activation of Lyn by de-phosphorylating the ‘inactivating’ phosphorylation site on the kinase. A signaling cascade whose activation is regulated by phosphatases would be quite novel, and work discerning the roles of phosphatases in this mechanosensing pathway is ongoing in our lab.

6.1.2 Upstream Regulation of Tyk2 in response to rigidity

We determined that the localization of Tyk2 was regulated by rigidity. Tyk2 membrane localization is also correlated with a less rigid collagen signature in human patient samples. These data discussed in chapter 4 indicate that Tyk2 membrane localization is correlated with its function in maintaining epithelial identity at low rigidities and may be playing a role in disease progression. Tyk2 has been described to localize to the membrane through interactions with cognate receptor proteins. We initially did not screen the cytokine receptors that Tyk2 has been reported to bind to since we did not believe Tyk2 activation was occurring in a canonical way. Tyk2 has been shown to interact with and mediate signaling through a number of different cytokine receptors, and an even larger number of these receptors are reported to interact with Tyk2, but a limited amount of data are available regarding the importance or effect of that interaction. Considering this information, the list was too long to screen one by one initially. However, given the importance of Tyk2 membrane localization, determining which receptor Tyk2 is interacting with at low rigidities is critical for understanding the mechanisms by which Tyk2 localization changes with rigidity and how this

localization change impacts downstream signaling. We are pursuing two methods to find this receptor: one is the IP-Mass spectrometry experiment discussed above to find novel Tyk2 interacting proteins. The other is to select a list of known Tyk2 interacting receptors by examining their expression in both Mcf10a and Eph4Ras cells, and then screening these receptors for a phenotype upon shRNA knockdown.

In addition to determining the receptor proteins that Tyk2 binds to, a number of additional means of Tyk2 regulation needs to be examined. There are several described post-translational modifications beyond phosphorylation of the activation loop for Tyk2. These post-translational modifications, which include additional tyrosine phosphorylations, serine phosphorylations, acetylation and ubiquitination, have been identified by proteomic studies, but their function, if any, in modulating signaling has not been investigated (Leitner et al., 2017). Many of these post-translational modifications are specific to Tyk2, and do not have a homologous modification in other Jak kinases. This provides the intriguing possibility that these modifications confer specific regulation on Tyk2, or even mediate interaction with specific proteins. Identifying any function of these modified residues in regulating Tyk2 with respect to rigidity and downstream mechanosensing would not only be beneficial to our further understanding of this pathway, but also to our understanding of Jak kinases.

There are several aspects of Tyk2 activation and regulation that are not fully elucidated and may be critical for our understanding of the role of Tyk2 in this mechanosensing pathway. These include the 'basal Tyk2 activity' we see at low stiffness, and the biological role for Tyk2 intra-molecular inhibition. This low level of activity for Jak kinases without cytokine stimulation has been described many times, but its function and/or the role it plays in signaling is not well described (Gauzzi et al., 1996). Some hypothesize that this basal activity is due to ligand independent receptor dimerization, but do not

speculate to its function in activating downstream signaling (Hammaren et al., 2019). In chapter 3 we describe a signaling pathway that requires constant Tyk2 activity, but appears to be independent of phosphorylation of the activation loop tyrosines 1054 and 1055. This indicated that the Tyk2 activity that is required for maintaining cytoplasmic Twist1 localization at low rigidities is likely independent of cytokine stimulation, and provides evidence that this 'basal activity' of Tyk2 has a functional role in mechanotransduction. Understanding what mediates this type of activity and how it is controlled within the cell is critical for us, but will also add to the understanding of Tyk2 activation and activity within the field, and hopefully open up further research into other biological consequences, which this cytokine independent activity could be regulating.

