Abstract

Cell-cell junctions regulate the form and function of epithelial tissues, in part, by mechanically coupling adjacent cells together. Unlike normal cells, pre-malignant cells are capable of mechanically uncoupling these junctions in response to motogenic factors such that the cells become invasive and, ultimately malignant. Therefore, I asked whether the mechanical responses of cell-cell junctions to increases in intracellular tension are altered in pre-malignant mammary epithelial cells in the absence of such motogenic factors. In an effort to answer this question I altered the intracellular tension on the cell-cell junctions of normal (EpH4) and pre-malignant oncogenic ras-transformed (EpRas) mammary epithelial cells either chronically, by altering the density of cells attached to a rigid substratum, or acutely, by physically extending (i.e. ‘stretching’) confluent monolayers of cells attached to a compliant silicone rubber substrate. When intracellular tension was chronically increased, the tension-sensitive protein zyxin relocalized to cell-cell junctions in normal, but not pre-malignant cells. The zyxin relocalization in normal cells was associated with a junctional increase in the phosphorylated form of myosin light chain 2 (MLC2) suggesting that it may involve actomyosin contractility. The same differential in zyxin relocalization and phosphorylated MLC2 accumulation occurred when the intracellular tension was acutely increased in the two cell types. This differential was blocked by Rho-ROCK inhibition which indicates that it may be dependent on actomyosin contractility. In addition, apical actin structure reorganization occurred when intracellular tension was acutely increased in the normal cells that did not occur in the pre-malignant cells. Taken together, these observations led me to conclude that the ability of cell-cell junctions to respond in a mechanosensory-appropriate manner to changes in intracellular tension is compromised in ras-transformed pre-malignant mammary epithelial cells. Acute pharmacologic inhibition of
oncogenic Ras-mediated increases in MAPK and/or PI3K signalling did not correct this compromised response. Therefore, this compromised mechanosensitivity, which may functionally contribute to the ability of pre-malignant cells to become invasive in response to motogenic factors, may be initiated by long term epigenetic changes that occur under conditions of stable oncogenic transformation.
Preface

The research program of this thesis was developed collaboratively by Dr. Calvin Roskelley, Dr. Marcia Graves, Dr. Spencer Freeman and the author, Tak Kwong Poon.

Experiments were designed by Tak Kwong Poon in consultation with Dr. Calvin Roskelley, Dr. Marcia Graves and Dr. Spencer Freeman. In the experiments from Figures 3.4, 3.7, 3.17 and 3.18, the staining procedure was performed by Pamela Dean. The remaining experiments and data analysis were carried out by Tak Kwong Poon.
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List of Abbreviations

2D - 2-dimensional
3D - 3-dimensional
Arp - actin related protein
BCA - bicinchoninic acid
BSA - bovine serum albumin
DAPI - 4’6-diamidino-2phenlyinode
DCIS - ductal carcinoma in situ
DDR1 - discoidin domain receptor 1
DMEM - Dulbecco’s modified Eagle’s medium
ECM - extracellular matrix
ECT2 - epithelial cell transforming 2
EDTA - ethylenediaminetetraacetic acid
EMT - epithelial to mesenchymal transition
EPLIN - epithelial protein lost in neoplasm
ERK - extracellular regulated kinase
f-actin - filamentous actin
FBS - fetal bovine serum
FRET - Förster resonance energy transfer
GAP - GTPase activating protein
GAPDH - glyceraldehyde 3-phosphate dehydrogenase
GEF - guanine nucleotide exchange factor
GPCR - G protein-coupled receptor
GTPase - guanosine triphosphatase
Hras - Harvey murine sarcoma virus oncogene
HRP - horseradish peroxidase
IgG - immunoglobulin G
INS - insulin
Kras - Kirsten murine sarcoma virus oncogene
LY - LY294002
MAPK - mitogen-activated protein kinase
Mena - mammalian Enabled
MLC - myosin light chain
MLCP - myosin light chain phosphatase
mTORC1 - mammalian target of rapamycin complex 1
NGS - normal goat serum
NMII - non-muscle myosin II
no. - number
PAGE - polyacrylamide gel electrophoresis
Par - partitioning defective
PBS - phosphate buffered saline
PD - PD98059
pERK - phosphorylated extracellular regulated kinase
PI3K - phosphoinositide 3-kinase
pMLC - phosphorylated myosin light chain
pMLC2 - phosphorylated myosin light chain 2
PVDF - polyvinylidene fluoride
Rho - ras homology
RIPA - radio-immunoprecipitation assay
ROCK - Rho-associated protein kinase
RTK - receptor tyrosine kinase
S - stretched
SDS - sodium dodecyl sulfate
TBS - tris buffered saline
TBS-T - tris buffered saline - tween
TGF-β1 - transforming growth factor β1
TSC1 - tuberous sclerosis protein 1
US - unstretched
VASP - vasodilator-stimulated phosphoprotein
v-Ha-ras - Harvey rat sarcoma viral oncogene homolog
ZO-1 - zonula occludens protein 1
Acknowledgements

I would like to take this opportunity to thank those that have made this possible. Firstly, I would like to thank my supervisor and mentor Dr. Calvin Roskelley, who generously allowed me to work in his laboratory where I was able to learn and explore cell biology in a supportive environment with a group of very talented individuals. Your guidance has been the highlight of this experience and I am forever grateful for the exceptional skills and knowledge that you have given me. I give many thanks to the members of my supervisory committee Dr. Christopher Maxwell and Dr. Wayne Vogl that provided helpful and valuable advice. I would also like to thank the past and present members of the Roskelley lab, namely Dr. Marcia Graves, Dr. Spencer Freeman, Pamela Dean, Jane Iwanyshyn, Dr. Jennifer McQueen and Erin Bell, who provided me with invaluable insights into every aspect of this journey through graduate school. Without their wisdom and kindness, and their expertise I so commonly came to rely on, I would have not known how to navigate the difficulties encountered.

I am grateful to all the friends I have made during graduate school, many of whom have provided me with both scientific and personal support. In particular, I would like to thank Leslie Chan who has enriched my experience here. I also need to thank my friends from outside graduate school who have supported me throughout the program. Last, but not least, I would like to thank my family, especially my mother Kam Poon for being there for me every step of the way.
Chapter 1: Introduction

1.1 Overview

Cancer disease progression in epithelial tissues is thought to first involve the transformation of normal tissue to a pre-malignant state by multiple oncogenic insults (Burstein et al., 2004). Evidence now suggests that increased mechanical stiffness in the extracellular matrix (ECM) surrounding pre-malignant cells then helps drive the cells into an invasive malignant state (Levental et al., 2009; Paszek et al., 2005). This progression is driven by the gradual loss or disruption of cell-cell junctions, structures that are well known to transmit mechanical cues into biochemical signals in normal epithelial cells (le Duc et al., 2010; Onder et al., 2008). Thus, as cell-cell junctions are still present in the pre-malignant state, I decided to ask if these mechanically sensitive structures have altered responses compared to those found in normal epithelia.

1.2 Mechanotransduction in normal and cancerous tissues

In normal tissues, the cytoskeleton and the extracellular environment are thought to be under a certain level of balanced mechanical tension which maintains cellular structural integrity, also termed as ‘cellular tensegrity’ (Ingber, 1993, 2003). In such a system, the cytoskeleton is under a tensional balance that regulates the shape of the cell. As a result, alterations in this tensional balance, which are most often generated by cytoskeletal and/or ECM modifications, drive changes in cell shape and phenotype. For example, experiments limiting cell spreading by varying concentrations of a non-adhesive coating on tissue culture plates show a negative correlation between cell height and proliferation (Folkman and Moscona, 1978). Additionally,
altering cell shape via adhesive island size or actomyosin contractility affects the ability of human mesenchymal stem cells to differentiate into an adipocytic or osteoblastic lineage (McBeath et al., 2004). Moreover, actomyosin dependent differentiation into muscular and neuronal cell lineages is modulated by manipulating extracellular stiffness (Engler et al., 2006).

Cells respond proportionally to an increase in ECM rigidity by contracting their cytoskeleton, thereby generating a tensional response that transduces mechanical signals into the cell (Lo et al., 2000; du Roure et al., 2005; Saez et al., 2005). These mechanical signals can then affect cellular processes directly or they can be converted into biochemical signals that can regulate gene expression (Mammoto et al., 2009). While maintaining the appropriate balance of tension-based mechanotransduction is critical for maintaining normal tissue form and function, it is often disrupted during cancer disease progression where it contributes the emergence of the invasive phenotype (Levental et al., 2009; Paszek et al., 2005).

1.3 Pre-malignant cells, epithelial to mesenchymal transition, and mechanical stiffness

Clinically, pre-malignant epithelial cells are hyperproliferative and they have altered morphologies that are often associated with changes in polarity but they are not yet invasive (Burstein et al., 2004). Therefore, the later acquisition of an invasive phenotype is a functionally important indication that a pre-malignant mass has moved forward on the spectrum towards malignancy (Arpino et al., 2005). Interestingly, the ability of pre-malignant cells to become invasive has been associated with an increase in the stiffness of the ECM. For example, increasing ECM stiffness by collagen fibre crosslinking causes the emergence of invasive features in pre-malignant mammary epithelial lesions (Levental et al., 2009). One of the best characterized means of epithelial cells becoming invasive is the epithelial to mesenchymal
transition (EMT) whereby the cells lose their cell-cell junctions, become migratory, and move through the matrix as single, mesenchyme-like cells (Lee et al., 2012; Sarrió et al., 2008; Thiery, 2002). During an EMT, the expression of cell-cell junction proteins, including the adherens junction protein E-cadherin, are repressed (Gregory et al., 2008; Onder et al., 2008; Vleminckx et al., 1991). This downregulation of E-cadherin is tension-dependent, presumably, at least in part, because changes in ECM stiffness are able to regulate its transcriptional suppressor Twist (Wei et al., 2015).

1.4 Mechanotransduction through the cytoskeleton

Mechanosensation and mechanoresponse, including changes in cell shape, occurs largely through the cytoskeleton by actomyosin contractility (Engler et al., 2006; McBeath et al., 2004; Wang et al., 2001). In addition, it is generally accepted that the balance of reciprocal forces between the extracellular environment and the cytoskeleton is actomyosin contractility based (Lo et al., 2000; Schwartz, 2010). Non-muscle myosin II (NMII) is an ATPase motor protein complex associated with actin filaments that generates contractile force on the actin cytoskeleton (Kovács et al., 2004; Shutova et al., 2012). Phosphorylation of the myosin light chain (MLC) subunit in NMII at serine 19 (pMLC(S19)) increases actomyosin contraction (Amano et al., 1996; Craig et al., 1983). One means of regulating this contractility is through the small GTPase RhoA which activates Rho-kinase (ROCK) and the citron kinase that phosphorylate MLC (Amano et al., 1996; Matsui et al., 1996; Yamashiro et al., 2003). Moreover, ROCK can also reduce myosin light chain phosphatase (MLCP)-mediated dephosphorylation of pMLC(S19) (Kimura et al., 1996). Although these studies have examined MLC in different species, it is assumed that the MLCs in the NMII complex have orthologues with protein sequence homology
between a number of species including chicken, human and mouse (Park et al., 2011; Vicente-Manzanares et al., 2009; Watanabe et al., 2007; Zavodny et al., 1990).

In addition to contractility, mechanoresponses can be manifested through changes in the structure of the actin cytoskeleton. For example, in fibroblasts, RhoA-mediated actomyosin contractility promotes the formation of basal actin stress fibres (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992) and the direct application of mechanical force to fibroblasts results in the widening of basal actin fibres and the reorientation of stress fibres (Yoshigi et al., 2005). Moreover, stress fibre thickening depends on zyxin-mediated recruitment of the vasodilator-stimulated phosphoprotein (VASP), an actin barbed-end anti-capping protein (Applewhite et al., 2007; Yoshigi et al., 2005). The association between applying physical force and subsequent RhoA contractility-dependent stress fibre formation has also been shown in endothelial cells (Kaunas et al., 2005). Apart from stress fibres, mechanically-stimulated formation of actin networks surrounding the nucleus depends on the activity of a formin-family protein (Shao et al., 2015). Therefore, an association exists between mechanosensation, mechanoresponse, actomyosin contractility and actin cytoskeletal reorganization. In a tissue context, interactions between the cytoskeleton and the extracellular environment require bridging structures which are provided by cell-ECM and cell-cell adhesions.

1.5 Mechanotransduction at cell junctions

The best characterized mechanoresponsive cell junctions are those that associate with the actin cytoskeleton (reviewed in Schwartz, 2010). Largely mediated by transmembrane proteins of the integrin family and associated with cytoskeletal linker proteins, mechanoresponsive cell-ECM junctions provide physical connections between the cytoskeleton and the glycoprotein
matrix on the basal surface of epithelial cells. Application of mechanical force can stimulate the maturation of cell-ECM junctions (Riveline et al., 2001) and, conversely, reducing actomyosin contractility results in the loss of cell-ECM adhesions (Chrzanoska-Wodnicka and Burridge, 1996). Force-induced relocalization of the focal adhesion protein vinculin to cell-ECM junctions is required for a cell to generate myosin contractility-mediated forces required for cell migration (Galbraith et al., 2002; Grashoff et al., 2010). Studies of mechanotransduction at cell-ECM adhesions have focused on proteins that unveil cryptic sites by the physical unfolding of proteins in response to increased tensional ‘stretch’. Talin is one such protein that interacts with integrins on one end and the actin cytoskeleton on the opposite end (Hemmings et al., 1996; Pfaff et al., 1998). *In vitro* experiments have revealed that when talin is mechanically pulled using forces in the physiological range, vinculin binding sites are exposed with functional consequence (del Rio et al., 2009). Vinculin bound to talin then recruits proteins such as the Arp2/3 complex to initiate actin reorganization at the cell-ECM adhesion (DeMali et al., 2002).

While much attention has been focused on studying cell-ECM junctions in mediating altered tensional response in a pre-malignant context, nothing is known about whether the mechanoresponse of cell-cell junction components can mediate such alterations in tensional homeostasis in pre-malignant cells (Levental et al., 2009; Maschler et al., 2005; Paszek et al., 2005). Tight junctions and adherens junctions are two types of cell-cell junctions associated with the actin cytoskeleton and both help to form physical linkages between adjacent epithelial cells. Tight junctions are apical to adherens junctions and contain transmembrane proteins (e.g. claudins, occludins; Furuse et al., 1993, 1998) linked to actin via scaffolding proteins (e.g. ZO-1; Itoh et al., 1993). While tight junctions are mechanoresponsive in the context of an endothelial cell, there is limited evidence to suggest that they are also sites of mechanotransduction in other
epithelial cell types (Tornavaca et al., 2015). Adherens junctions on the other hand are well-characterized mechanotransducers in normal epithelial cells. These junctions contain transmembrane proteins from the cadherin family linked to actin via protein linkers from the catenin family (Aberle et al., 1994; Jou et al., 1995; Takeichi et al., 1981). In particular, the classical cadherin subtype E-cadherin is well known to transmit physical cues through connections to the actin cytoskeleton to elicit a cellular response via homotypic interactions between the same cadherin subtypes on adjacent cells.