Understanding the specific mechanisms underlying Tyk2 activation and the biological role for Tyk2 intra-molecular inhibition are related, and are potentially both related to discerning the mechanisms controlling basal Tyk2 activity. The most intriguing of these is understanding the role that the Tyk2 kinase-like domain (KLD) or pseudokinase domain has on inhibiting the kinase domain, and under what biological contexts this is relevant. The inhibitory effect that the KLD has is very well described in an *in vitro* manner, but not well described in the context of the cell (Lupardus et al., 2014b). The interaction of the KLD and the kinase domain renders the kinase domain completely inactive, and it is hypothesized that ligand binding or receptor dimerization or some other uncharacterized structural change that occurs during Tyk2 activation changes the conformation such that these two domains no longer interact, which opens up the kinase domain for phosphorylation and for substrate binding. An interesting possibility, however, is that this intramolecular interaction is what controls basal activity, and that phosphorylation of the activation loop is responsible for the ligand induced robust activation of the kinase. Under this hypothesis, Tyk2 at low rigidity is basally active due to dissociation of the KLD domain and the kinase domain, and at high

rigidity some form of conformational change folds the KLD domain back onto the kinase domain – rendering it completely inactive. A significant amount of work needs to done to determine whether this is the case, however, including more mutational experiments and ideally more structural information about Tyk2 from low and high rigidities.

6.1.3 The potential function of Tyk2 as a general mechanoregulatory protein

The work described in chapters 2-4 is the only research regarding the role of Tyk2 in mechanosensing, but this does not limit the role of Tyk2 in mechanoregulation to just the Twist1 pathway we study. There are a number of mechanically regulated pathways in epithelial cells, and what role, if any, Tyk2 plays in those needs to be addressed. As an example, Yap is another transcription factor that translocates to the nucleus in response to rigidity (Dupont et al., 2011). Previous work in the lab indicated that the regulatory mechanisms governing Yap translocation are different from those that regulate Twist1. Yap translocation appears to be mediated by changes in the actin cytoskeleton and can be additionally induced by changes in cell shape or actin dynamics (Dupont et al., 2011). While these regulators do not affect Twist1 translocation, that does not eliminate the possibility that these regulators of Yap translocation may also regulate Tyk2. We have not yet examined whether changes in cell shape and size through micropatterning alter Tyk2 activity or localization, but these experiments are important to understand whether the regulation of Tyk2 is specific to the rigidities that regulate Twist1 or is regulated by other factors. Additionally, we can examine the role of Tyk2 in regulating other mechanotransduction pathways. For example, does modulating Tyk2 activity regulate Yap translocation or focal adhesion dynamics?

Mechanosensing is not limited to epithelial cells, and there are mechanically regulated signaling pathways within the immune system as well. The Jak-stat pathway has

not been described to be regulated by mechanical signals in the immune system, but the mechanical regulation of other immune components has been well described. Activation of both B cells and T cells has been shown to be a mechanically regulated event (Upadhyaya, 2017). Tension on the bond between T cell receptor or B cell receptor and the ligand induces a stronger adhesion to the target and a stronger response. Integrin mediated adhesion is also critical for various stages of T cells activation, including both migration through tissues and rolling migration along endothelial cells within circulation (Rossy et al., 2018). Certain immune cell subtypes have also been shown to undergo phenotypic changes when cultured on different substrate rigidities – although the range of rigidities utilized in such studies was in the 100kPa to megapascal range, and likely far out of range for any of the rigidity sensing mechanism we study (Upadhyaya, 2017). That being said, activation of the release of certain cytokines occurs under very high rigidity conditions, indicating that mechanosensing signaling that has yet to be described may be playing a role in these immune cells. The possibility that Tyk2 is involved in some of that uncharacterized mechanosensing remains to be tested. Understanding whether mechanical regulation of Tyk2 can occur in the immune system, and what effect this may have biologically could be a very interesting avenue of future research.

6.2 The role of Tyk2 in cancer progression and dissemination

6.2.1 In vivo model for assaying the role of Tyk2 at different steps in tumor development

Our experiments conducted to understand the role of Tyk2 within epithelial tumors have thus far been limited to orthotopic models in immunocompromised mice with Tyk2 loss specific to the implanted tumor cells. This type of model offers some advantages with respect to understanding the function of Tyk2 within a solid tumor independent of the systemic effects of Tyk2 loss. Tyk2 deficient animals, for example, are much more susceptible to