Evidence for the transmission of physical cues by adherens junctions in epithelial cells has been demonstrated in a number of studies. For example, Rho-mediated contractility is modulated at E-cadherin-mediated adherens junctions (Hidalgo-Carcedo et al., 2011; Noren et al., 2003; Ratheesh et al., 2012). Another study involved the measurement of the physical force applied to molecules of an E-cadherin-based Förster resonance energy transfer (FRET) sensor. This method was used to demonstrate that E-cadherin located within adherens junctions is physically extended when cells are actively pulled apart along a single axis using microneedles (Borghi et al., 2012). Linkage to the actin cytoskeleton and contractility in response to increased tension was critical to this response as removal of α-catenin from the cells or treatment with inhibitors of actomyosin contractility and actin polymerization prevented E-cadherin’s physical extension (Borghi et al., 2012). Other studies using mechanical stimulation via E-cadherin coated magnetic beads demonstrated that homotypic E-cadherin interactions are required for an actomyosin dependent mechanical response (le Duc et al., 2010; Tabdili et al., 2012). Finally, magnetic and physical tugging on cells demonstrated a requirement for E-cadherin junctions to physically pull on an α-catenin FRET sensor (Kim et al., 2015).
There is experimental evidence that actomyosin-dependent contractile responses at adherens junctions can be controlled by regulators of RhoA. For example, p190RhoGAP recruitment to adherens junctions by the discoidin domain receptor 1 (DDR1)-Par3/Par6 polarity complex leads to an expected inhibition of contractility there (Hidalgo-Carcedo et al., 2011). Conversely, the α-catenin associated protein centralspindlin recruits the Rho guanine nucleotide exchange factor (RhoGEF) epithelial cell transforming 2 (ECT2) to adherens junctions and promotes actomyosin contractility (Ratheesh et al., 2012). Centralspindlin also acts to inhibit p190RhoGAP from being localized at adherens junctions to reinforce the contractile response (Ratheesh et al., 2012). Thus, actomyosin contractility at adherens junctions is regulated and likely in equilibrium in normal epithelia and this regulation may be altered depending on the functional context of the linked cells in the epithelium (Lecuit and Yap, 2015). I postulate that one such context may be oncogenic transformation.

Proteins that can directly alter actin-based cytoskeletal structures also localize to adherens junctions with functional consequence. For example, actin polymerization regulators such as the Arp2/3 complex help maintain the contractile circumferential actin network at the belt-like zonula adherens that encircle individual epithelial cells that are surrounded by multiple neighbours (Kovacs et al., 2011; Vasioukhin et al., 2000; Verma et al., 2012). Additionally, α-catenin contains tension-sensitive vinculin binding sites (Yao et al., 2014) and vinculin binding facilitates the recruitment of Mena/VASP to the adherens junction where it organizes a mechanically-sensitive pool of actin fibres (Leerberg et al., 2014).

The protein zyxin can be found at multiple subcellular locations including the nucleus, focal adhesions, along stress fibres and at cell-cell junctions in cells under high tension (Call et al., 2011; Crawford and Beckerle, 1991; Mori et al., 2009; Suresh Babu et al., 2012; Uemura et
al., 2011; Yoshigi et al., 2005). The latter localization is likely mediated by the fact that zyxin contains Mena/VASP binding sites, an α-actinin binding site and LIM domains that facilitate an association with actin filaments (see Fig 1.1; Hoffman et al., 2012). Notably, zyxin is involved in actin stress fibre repair in cells under high tension due to its interaction α-actinin and VASP (Smith et al., 2010). While the function of zyxin at cell-cell junctions remains controversial, its localization there serves as a useful marker of an intact junctional response to tension (see Fig 1.2; Mori et al., 2009; Sperry et al., 2010). Thus, I compared zyxin localization under different tensional conditions in normal and pre-malignant mammary epithelial cells.

### 1.6 An experimental model of pre-malignant mammary epithelial cell transformation

While invasive epithelially-derived malignant cells that have undergone EMT lose their epithelial cell-cell junctions, non-invasive pre-malignant epithelial cells retain E-cadherin expression and they continue to form adherens junctions (Oft et al., 1996; Thiery, 2002). For example, in the clinical samples of non-invasive pre-malignant breast ductal carcinoma in situ (DCIS) show membranous localization of E-cadherin and the associated adherens junction protein β-catenin (Acs et al., 2001; Karayiannakis et al., 2001). In an effort to mimic this pre-malignant change experimentally, Hartmut Beug’s group in Vienna transformed normal mouse mammary epithelial cells with a constitutively activated form of the ras oncogene (Oft et al., 1996; Roop et al., 1986).

Ras proteins are small GTPases that normally act downstream of G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) to activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (Kamata and Feramisco, 1984; McGrath et al., 1984; Moodie et al., 1993; Mulcahy et al., 1985; Rodriguez-
Viciana et al., 1994). MAPK and PI3K signalling controls a wide variety of cellular functions that include growth, metabolism, motility and differentiation. Multiple forms of Ras proteins with high sequence homology have been identified including Hras (Ha-ras), Kras and Nras (Ellis et al., 1981; Hall et al., 1983). Oncogenic viral ras genes often contain a valine 12 point mutation (G12V) that constitutively activates them and, ultimately, their downstream effectors (Kamata and Feramisco, 1984; McGrath et al., 1984; Sweet et al., 1984).

Pre-malignant mammary epithelial cells transformed with oncogenic ras are hyper-proliferative, but they retain their epithelial characteristics (Janda et al., 2002a). For example, they still take on classical epithelial cobblestone morphologies when grown as monolayers in 2-dimensions and they express E-cadherin and form adherens junctions (Ayollo et al., 2009; Hogan et al., 2009; Vleminckx et al., 1991). They are also capable of forming alveolus-like central lumens, a hallmark of epithelial polarity, when they are cultured as spheroids in 3-dimensions (Janda et al., 2002a, 2002b).

The normal mammary epithelial cell line EpH4 was clonally derived from a population of spontaneously immortalized mammary gland cells generated from pregnant mice (Fialka et al., 1996; Reichmann et al., 1989, 1992). When they are maintained as a 3-dimensional (3D) culture on collagen gels or basement membrane-like Matrigel, EpH4 cells form tubular and alveolar structures that closely resemble those found in vivo (Oft et al., 1996). When treated with lactogenic hormones of pregnancy, EpH4 alveoli also differentiate and secrete a β-casein - containing milk-like substance into their central lumens (Niemann et al., 1998). In 2-dimensional (2D) culture EpH4 cells form typical cobblestone epithelial monolayers with adherens and tight junctions (Fialka et al., 1996). To generate pre-malignant EpRas cells, EpH4 cells were transformed with a constitutively activated viral form of the Ha-ras oncogene (Oft et al., 1996).
EpRas cells form enlarged alveolus-like structures in 3D culture because they have an increased rate of proliferation compared to normal EpH4 cells (Janda et al., 2002a; Oft et al., 1996). However, under 2D conditions EpRas cells are highly similar to normal cells morphologically and they still form adherens and tight junctions (Oft et al., 1996). Importantly, EpH4 and EpRas cells are differentially responsive to TGF-β1 treatment. In normal EpH4 cells TGF-β1 induces apoptosis while it causes pre-malignant EpRas cells to undergo an EMT (Oft et al., 1996). Therefore, I concluded that it would be possible to compare EpH4 and EpRas cells maintained in 2D culture to determine if pre-malignant transformation compromises the ability of cell-cell junctions to respond appropriately to increases in intracellular tension, both chronic and acute, in the absence of TGF-B1.

1.7 Modulating intracellular tension experimentally

Modulating the density of epithelial cells attached to rigid planar substrata (e.g. fibronectin-coated tissue culture plastic or glass coverslips) alters the traction forces experienced by cells maintained in 2D culture. Sparsely plated cells maintained as subconfluent islands are subject to high traction forces, particularly at the island edges, such that they experience increased intracellular tension compared to cells maintained as a dense confluent monolayer (Han et al., 2012; du Roure et al., 2005; Saez et al., 2010, 2005). Thus, this differential plating paradigm on rigid substrata is a method of manipulating intracellular tension in a ‘chronic’ fashion. Intracellular tension (more specifically cytoskeletal tension) can also be modulated by plating cells on compliant (i.e. flexible) silicone rubber substrata and then stretching the substrata for short periods of time (Gavara et al., 2008; Pourati et al., 1998). This is done by plating the substrata on a Teflon post and then applying downward vacuum force on the edge of the rubber substratum. Stretching the ECM-coated silicone rubber increases the intracellular tension of the
cells by physically pulling on the cytoskeleton through cell-cell and cell-ECM adhesions in an ‘acute’ manner.