certain tumors, which is due primarily to decreased immunosurveillance from Tyk2 loss in NK cells and T cells (Prchal-Murphy et al., 2015). Thus, taking a whole body Tyk2 knockout mouse and attempting to parse out the effects specific to Tyk2 loss in the tumor, and not in the immune system would be very difficult. However, the system employed here does not provide any information regarding the role of Tyk2 in normal breast tissue development and homeostasis or the role of Tyk2 in early lesion development. Considering the role Tyk2 is playing in maintaining epithelial status in breast epithelial cells *in vitro*, loss of Tyk2 expression in the breast may lead to developmental errors, errors in branching morphogenesis and also may predispose mice to breast tumor formation, or lead to faster disease progression during breast tumor development. One way in which we are planning to address some of these questions is by generating a mouse with Tyk2 specific deletion within the breast by crossing a floxed Tyk2 mouse to a breast specific MMTV-Cre mouse (Vielnascher et al., 2014). This will not only enable us to visualize the role of Tyk2 in breast development within these mice, but will also allow us to understand how Tyk2 functions early in breast tumor development, and later in metastatic disease. This is particularly interesting because translocation of Tyk2 from the membrane to the cytoplasm occurs early on during breast lesion development in human patient samples. Utilizing a genetic mouse model will allow us to ask questions about the role of Tyk2 in these early lesions, and whether the loss of Tyk2 can lead to more invasion in these early stages.

6.2.2 Potential use of Tyk2 as a biomarker in early breast lesion development

As mentioned in the previous section and in chapter 4, Tyk2 localization to the membrane is robust in normal breast tissue and early ductal intraepithelial neoplasia samples, but becomes diffused throughout the cytoplasm in DCIS and breast tumor samples. This localization change happens early in lesion development, and is correlated with a

change in the architecture of the collagen within the ECM. There are very few markers available to differentiate patients who have DIN1B and patients that have early stage DCIS. The primary factor in deciding how to diagnose these patients is the size of lesion (Bendifallah et al., 2012). This has led to a lot of concern over misdiagnosis in patients with these lesions with a fear of incorrectly diagnosing a patients with cancer and implementing a disproportionally aggressive treatment plan, or incorrectly characterizing patients with a DIN lesion who actually have early stage DCIS. The number of these early lesions identified in patients has increased with the increase in mammographic screening for breast cancer, and the field has subsequently done a lot of work to fine-tune the parameters for diagnosing each of these stages, but there are still no molecular markers that define these states. There is also not a good way of predicting which patients will progress from DIN1B lesions to DCIS, or from DCIS lesions to invasive cancer. There have been several clinical papers that discuss attempts to define at DIN1B patients who are at a higher risk for eventual tumor development, but none have identified a definitive marker (Cole et al., 2010). The results we present regarding the differential localization of Tyk2 pose an interesting hypothesis that Tyk2 cytoplasmic localization may be a biomarker for DIN lesions that could progress to DCIS and even invasive carcinoma. A study analyzing DIN lesions for Tyk2 localization that are isolated from patients with disease progression follow-up information is needed to determine whether Tyk2 localization could be a prognostic biomarker. With this information, we would be able to determine whether Tyk2 membrane localization in the original lesion correlates to stable disease and whether Tyk2 cytoplasmic localization correlates with progression to DCIS or invasive carcinomas. These studies could determine whether Tyk2 localization could serve as a critical marker for determining risk stratification in these patients and subsequently what type of treatment is appropriate.