1.8 Hypothesis

I hypothesize that the cell-cell junctions of pre-malignant ras oncogene-transformed mammary epithelia have an altered ability to respond to increases in intracellular tension compared to those responses that occur in normal mammary epithelial cells.

1.9 Objectives

To characterize the ability of cell-cell junctions to respond to chronic modulation of intracellular tension in normal and pre-malignant cells, normal EpH4 and pre-malignant EpRas cells were plated on a rigid substratum at different densities and changes in the localization of tension-sensitive protein zyxin and the contractility marker pMLC2(S19) were assessed. To characterize the ability of cell-cell junctions to respond to acute modulation of intracellular tension, cells plated on flexible silicone rubber substrata were stretched and zyxin and pMLC2(S19) localization were assessed. To study whether increased MAPK and/or PI3K signalling downstream of the ras oncogene are responsible for any observed alteration in the tensional response of pre-malignant cells, pharmacological inhibitors of MAPK and PI3K were used in an effort to ‘normalize’ the response.
Figure 1.1: The domains associated with tensional response and actin repair of the protein zyxin.

The protein zyxin contains a site to bind $\alpha$-actinin near its N-terminus and has 4 ActA repeats with which Mena/VASP proteins can associate. The LIM domains located closer to the C-terminus are required for the localization of zyxin to sites of mechanically-induced actin stress fibre damage. The ability of zyxin to recruit $\alpha$-actinin and Mena/VASP proteins is associated with its capacity to repair stress fibres.
Figure 1.2: The composition of select actin-associated cell-cell junctions.
The apical-most cell-cell junctions in polarized epithelia are the tight junctions that are composed of transmembrane proteins (e.g. claudins and occludins) attached to adaptor proteins (e.g. ZO-1) linked to the actin cytoskeleton. Localized basal to tight junctions, the adherens junctions contain calcium-dependent transmembrane proteins from the cadherin family such as E-cadherin that are attached to the actin cytoskeleton via a number of cytoskeletal linkers (α-catenin, β-catenin). While both tight and adherens junctions are associated with the actin cytoskeleton, adherens junctions are better characterized to transduce external mechanical signals into an internal contractile response. These adherens junctions transmit extracellular mechanical signals to the actin cytoskeleton that is thought to respond by generating intracellular (contractile) force through the action of myosin motors (e.g. NMII). The protein zyxin localizes to cell-cell junctions under increased tension presumably through its association with junction-associated actin.
Chapter 2: Materials and Methods

2.1 Cell culture

Normal mouse mammary epithelial EpH4 cells (Fialka et al., 1996) and v-Ha-ras transformed pre-malignant EpRas cells (Oft et al., 1996) were routinely cultured in DMEM/F12 media (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS; Hyclone/Thermo Scientific, South Logan, UT) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO) and maintained at 37°C in a humidified incubator with 5% CO2. For passaging, cells were quickly rinsed once with 10 mL Ca²⁺-free DMEM/F12 (Sigma-Aldrich, St. Louis, MO) warmed to 37°C, a new 10 mL wash of Ca²⁺-free DMEM/F12 warmed to 37°C was added, and the cells were then incubated at 37°C for 10 minutes. Afterwards, the Ca²⁺-free DMEM/F12 was removed and 2 mL of low concentration 0.05% Trypsin-EDTA (Life Technologies/Thermo Fisher Scientific, Waltham, MA) was added for approximately 3 minutes to detach cells from the substratum and minimize phenotypic drift due to adhesion protein damage.

2.2 Fibronectin coating of glass coverslips, tissue culture plastic plates, and silicone-rubber bottomed plates

No.1, 18 mm round coverslips (VWR International, Radnor, PA) were sterilized with 70% ethanol, placed in a 20 mm diameter 12 well plate (Falcon/Corning, Corning, NY) and rinsed with sterile phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO). Fibronectin (FN; Sigma-Aldrich, St. Louis, MO) diluted in PBS was added to the sterile coverslips, tissue culture plastic bottomed wells, or silicone rubber-bottomed BioFlex plates (Flexcell International Corporation, Hillsborough, NC) at 2.5 μg/cm² and incubated at 37°C for 1 hour followed by
successive rinses with DMEM/F12 with 2 mM L-glutamine and DMEM/F12 with 1% FBS and 2 mM L-glutamine.

2.3 Plating cells on fibronectin-coated coverslips or plates

After trypsinization, cells were counted using a hemocytometer. For sparse conditions, 12,000 or 30,000 cells were plated in 20 mm wells with 18 mm coverslips or 35 mm wells (tissue culture plastic or silicone rubber-bottomed BioFlex plates), respectively. For dense conditions, 60,000 cells or 150,000 cells were plated into 20 mm wells with 18 mm coverslips or 35 mm wells, respectively. Cells were plated in 1 mL, 2 mL or 3 mL of 1% FBS 2 mM L-glutamine DMEM/F12 in 20 mm wells, 35 mm wells tissue culture or 35 mm wells BioFlex plates, respectively, and cells were allowed to adhere overnight. Media was then changed to complete 5% FBS-containing and the cells were allowed to grow for a total of 3 days after plating, before experiments and analyses were initiated.

2.4 Application of mechanical force to cells on flexible rubber substrates

Stretch-induced tension was applied to FN-coated silicone rubber substrata using a Flexcell FX-5000 apparatus (Flexcell International Corporation, Hillsborough, NC). Vacuum force was applied through a Flexcell baseplate (Flexcell International Corporation, Hillsborough, NC) that provides a stable platform on which 35 mm diameter silicone rubber-bottomed plates can be placed. The baseplate contains 25 mm diameter cylindrical posts that sit beneath each silicone rubber-bottomed well placed onto a baseplate. Once a downward vacuum is activated, the silicone rubber is pulled down on the periphery of the well (i.e. beyond the cylindrical posts) and this stretches the portion of the substratum lying over the posts, applying 2D equally distributed tension to the attached cells in all directions. FlexSoft FX-5000 software (Flexcell
International Corporation, Hillsborough, NC) was used to apply a constant of vacuum force-induced 10% elongation stretch. Unstretched silicone rubber-bottomed plates were not inserted into the baseplate but were placed in the same humidified 37°C incubator in parallel until the end of each experiment.

2.5 **Blebbistatin treatment**

The actomyosin contractility inhibitor blebbistatin (EMD Millipore, Billerica, MA) was reconstituted in DMSO at a stock concentration of 100 mM. Cells were rinsed once with complete media, and then pre-treated for 30 minutes with 50 μM blebbistatin diluted in complete media before starting the experiments for the times described in the results and figure legends.

2.6 **Y-27632 treatment**

The ROCK inhibitor Y-27632 (Sigma, St. Louis, MO) was reconstituted in distilled and deionized water (ddH₂O) at a stock concentration of 10 mM. Cells were rinsed once with complete media, and then pre-treated for 45 minutes with 10 μM Y-27632 diluted in complete media before starting experiments for the times described.

2.7 **MAPK and PI3K inhibitor/stimulator treatments**

The MAPK inhibitor PD98059 (Calbiochem/EMD Millipore, Billerica, MA) and PI3K inhibitor LY294002 (Calbiochem/EMD Millipore, Billerica, MA) were both reconstituted in DMSO at a stock concentration of 20 μM. Insulin (Sigma, St. Louis, MO), which stimulates both MAPK and PI3K in EpH4 cells, was dissolved in 4 mM HCl at a stock concentration of 5 mg/mL. Forty μM PD98059, 20 μM LY294002, 5 μg/uL insulin or a combination of 40 μM PD98095 with 20 μM LY294002 or all 3 compounds at the concentrations listed above, were
generated by dilution in 1% FBS DMEM/F12 with 2 mM L-glutamine. Cells were pre-treated for 1 hour before starting experiments for the times described.

2.8 Western blot analysis

Cells were placed on ice, rinsed with PBS and 50 μL of ice cold radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM tris pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with Complete Mini Ultra Tablet protease inhibitors (Roche, Basel, Switzerland) and 1:100 dilution of both phosphatase inhibitor cocktails 2 and 3 (Sigma, St. Louis, MO), was then applied. Cells were collected using a cell scraper and the resulting unclarified cells lysates were transferred to an ice cold 1.5 mL centrifuge tube, and kept on ice for a total of 30 minutes with vortexing every 5 minutes for 5 seconds. Lysates were then clarified by centrifugation at 19,000 x g for 15 minutes at 4°C.

The protein concentration of the clarified whole cell lysates was determined by the bicinchoninic acid (BCA) assay using a commercially available kit (Pierce/Thermo Scientific, Rockford, IL). Protein concentration was normalized by dilution in fully denaturing sample buffer containing β-mercaptoethanol. The samples were then boiled and separated by SDS-polyacrylamide gel electrophoresis (PAGE) alongside a Kaleidoscope molecular weight ladder (Bio-Rad, Hercules, CA). The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) for 1 hour at 100 volts in transfer buffer (200 mM glycine, 25 mM tris, 20% methanol) at room temperature. The PVDF membranes were then removed, allowed to air dry, rehydrated in methanol, washed once in dH₂O and finally washed once in tris buffered saline containing Tween-20 (TBS-T; 50 mM Tris, 30 mM NaCl,
0.1% Tween-20). The membranes were then blocked in 5% skim milk powder in TBS-T for 1 hour at room temperature. Primary antibodies were prepared in 1% bovine serum albumin (BSA; Fisher Scientific, Fair Lawn, NJ) in TBS-T with 0.02% sodium azide and used at the concentrations, incubation times and temperatures indicated in Table 2.1.

After primary antibody incubation, membranes were rinsed 3 times with TBS-T and then washed 3 times for 5 minutes in TBS-T. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted 1:10000 in 1% BSA TBS-T, and then added to the membranes for 1 hour at room temperature. Membranes were then quickly rinsed 3 times with TBS-T, washed 3 times for 5 minutes each wash in TBS-T. Membranes were then transferred into TBS, and quickly rinsed 3 times in TBS and then washed 3 times for 5 minutes in TBS. Excess TBS was removed and membranes were incubated for 3 minutes in Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA) and signal was detected using DiaFilm autoradiography film (Diamed, Mississauga, ON) that was developed using a Kodak X-OMAT 2000A (Eastman Kodak Company, Rochester, NY) film processor. Molecular weight of the detected proteins was estimated by comparison to the protein ladder.

To strip western blots, 10 mL stripping buffer (62.5 mM tris pH 6.7, 2% SDS, 1.5% β-mercaptoethanol) was added to the PVDF membrane for 10 minutes in a 50°C water bath. Afterward, blots were rinsed 3 times with TBS-T and then washed 3 times for 10 minutes at room temperature, re-blocked and probed with a second set of primary and secondary antibodies as described above.
2.9 Immunocytochemistry (immunostaining)

Cells were rinsed with warmed PBS and then fixed in 4% paraformaldehyde diluted in PBS at 37°C for 20 minutes. Cells on silicone rubber-bottomed plates were fixed with (stretched) or without (unstretched) the vacuum on in a 37°C incubator. After fixation the silicone rubber was cut from the wells using a razor blade and all cells, either on coverslips or silicone rubber, were washed once for 5 minutes with PBS. Depending on the protein to be visualized, detergent extraction was then performed at room temperature according to the concentrations and length of time listed in Table 2.2. The cells were then washed 3 times for 5 minutes each with PBS before being blocked in a solution containing 1% BSA and 10% normal goat serum (NGS) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 1 hour at room temperature. Primary antibodies diluted in 1% BSA/ PBS were then applied overnight, at 4°C, at the concentrations listed in Table 2.2.

After primary antibodies were removed, the cells were washed 3 times for 7 minutes each with PBS before secondary antibodies were applied and incubated for 1 hour at room temperature. Secondary antibodies were diluted in 1% BSA/ PBS at the concentration listed in Table 2.3, together with a 1:300 dilution of rhodamine phalloidin (Life Technologies/Thermo Fisher Scientific, Waltham, MA) to illuminate filamentous (f-) actin.

After secondary antibody incubation, stained cells were washed 3 times for 7 minutes with PBS and stained with 1 μg/mL 4’6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) diluted in 1% BSA/ PBS to illuminate nuclei. Samples were then further washed 4 times for 7 minutes each with PBS and mounted on slides with ProLong Gold (Life Technologies/Thermo Fisher Scientific, Waltham, MA) to reduce photobleaching during imaging. Note that while cells
on 18 mm coverslips were mounted cell-side-down onto slides, silicone rubber membranes were mounted cell-side-up and then covered with a square 22 mm x 22 mm No. 1 size coverslip (Fisher Scientific/Thermo Fisher Scientific, Waltham, MA). All coverslips were then sealed with nail polish.

2.10 Imaging

Stained cells were imaged using either a Leica Sp5 scanning laser confocal microscope (Leica Microsystems, Wetzlar, Germany) or a 3i spinning disk confocal system (3i/Intelligent Imaging Innovations, Denver, CO). A Leica (Leica Microsystems, Wetzlar, Germany) 63x oil immersion objective was used on the Leica Sp5 microscope. A Zeiss (Carl Zeiss, Jena, Germany) 63x oil immersion objective was used on the 3i system which also included a 2.5x built-in optical zoom to the camera. Tonal level adjustments were made to images in panels to clarify features and the same tonal level adjustments were made between comparable images within one panel in the same channel to preserve intensity information unless otherwise stated.

2.11 Image quantification of zyxin at apical cell-cell junctions

CellProfiler (Carpenter et al., 2006; Kamentsky et al., 2011) was used to quantify images for zyxin localization to intercellular junctions. Images were taken on 3 channels: 488, 568 and 647. Tonal levels were adjusted for each image in the exact same manner to ensure that intensity data was not lost or misrepresented in each quantified image. Six consecutive apical Z-slices were selected. A mask of the E-cadherin channel was generated to outline the location of the cell-cell junctions per Z slice. Mean zyxin intensity at cell-cell junctions was measured by determining pixel coincidence with the area covered by the E-cadherin mask. Means of the 6 slices per image were then determined which were then averaged over 5 non-overlapping fields.
Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA) using an unpaired t-test with a 95% confidence interval.