6.2.3 Potential therapeutic pitfalls for targeting Tyk2 in autoimmune disorders

Tyk2 has been implicated in immune disorders for many years. While the other Jak kinases are implicated in a variety of disease, including cancer, the focus for targeting Tyk2 has been primarily on autoimmune disorders. There are several major pharmaceutical companies currently developing Tyk2 specific inhibitors to treat autoimmune disorders such as alopecia, Crohns' disease and psoriasis (among others). One motivation that prompted pharmaceutical companies to change focus from utilizing pan-Jak inhibitors to targeting specific Jak kinases comes from the significant side effects that most of the pan-Jak inhibitors cause (Burke et al., 2019). Until recently, designing inhibitors to target one Jak kinase as opposed to all four was very difficult, as the vast majority of these inhibitors are ATP-competitive inhibitors which bind to the kinase domain. These regions are very similar among the Jak kinases, and specificity for one cannot be achieved well this way. Through the recent structural and functional studies detailing the inhibitory function of the pseudokinase domain (KLD), many of these pharmaceutical companies have shifted their focus to generating inhibitors that bind to and stabilize this domain, locking the protein into an autoinhibited conformation. The KLD of Jaks are distinct enough to allow for very specific inhibitor generation. As a result, companies such as Pfizer, Bristol-Meyers Squibb (BMS) and Nimbus/Celgene, which is the company we received our inhibitors from, have begun phase I/II/IIItrials for a number of their Tyk2 specific inhibitors (Armstrong, 2018)

Generally each company is focused on targeting a different autoimmune disorder, but the rationale for Tyk2 specific inhibition is similar for all of these drugs – and that is limiting off-target effects, and thus limiting side-effects. BMS in particular sites the isolation of a particular Tyk2 mutant as inspiration for their drug – the P1104A/V mutant (Burke et al., 2019). This mutation is within the substrate binding groove of the kinase domain of Tyk2, and

it prevents receptor-mediated Tyk2 activation (Kaminker et al., 2007). Germline homozygosity for this mutant Tyk2 allele is relatively common among Europeans, and has been shown to cause susceptibility to tuberculosis infections specifically, but not other types of infection (Boisson-Dupuis et al., 2018; Kerner et al., 2019). This Tyk2 allele also confers resistance to several autoimmune disorders by impairing specific cytokine signaling (Dendrou et al., 2016; Gorman et al., 2019). This obviously, makes Tyk2 into a very intriguing target for pharmaceutical companies designing drugs for autoimmune disease. However, this mutation was initially identified as a colon and breast cancer associated germline mutation and was later also shown to be associated with AML. These studies utilized computational algorithms to determine that this mutation was causally related to disease, but no functional studies on how this LOF allele may be aiding tumor development have been undertaken (Kaminker et al., 2007).

Results discussed in this dissertation, however, provide a rationale for Tyk2 deactivation being a potential driver for tumor progression and metastasis. We showed that loss of Tyk2 leads to increased EMT, increase invasion and increased metastatic disease in mouse models. This could potentially be a serious side-effect of drugs that specifically target Tyk2 that has not been considered, and at least one of these Tyk2 specific inhibitors is currently undergoing phase III trials. Given what we have discovered about the role of Tyk2 in our models *in vitro* and our *in vivo* experiments and correlations in human data, we caution companies designing drugs for Tyk2 specific inhibition to watch for long-term oncogenic effects, especially in patients who are at all pre-disposed to breast cancer or patients that have any DCIS or even DIN lesions. Much more work needs to be done to determine whether alterations in Tyk2 activity or localization are correlated with cancer progression in other types solid tumors. Investigation into whether this is the case could be critical for understanding how much cancer risk these Tyk2 inhibitors may be causing as some patients

with autoimmune disease are at a higher risk for certain tumors. For example Crohn's disease patients are at a much higher risk for colon cancer. While we have no evidence to suggest Tyk2 inhibition could lead to increased tumor invasiveness in colon cancer, it is worth investigating to determine if treating these patients with a Tyk2 inhibitor could increase their risk for colon cancer, or for developing more aggressive and invasive colon cancer.

6.3 Mechanosensing in epithelial cells

6.3.1 Mechanosensory modules in 2D

The mechanotransduction pathway discussed within this dissertation is, as far as we have been able to determine, quite independent from the well-described mechanotransduction pathways that mediate cell adhesion and migration in 2D. In this type of mechanosensing, integrin molecules at the cell surface interact with ECM proteins, and complex modules on the cytoplasmic side of these integrin proteins relay mechanical information from the ECM to the actin cytoskeleton, and activate various signaling pathways involved in cell survival and cell migration – among other more cell type specific signaling (Moore et al., 2010). Data not described here, but observed in the lab and included in other papers indicates that Twist1 translocation is initiated by increases in rigidity, but appears to be independent of integrin signaling and other integral parts of this type of mechanotransduction such as actomyosin contractility, cell shape, and focal adhesion kinase (FAK) activation, which is a critical mediator of rigidity-dependent adhesion stabilization and maturation (Galbraith et al., 2002; Moore et al., 2010; Northcott et al., 2018). The work done to determine that these mechanosensing pathways are completely independent from each other has not been exhaustive, however. In particular, the relationship between Tyk2 and many of these mechanosensing proteins has yet to be investigated.

6.3.2 Potential interplay between 2D mechanosensing and Twist1 mechanosensing pathway

Very elegant work detailing the specific molecular events that are responsible for sensing rigidity during cell spreading and adherence has been done by the group of Mike Sheetz. Work done in the Sheetz lab and others have shown that a molecular module they call a ‘contractile unit’ is critical for rigidity sensing, and that loss of different components of this module cause cells to lose their rigidity sensing capabilities, and allows cells to survive at much lower rigidities (Elosegui-Artola et al., 2016; Wolfenson et al., 2016). In a recent paper, they described the importance for the loss of this contractile unit in cell transformation and suggest that this loss is critical for cancer cells to grow on any surface, regardless of rigidity, and may be important for anoikis resistance in the circulation during metastatic spread (Yang et al., 2019). This work is interesting to us as it seems to be in contradiction to our studies, where increases in rigidity are not only sensed by tumor cells, but lead to an increase in tumor aggressiveness.

However, there are a number of differences in the modeling and type of rigidity sensing that our respective labs are examining that may explain some of these contradictions. The Sheetz lab exclusively examines cells function and molecular dynamics in a 2D environment – as almost all mechanosensing work is done. Additionally, they primarily work with much higher rigidities – comparing 1000Pa to 5 or 10 thousand Pascals. This is for a number of reasons. First, the 2D environment allows for much more precise molecular studies – for example using micropillars to study single molecules within a contractile unit was necessary for them to determine how the contractile unit functions and what the key molecular components are. Higher rigidities are utilized in their system because most cells actually die at lower rigidities when cultured in 2D. This was the basis for their work regarding cancer cells and their loss of contractile unit formation – because cancer cells

can survive when seeded at low rigidities in 2D, and their recent paper determine that this was because of a loss of contractile unit formation (Yang et al., 2019).

In our system – Mcf10a cells only survive and form acini structures at such low rigidities when seeded in 3D (with Matrigel). If the Matrigel overlay is omitted, these cells die within a few days. Are the mechanosensing mechanisms that we are looking at different from those that the Sheetz lab has defined, or are they similar but we are seeing different results due to different culture methods? In order to begin determining which of these hypotheses is correct, we are planning to knock down different contractile unit components – starting with tropomyosin 2.1 (Tpm2.1) as this protein appears to be critical for contractile unit formation, but not as critical for other cellular processes like the contractile unit protein myosin for example (Yang et al., 2019). We will example these Tpm2.1 knockdown cells for their phenotype in our 3D culture at low and high rigidities and for Twist1 localization to determine whether Tpm2.1 is necessary for the Twist1 mechanosensing pathway. We will also plate these cells in a 2D system at low and high rigidities to see whether loss of Tpm2.1 confers the ability to survive in 2D at low rigidities in these cells, as the Sheetz lab reported. These experiments will also determine if the mechanically induced translocation of Twist1 still occurs at high rigidity in 2D without Tpm2.1. Together these two experiments will tell us if our signaling pathway is independent of contractile unit formation or if it is related to mechanosensing mechanisms described by the Sheetz lab.

Another intriguing possibility is that loss of Tpm2.1 allows for cell survival at 2D, but leaves our mechanosensing pathway intact. This would allow us to culture cells in 2D environments which would not only make a lot of our work easier technically, but would also allow us to do more complex imaging and molecular dynamics experiments through live cell imaging. We could, for example, determine when and how different proteins are recruited to

or lost from the membrane or visualize Twist1 localization in real time. This would allow us to gain a much more detailed understanding of how this Twist1 mechanosensing pathway is regulated.