**Table 2.1 Primary antibodies used for western blotting**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Incubation Length/ Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Akt (total protein), Rabbit</td>
<td>Cell Signalling Technologies, Danvers, MA</td>
<td>1:1000</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>Anti- extracellular regulated kinase 1 and 2 (ERK1/2; total protein), Rabbit</td>
<td>Santa Cruz Biotechnology, Dallas, TX</td>
<td>1:2000</td>
<td>Overnight at 4°C</td>
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<tr>
<td>Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Mouse</td>
<td>EMD Millipore, Billerica, MA</td>
<td>1:20000</td>
<td>Overnight at 4°C</td>
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<tr>
<td>Anti-phosphorylated Akt at serine 473 (pAkt(S473)) Rabbit</td>
<td>Cell Signalling Technologies, Danvers, MA</td>
<td>1:2000</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>Anti-phosphorylated extracellular regulated kinase 1 and 2 at threonine 202 and tyrosine 204 (pERK1/2 (T202/Y204)), Rabbit</td>
<td>Cell Signalling Technologies, Danvers, MA</td>
<td>1:4000</td>
<td>Overnight at 4°C</td>
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<tr>
<td>Anti-zyxin (clone B71), Rabbit</td>
<td>Gift from Dr. Mary Beckerle’s Lab</td>
<td>1:10000</td>
<td>1 hour at room temperature</td>
</tr>
</tbody>
</table>
Table 2.2 Primary antibodies used for immunocytochemistry

<table>
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<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-E-cadherin, Mouse</td>
<td>BD Biosciences, San Jose, CA</td>
<td>1:500</td>
<td>0.5% triton X-100 in PBS for 10 minutes</td>
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<tr>
<td>Anti-phosphorylated myosin light chain 2 at serine 19 (pMLC2(S19)), Rabbit</td>
<td>Cell Signalling Technologies, Danvers, MA</td>
<td>1:50</td>
<td>0.2% triton X-100 in PBS for 10 minutes</td>
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<tr>
<td>Anti-zonula occludens protein 1(ZO-1), Rabbit</td>
<td>Invitrogen/Thermo Fisher Scientific, Camarillo, CA</td>
<td>1:100</td>
<td>0.5% triton X-100 in PBS for 10 minutes</td>
</tr>
<tr>
<td>Anti-zonula occludens protein 1 (ZO-1), Rat</td>
<td>Santa Cruz Biotechnology, Dallas, TX</td>
<td>1:100</td>
<td>0.5% triton X-100 in PBS for 10 minutes</td>
</tr>
<tr>
<td>Anti-zyxin (clone B71), Rabbit</td>
<td>Gift from Dr. Mary Beckerle’s Lab</td>
<td>1:600</td>
<td>0.5% triton X-100 in PBS for 10 minutes</td>
</tr>
</tbody>
</table>

Table 2.3 Secondary antibodies used for immunocytochemistry

<table>
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<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Mouse IgG H&amp;L (Alexa Fluor 488) Goat</td>
<td>Life Technologies/Thermo Fisher Scientific, Waltham, MA</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-Mouse IgG H&amp;L (Alexa Fluor 647) Goat</td>
<td>Life Technologies/Thermo Fisher Scientific, Waltham, MA</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-Rabbit IgG H&amp;L (Alexa Fluor 488) Goat</td>
<td>Life Technologies/Thermo Fisher Scientific, Waltham, MA</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-Rabbit IgG H&amp;L (Alexa Fluor 647) Goat</td>
<td>Life Technologies/Thermo Fisher Scientific, Waltham, MA</td>
<td>1:200</td>
</tr>
</tbody>
</table>
Chapter 3: Results

3.1 The ability of cell-cell junctions to respond to chronic increases in intracellular tension is compromised in pre-malignant, ras-transformed mammary epithelial cells maintained on rigid substrata

The LIM domain-containing protein zyxin is known to dynamically localize to sites of increased intracellular tension (Mori et al., 2009; Smith et al., 2014). Therefore, I first set out to determine if the relocalization of zyxin could be used to indicate differential responses to changes in intracellular tension at cell-cell junctions in normal EpH4 compared to pre-malignant EpRas mammary epithelial cells.

To generate chronic differences in intracellular tension using differential cell density, I maintained normal (EpH4) and ras-transformed (EpRas) mammary epithelial cells under subconfluent (i.e. island) and confluent conditions in 2D culture on a fibronectin (FN)-coated stiff glass substratum for three days and then assessed zyxin localization by immunofluorescence (Fig 3.1). Cell-cell junctions were simultaneously visualized using actin, E-cadherin (adherens junction specific), and ZO-1 (tight junction specific) in both the basal (i.e. near the site of cell attachment to FN on the coverslip) and apical (i.e. near the free surface of the cell) domains using confocal microscopy.

When they were maintained as confluent monolayers (i.e. lower traction force/tension state; see detailed explanation in section 1.7 of the Introduction, above), normal EpH4 mammary epithelial cells formed E-cadherin-containing adherens junctions that could be seen both apically and basally (Fig 3.2A). Zyxin was not prominently localized to these junctions under these
conditions, but instead was punctate and more prominent in the basal domain in what appeared to be small cell-ECM contacts at the cell-FN substratum interface (Fig 3.2A).

When they were maintained as subconfluent islands (i.e. presumed higher traction force/tension state), normal EpH4 mammary epithelial cells formed E-cadherin-containing adherens junctions that were more prominent in the apical than basal domains (Fig 3.2B). In the basal domain, zyxin was associated with adhesions at the cell-ECM interface and in the apical domain, zyxin was clearly associated with E-cadherin-containing cell-cell junctions (Fig 3.2B). To confirm that zyxin was indeed at cell-cell junctions in the apical domain I co-stained for the tight junction associated protein ZO-1. Here, again, zyxin was co-localized ZO-1-marked cell-cell (i.e. tight) junctions in the apical domain within the island, but not in the confluent monolayers (Fig 3.3). Taken together, these observations suggest that the relocalization of zyxin can be used as an indicator of a functional response to chronically increased intracellular tension at cell-cell junctions, specifically in the apical domain.

Intracellular tension, particularly that which is associated with the actin portion of the cytoskeleton, is often mediated by actomyosin contractility which can be monitored by the presence of phosphorylated myosin light chain 2 (pMLC2) at serine 19 (Ikebe and Hartshorne, 1985; Ikebe et al., 1988; Rayment, 1996; Vicente-Manzanares et al., 2009). When normal EpH4 cells were maintained under low tension as confluent monolayers, pMLC2 was not prominently associated with cell-cell junctions. Instead, it was punctate and mostly confined to the basal domain, presumably in large part associated with cell-ECM contacts (Fig 3.4A). In contrast, when cells were maintained under higher tension as subconfluent, sparse islands, pMLC2 was prominent at actin fibres in the basal domain and at cell-cell junctions in the apical domain (Fig
These observations support the conclusion that cell-cell junctions in the apical domain of normal mammary epithelial cells respond to chronic increases in intracellular tension.

When pre-malignant, ras-transformed mammary epithelial cells (EpRas) were maintained as confluent monolayers (i.e. lower traction force/intracellular tension), E-cadherin localized to cell-cell borders and it was present in both the apical and basal domains, presumably because these cells are very flat (compare Z-orientation images Fig 3.9B and 3.11B), although this localization was less linear than in the normal EpH4 cells (Fig 3.5A). This confirms that, as has been published previously (Oft et al., 1996) these ras-transformed cells are still able to form adherens junctions. Under these conditions, little zyxin was associated with EpRas cell-cell junctions in either the apical or basal domains (Fig 3.5A). When the pre-malignant cells were maintained in sparse, separated islands (higher traction force/intracellular tension), zyxin localized to cell-ECM adhesions in the basal domain and in the apical domain there were small amounts of zyxin that were found in close proximity to E-cadherin (Fig 3.5B). However, zyxin was not prominently associated with ZO-1 in the apical domain under these higher traction force conditions (Fig 3.6B) which suggests that the ability of cell-cell junctions to respond efficiently to chronic increases in intracellular tension are likely compromised in the pre-malignant ras-transformed cells. This tentative conclusion was supported by the pMLC2 staining. Specifically, while pMLC2 was prominent along actin fibres in the basal domain, it did not efficiently associate with cell junctional actin in the apical domain of cells maintained as subconfluent islands under high tension conditions (Fig 3.7).
3.2 The ability of cell-cell junctions to respond to chronic increases in intracellular tension is compromised in pre-malignant, ras-transformed mammary epithelial cells maintained on flexible substrata

Up to this point, I had chronically modulated tension on a rigid substratum. As a prelude to carrying out acute experiments (see below), I assessed the ability of the cell-cell junctions of normal EpH4 and pre-malignant EpRas cells to respond to chronic differences in intracellular tension when they were plated on flexible FN-coated silicone rubber substrata. When normal EpH4 cells were maintained on silicone rubber as confluent monolayers (low traction force/low intracellular tension), little zyxin was localized to cell-cell junctions at either the basal or apical domains (Fig 3.8, 3.9). In contrast, when normal cells were maintained as subconfluent islands (higher traction force/higher intracellular tension), zyxin was relocalized to basal cell-ECM junctions and apical cell-cell junctions (Fig 3.8, 3.9). This relocalization to basal and apical junctions in the subconfluent island cultures was clear in Z-orientation images (Fig 3.9B). Thus, as was the case on rigid substrata, the cell-cell junctions of normal cells were able to respond to chronic increases in intracellular tension when they were maintained on flexible substrata.

When premalignant EpRas cells were maintained on silicone rubber as confluent monolayers (low traction force/low intracellular tension), little zyxin was localized to cell-cell junctions at either the basal or apical domains (Fig 3.10, 3.11). When EpRas cells were instead maintained as subconfluent islands (higher traction force/higher intracellular tension) zyxin localized to basal cell-ECM adhesions and basal actin fibres but it did not prominently localize to apical cell-cell junctions (Fig 3.10, 3.11). Thus, as was the case on rigid substrata, the ability of cell-cell junctions to respond efficiently to chronic increases in intracellular tension was
compromised in pre-malignant, ras-transformed cells when they are maintained on flexible substrata.

3.3 The ability of cell-cell junctions to respond to acute increases in intracellular tension is compromised in pre-malignant, ras-transformed mammary epithelial cells maintained on flexible substrata

When cells are attached to ECM-coated flexible silicone rubber substrata their intracellular tension (more specifically cytoskeletal tension) can be acutely increased by stretching that substratum (Gavara et al., 2008; Pourati et al., 1998). To carry out such experiments I plated the cells as confluent monolayers on FN-coated silicone rubber membranes and used a downward-directed vacuum to stretch the membrane by 10% in all directions (equibiaxial stretch) for five minutes using a Flexcell apparatus (Fig 3.12; see Materials and Methods for details). When this acute increase in intracellular tension was applied to normal EpH4 cells (i.e. the ‘stretched’ condition) zyxin relocalized to cell-cell junctions in the apical domain in a statistically significant manner (Fig 3.13A). Interestingly, there was much less shifting of zyxin to cell-ECM junctions in the basal domain under these short-term acute conditions of increased intracellular tension compared to that which was observed when tension was increased chronically in subconfluent island culture (compare Fig 3.8A and 3.13A, basal domains). When an acute increase in intracellular tension (i.e. stretched) was applied to pre-malignant EpRas cells, there was no significant increase in zyxin relocalization to apical cell-cell junctions (Fig 3.13B). While there was an apparent increase in cytoplasmic zyxin in the higher tension condition, particularly in the apical domain, there was no overall increase in total zyxin in the cells as shown by Western blotting (Fig 3.14). This suggests that zyxin may associate with non-junctional cytoskeleton-anchored, insoluble moieties in the cell cytoplasm in the stretched...
condition. Regardless, taken together, these observations indicate that the ability of cell-cell junctions to respond efficiently to acute increases in intracellular tension is compromised in pre-malignant, ras-transformed cells. Thus, I was able to replicate this defect in the pre-malignant cells under three different conditions: chronically on rigid glass, chronically on silicone rubber, and acutely on silicone rubber.

I next asked if there were any structural changes in the actin cytoskeleton observable at the light microscopic level when I acutely increased intracellular tension by stretching the rubber substratum given that actomyosin contractility is responsible for resisting such physically-mediated perturbations in intracellular tension (Paszek et al., 2005; Wozniak et al., 2003). While there were no changes in the staining pattern of f-actin on the basal surface of normal EpH4 mammary epithelial cells after stretch was applied (Figure 3.15A and B), there was a change in the staining pattern on the apical surface of the normal cells. Specifically, the non-junctional apical actin cytoskeleton was converted from a punctate, microvillus-like pattern in the resting low tension/unstretched condition to bundles that resembled stress-fibre-like structures in the high tension/stretched condition. This switch in the non-junctional actin-patterning coincided with the expected relocalization of zyxin (Fig 3.15A) and the accumulation of the contractility marker pMLC2 (Fig 3.16A). Importantly, this change in f-actin structure did not occur in the pre-malignant EpRas cells as there was no stretch/tension-mediated induction of apical actin bundles/stress fibres, zyxin relocalization (Fig 3.15C and D), or accumulation of pMLC2 (Fig 3.16C). This suggests that there may also be a defect in the ability of the contractile actin cytoskeleton to respond appropriately to acute increases in intracellular tension in the apical domain of pre-malignant cells.
To directly test whether actomyosin-based contractility plays a role in the ability of normal EpH4 cells to respond appropriately to acute increases in intracellular tension, I pharmacologically inhibited the Rho-associated protein kinase (ROCK) as it drives the critical Ser 19 phosphorylation of MLC2 (Amano et al., 1996; Vicente-Manzanares et al., 2009). To do this, cells were maintained as confluent monolayers on flexible FN-coated rubber membranes, pre-treated for 45 minutes with 10 \( \mu \text{M} \) of the Y-27632 ROCK inhibitor and then stretched. pMLC2 accumulated, as expected, at apical intercellular regions of normal EpH4 cells after stretch in control conditions and this was blocked by Y-27632 pre-treatment (Fig 3.17A). Y-27632 pre-treatment also reduced the stretch-mediated formation of apical actin bundles/stress fibres (Fig 3.17B, 3.18B) and the relocalization of zyxin to apical cell-cell junctions in normal EpH4 cells (Fig 3.18A). Y-27632 pre-treatment of pre-malignant EpRas cells caused an overall reduction of pMLC2 staining (Fig 3.17C) but it did not affect apical localization of actin (Fig 3.17D) or zyxin (Fig 3.18C and D). Taken together, these results suggest that ROCK-induced actomyosin contractility plays a functional role in the tensional response at cell-cell junctions in normal mammary epithelial cells.

3.4 **Short term MAPK and PI3K signalling inhibition does not restore the ability of pre-malignant EpRas cells to respond to acute increases in intracellular tension at cell-cell junctions**

Constitutively-active oncogenic forms of the ras oncogene, including the v-Ha-ras form used to transform EpRas cells to pre-malignancy, upregulate activity of both MAPK and PI3K signalling pathways (Oft et al., 1996). To determine if the constitutive activation of either, or both, of these pathways contributes to the compromised intracellular tension response of EpRas cells, I pharmacologically inhibited the pathways using PD98059 (MAPK inhibition) and
LY294002 (PI3K inhibition), either alone or together, and then examined the localization of zyxin after cells attached to silicone rubber substrata were stretched.

Pathway activation by the v-Ha-ras oncogene in EpRas cells was confirmed by western blotting using antibodies against phosphorylation specific forms of ERK1/2 (T202/Y204; pERK1/2) for MAPK signalling, and Akt (S473; pAkt) for PI3K signalling (Fig 3.19). Specifically, pre-malignant EpRas cells had constitutively increased levels of pERK1/2 and pAkt compared to normal EpH4 cells in control, untreated conditions (Fig 3.19A and B). This difference between the two cell lines, especially for PI3K/Akt signaling, was further exacerbated by insulin treatment which stimulates both pathways in EpH4 and EpRas cells. Individual pathway inhibition using 40 μM PD98059 and 20 μM LY294002 resulted in effective inhibition of affected pathways in all cells, including EpRas. Furthermore, simultaneous treatment with PD98059 and LY294002 effectively inhibited both MAPK and PI3K signalling pathways concurrently (Fig 3.19A and B).