6.4 Mechanosensing in tumor progression and metastasis

6.4.1 Cellular responses to tunable changes in rigidity

In the 3D culture system we utilized for the work described in this dissertation we are able to tune the rigidity that the cells are seeded on through altering the rigidity of the PA hydrogel. This rigidity is static and cannot be altered once the cells are seeded. As such, we do not know how dynamic the EMT induced in these mammary cells is. EMT is generally a dynamic process, as discussed in the introduction, during tumor progression cells often undergo a partial EMT, and the process reverts with an MET at metastatic sites to allow for metastatic outgrowth (Tsai et al., 2012). We have yet to determine whether the invasion stimulated by increases in rigidity is reversible. In chapter 2 we have showed that this EMT is inducible after acini formation by treating the cells with a Tyk2 inhibitor several days after seeding. The reverse experiment – that is to determine whether the cells can go back to forming acini after being stimulated to invade – is not technically feasible with currently available hydrogels. Treatment with a Lyn inhibitor, for example, after the cells have been in culture for a few days and have already adopted an invasive morphology, cannot cause the cells to revert back to an acini structure. This suggests that this is not reversible. However, the fact that the cells can form acini when plated in soft environments after being cultured on stiff plastic dishes does suggests that there is plasticity in their ability to react to different rigidities. This is complicated by the fact that they are trypsinized and thus exist in suspension (an environment that provides no cell attachment and subsequently no rigidity sensing) before being plated in the low rigidity environment. The direct transition would be

much more interesting to examine. We are currently working to adapt a tunable gel system to our culture methods. These methacrylated hyaluronic acid hydrogels become stiffer with UV treatment (Ondeck and Engler, 2016). Adapting these gels to our 3D culture system will allow us to assess cell changes upon stiffening of the environment, and will hopefully allow us to examine the dynamics of different proteins upon ECM stiffening.

6.4.2 Implications for tumor development and metastasis

As a tumor cell goes through the metastatic cascade, the cell experiences a variety of cues from the changing environment, including a variety of different substrate rigidities. This is one reason why analyzing how cells change when going from one rigidity to another is interesting because it could provide clues to how cells may respond at different locations while metastasizing. For example, the tumor environment becomes stiffer, due to a variety of factors discussed in the introduction. This can, in turn, activate EMT and stimulate tumor cell invasion. The tumor cells then enter the circulation where they experience an environment of very low rigidity but very high shear stress. Do tumor cells that have come from a stiffer environment react to shear stress differently? Do they react to effectively being in suspension differently?

Another important question is how rigidity-induced EMT affects tumor cells that have metastasized to distant sites. Common metastatic sites for breast cancer include the bone (which is the most common), the lung, the liver and the brain. These tissues are very heterogeneous in terms of their rigidity – bone is very stiff and lung and brain tissue is very soft. There are a number of mechanisms which may play a role in where breast cancer cells metastasize to, and also a number of factors which contribute to their ability to survive and grow at those secondary sites. What controls these processes in not well-defined, however, and is probably governed by many factors in combination. One factor which has not been

examined well is the rigidity of that metastatic environment and how it relates to the rigidity of the primary tumor and the primary tumor microenvironment. One MMTV-PyMT mouse study looked at the lung metastatic microenvironment and found it to be quite soft while the tumor stroma became quite stiff over time (Plodinec et al., 2012). One possibility is that the rigidity of the secondary environment is coordinating with other effectors to stimulate MET and lead to outgrowth of the disseminated tumor cells. Several studies, including some from our lab, have shown that cells that are in a more mesenchymal state because of the activation of EMT-TFs can metastasize, but these micro-metastases remain in a dormant state and cannot grow past a couple of cells in size until the EMT inducing factor is removed and the cells are allowed to undergo MET and revert back to a more epithelial state (Banyard and Bielenberg, 2015; Tsai et al., 2012; Zhang et al., 2016). This is supported by data showing that metastases are more epithelial while the induction of a partial EMT is, if not required for, greatly enhances metastatic dissemination (Banyard and Bielenberg, 2015). Our model indicates that when cells are placed in a softer environment, they behave more like epithelial cells, and while we have not been able to show that an MET-like reversion is possible in our *in vitro* system, there is a possibility that a lower rigidity environment can stimulate mesenchymal to epithelial transition (MET) and aid in metastatic outgrowth. This will need to be examined carefully if inhibition or targeting of this mechanically induced EMT is ever attempted therapeutically– it could lead to outgrowth of already disseminated tumor cells while attempting to stop the further dissemination of tumor cells which may end up killing a patient instead of preventing metastatic disease.

6.4.3 Mechanoregulation of Tyk2/Twist in other cancer types

Our results, with respect to the role of Tyk2 in regulating Twist1 mechanosensing, have thus far been examined only in breast epithelial cells and breast tumor metastasis.

Whether Tyk2 is playing a similar role in maintaining an epithelial state in other epithelial tumors remains to be investigated. Unpublished data from our lab shows that Twist1 translocation in response to rigidity appears to also occur in Ewing sarcoma cells – indicating that this pathway may be more general and not specific to breast epithelial cells. Tyk2 expression in different types of solid tumors varies between studies – some groups find Tyk2 to be upregulated in tumors and some find it to be downregulated, with little evidence that certain types of solid tumor may react in the same way as breast tumors do. Information about Tyk2 localization in tumors is practically non-existent within the literature. However, examining the IHC for different normal and cancerous tissues types on the human protein atlas suggests that breast tissue may be specific in terms of Tyk2 translocation off of the membrane in tumors. The exception being cervical cancer – where the majority of cervical squamous cell carcinomas have lost Tyk2 membrane staining which is very strong in the normal cervix samples analyzed (Uhlen et al., 2010). The IHC data from the human protein atlas is limited however, and it is possible that several epithelial cancers may show this localization change, or it may be critical for a specific type of cancer within a tissue, but not all cancer subtypes. Examination of Tyk2 expression and localization within early stage lesions of other types of tumors has also not been performed. Given the early stage at which Tyk2 localization appears to change, it would be interesting to determine whether this is common among other early stage lesions. A much more methodical examination of these epithelial cancers and early stage lesions for Tyk2 localization needs to be performed to truly understand whether Tyk2 mis-localization is a common feature of carcinoma progression or specific to the breast.

Determining whether Twist1 localization responds to rigidity in additional tumor types is critical to understand the relevance of this pathway for other types of cancer. Additionally, tissues throughout the body have very different ‘normal’ rigidities. It is possible that the

threshold of rigidity that cells respond to differs depending on the rigidity that they normally perceive within the body. Determining whether Twist1 translocation occurs in different cells types, and if the rigidity at which this translocation occurs will aid in our understanding of the overall relevance of this pathway for tumor invasion and metastasis. This will also help us understand the mechanistic regulation for this pathway – that is whether it is an absolute value of a rigidity that induces Twist1 translocation or whether it is a fold change from the normal rigidity of a particular tissue that causes induction of this mechanosensing pathway.

6.5 Concluding remarks

In this dissertation I have described the role of Tyk2 is regulating the stiffness induced translocation of Twist1 and subsequently the induction of EMT, invasion and metastasis. I have shown that the loss of Tyk2 stimulated increased nuclear Twist1 translocation and increased EMT and invasion at lower rigidities. The role of Tyk2 in maintaining epithelial identity by regulating this pathway requires Tyk2 kinase activity, as inhibition of Tyk2 led to a similar increase in invasion and metastasis through Twist1 translocation. Discovering the substrates of Tyk2 that mediate Tyk2's effects on Twist1 mechanosensing is a critical next step in this research. I have shown that Tyk2 inhibition leads to increased Lyn activity and an increase in EphA2 phosphorylation on the relevant serine. However, I have yet to directly link Tyk2 activity and the Lyn/EphA2/Twist1 mechanosensing pathway. Methods to identify intermediary proteins that connect these pathways have already been discussed. Identifying these proteins will be critical to understand whether the inhibitory functions of Tyk2 and the activating functions of Lyn are tied closely together, or whether there are separate signals which cooperate to regulate Twist1 phosphorylation and EMT activation.

In addition to describing the role of Tyk2 presence and activity in regulating this Twist1 mechanosensing pathway, I have also shown that Tyk2 localization appears to be

regulated by rigidity. Tyk2 localizes to the membrane at low rigidities and becomes more diffuse and cytoplasmic at high rigidities. This localization change appears to be dependent on rigidity and independent of other cell morphology changes that occur during EMT such as polarity loss and loss of cell-cell junctions. This localization change was robust, but the cause of the localization change and whether it was relevant to Tyk2 signaling or a result of a loss of Tyk2 activity still remains to be determined. Tyk2 dissociation from cognate receptors has not been described as a means of regulation for Tyk2 in the literature. This could prove to be a novel regulatory mechanism for Jak kinases. To understand how Tyk2 localization is changing, we will need to determine what transmembrane proteins Tyk2 is interacting with in mammary epithelial cells, and which of these proteins is relevant for regulating Tyk2 activity and localization with respect to rigidity. There is a distinct possibility that Tyk2 is interacting with a variety of receptors in our cell types of interest – Tyk2 has been described to interact with many receptors and there is no indication in the literature that this interaction is limited to one receptor type in a single cell. Determining which receptor interaction is relevant for regulating downstream signaling in this mechanotransduction pathway will be critical. Perturbing the ability of Tyk2 to associate with its relevant receptor will also enable us to understand how critical Tyk2 receptor association, and subsequently membrane association, is for Tyk2 signaling in this mechanosensing pathway.

I also show that Tyk2 cytoplasmic localization was found in all breast tumor samples analyzed, while Tyk2 membrane localization was seen in all normal breast epithelial cells analyzed. This stark contrast in localization mirrors what we saw in our 3D culture system at low and high rigidities. I additionally show that Tyk2 cytoplasm localization occurs early on in premalignant breast lesions. This very early translocation event provides the possibility that Tyk2 translocation could be a marker to identify patients with low grade DIN1a or DIN1b lesions that are at a higher risk for developing DCIS or invasive ductal carcinoma. This is still

hypothetical as we do not have any information on the outcomes of the patients for whom we received samples and performed Tyk2 staining. Collaboration with pathologists and ongoing research regarding risk stratification for early lesions will be critical in determining whether Tyk2 localization has any value as a prognostic marker, or whether this localization change we are seeing is either unrelated to unable to predict disease progression.

Our work also provides strong evidence that Tyk2 loss or inhibition could be oncogenic or lead to enhanced tumor invasion and metastasis. This information is critical as research into the role of Tyk2 in cancer has only within the last couple of years really begun and there is still little consensus within this growing field about whether Tyk2 is an oncogene like other Jak kinases, or whether Tyk2 is actually tumor suppressive. I believe the function of Tyk2 in cancer is very context dependent, and the role of Tyk2 in hematologic malignancies may be very different than the role of Tyk2 we have described here. However, given the research contained in this dissertation and the experiments conducted in solid tumors within the literature, it seems clear that in the context of breast cancer, loss of Tyk2 or loss of Tyk2 activity, can stimulate tumor progression and metastasis. This discovery is important for a number of reasons. One reason is that Tyk2 has become a very popular target recently for pharmaceutical companies in the treatment of various autoimmune diseases. These inhibitors in development are given orally, and are subsequently acting systemically to inhibit Tyk2. Patients treated for autoimmune diseases will likely be on these inhibitors for life. Given our research, systemically inhibiting Tyk2 for prolonged periods of time would seriously impose a risk on patients who harbor any breast lesions or are prone to breast cancer as Tyk2 inhibition can stimulated invasion and metastatic disease.

Understanding the mechanisms that drive metastatic disease is very important as metastatic disease is usually what causes lethality in patients, not the primary tumor.

Elucidating the molecular pathways that drive metastasis can hopefully lead to better drug design and targeting of this process to better treat or inhibit metastatic diseases. The loss of Tyk2 activity or expression appears to be a potent driver of EMT and invasion, though targeting of Tyk2 itself seems infeasible as it would need to be reactivated or upregulated which would most likely cause a lot systemic immune dysfunction. However, understanding in full the mechanoregulation of Twist1 translocation and EMT induction in response to rigidity will provide us with a much better understanding of how best to potentially target this critical process in metastasis.

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