I next asked whether pharmacological inhibition of MAPK and/or PI3K signaling would rescue the ability of pre-malignant EpRas cells to respond appropriately to acute increases in intracellular tension. To do this, cells were maintained as monolayers on silicone rubber plates, pretreated with PD98059 and/or LY294002 and then stretched (compared to unstretched on rubber). The increase in stretch-induced intracellular tension did not appreciably increase signalling in MAPK or PI3K pathways in EpRas cells, but the inhibitors effectively dampened both, when added either individually or together (Fig 3.20A and B). The pharmacologic inhibition of MAPK (Fig 3.20C) and PI3K (Fig 3.20D) individually did not result in the recovery of zyxin localization to apical intercellular junctions when the pre-malignant cells were stretched. The same was true when both MAPK and PI3K pathways were inhibited together (Fig
3.20E). Therefore, short term pharmacologic inhibition of the MAPK and PI3K signalling that is constitutively activated by the v-Ha-Ras oncogene, does not rescue the compromised response to increased intracellular tension in pre-malignant ras-transformed EpRas mammary epithelial cells.
Figure 3.1: Experimental system for the modulation of tension using traction forces via differential density plating.
A) Normal EpH4 and pre-malignant EpRas cells were plated in either dense (50,000 cells) or sparse (12,000 cells) conditions on fibronectin-coated glass coverslips and allowed to grow for 3 days. The resulting cells in dense conditions were under lower intracellular tension while those in sparse conditions which were under higher intracellular tension due to differences in traction forces. B) Phase contrast images of normal and pre-malignant cells grown in dense (lower tension) or sparse (higher tension) conditions. Scale bar = 20 μm.
Figure 3.2: Zyxin localizes to apical E-cadherin-containing cell-cell junctions in normal mammary epithelial cells attached to a rigid substratum under chronic high tension conditions.
Normal EpH4 mammary epithelial cells plated on fibronectin-coated glass coverslips were co-immunostained for zyxin (green) and E-cadherin (red). Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. A) In high density monolayer cultures (chronic lower tension conditions) the adherens junction component E-cadherin was present at sites of cell-cell interaction in both apical and basal domains. Zyxin was punctate on the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. B) In sparse monolayer cultures (chronic higher tension conditions) E-cadherin was more prominent at sites of cell-cell interaction in the apical domain. In the basal domain zyxin was localized in what appears to be cell-ECM junctions (red arrows). In the apical domain zyxin co-localized with E-cadherin at sites of cell-cell interaction (orange arrows). Scale bar = 10 μm.
Figure 3.3: Zyxin localizes to apical ZO-1-containing cell-cell junctions in normal mammmary epithelial cells attached to a rigid substratum under chronic high tension conditions.
Normal EpH4 mammary epithelial cells plated on fibronectin-coated glass coverslips were co-immunostained for zyxin (green) and ZO-1(red). Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. **A)** In high density monolayer cultures (chronic lower tension conditions) the tight junction component ZO-1 was present at sites of cell-cell interaction in apical domains only. Zyxin was punctate on the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. **B)** In sparse monolayer cultures (chronic higher tension conditions) ZO-1 was prominent at apical sites of cell-cell interaction only. In the basal domain zyxin was localized to what were presumed to be cell-ECM junctions (red arrows). In the apical domain zyxin co-localized with ZO-1 at sites of cell-cell interaction (orange arrows).

Scale bar = 10 μm.
Figure 3.4: Phosphorylated myosin light chain 2 (pMLC2) localizes to apical cell-cell junctions in normal mammary epithelial cells attached to a rigid substratum under chronic high tension conditions.
Normal EpH4 mammary epithelial cells plated on fibronectin-coated glass coverslips were immunostained for pMLC2 at serine 19 (green) and co-stained for f-actin using rhodamine phalloidin (red). **A** In high density monolayer cultures (chronic lower tension conditions) pMLC2 did not prominently co-localize with cell junctional actin in either the apical or basal domain. **B** In sparse monolayer cultures (chronic higher tension conditions) pMLC2 co-localized with cell junctional actin in the apical domain, particularly at cell corners where multiple cells meet (white arrows). In the basal domain pMLC2 localized with cytoplasmic f-actin that resembled stress fibres (white arrows). Scale bar = 10 μm.
Figure 3.5: Zyxin weakly localizes to apical E-cadherin-containing cell-cell junctions in pre-malignant mammary epithelial cells attached to a rigid substratum under chronic high tension conditions.
Pre-malignant ras-transformed EpRas mammary epithelial cells plated on fibronectin-coated glass coverslips were co-immunostained for zyxin (green) and E-cadherin (red). Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. **A)** In high density monolayer cultures (chronic lower tension conditions) the adherens junction component E-cadherin was present at sites of cell-cell interaction in both apical and basal domains. There was also some punctate E-cadherin. Zyxin was punctate in the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. **B)** In sparse monolayer cultures (chronic higher tension conditions) E-cadherin was prominent at sites of cell-cell interaction in the apical domain and less so in the basal domain. In the basal domain zyxin was localized in what were presumed to be cell-ECM junctions (red arrows). In the apical domain small amounts of zyxin were co-localized with E-cadherin at sites of cell-cell interaction (orange arrows). Scale bar = 10 μm.
Figure 3.6: Zyxin does not localize to apical ZO-1-containing cell-cell junctions in pre-malignant mammary epithelial cells attached to a rigid substratum under chronic high tension conditions.
Pre-malignant ras-transformed EpRas mammary epithelial cells plated on fibronectin-coated glass coverslips were co-immunostained for zyxin (green) and ZO-1 (red). Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. **A** In high density monolayer cultures (chronic lower tension conditions) the tight junction component ZO-1 was present at sites of cell-cell interaction in the apical domain only. Zyxin was punctate in the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. **B** In sparse monolayer cultures (chronic higher tension conditions) ZO-1 was prominent at sites of cell-cell interaction in the apical domain only. In the basal domain zyxin was localized in what were presumed to be cell-ECM junctions (red arrows). In the apical domain zyxin was not co-localized with ZO-1 at sites of cell-cell interaction. Scale bar = 10 μm.
Figure 3.7: Phosphorylated myosin light chain 2 (pMLC2) does not efficiently localize to apical cell-cell junctions in pre-malignant mammary epithelial cells attached to a rigid substratum under chronic high tension conditions.
Pre-malignant ras-transformed EpRas mammary epithelial cells plated on fibronectin-coated glass coverslips were immunostained for pMLC2 at serine 19 (green) and co-stained for f-actin using rhodamine phalloidin (red). A) In high density monolayer cultures (chronic lower tension conditions) pMLC2 did not co-localize with cell junctional actin in either the apical or basal domain. B) In sparse monolayer cultures (chronic higher tension conditions) pMLC2 did not efficiently co-localize with cell junctional actin in the apical domain where it was punctate. In the basal domain pMLC2 localized with cytoplasmic f-actin that resembled stress fibres (white arrows). Scale bar = 10 μm.
Figure 3.8: Zyxin localizes to apical E-cadherin-containing cell-cell junctions in normal mammary epithelial cells attached to a flexible substratum under chronic high tension conditions.
Normal EpH4 mammary epithelial cells plated on fibronectin-coated silicone rubber membranes were co-immunostained for zyxin (green) and E-cadherin (red). They were also co-stained for f-actin with rhodamine phalloidin (blue). Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. In high density monolayer cultures (chronic lower tension conditions) E-cadherin and f-actin were present at sites of cell-cell interaction in both apical and basal domains. Zyxin was punctate on the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. In sparse monolayer cultures (chronic higher tension conditions) E-cadherin and f-actin were more prominent at sites of cell-cell interaction in the apical domain. In the basal domain zyxin was localized in what appears to be cell-ECM junctions (red arrows). In the apical domain zyxin co-localized with E-cadherin and f-actin at sites of cell-cell interaction (orange arrows). Scale bar = 10 μm.
Figure 3.9: Zyxin localizes to apical ZO-1-containing cell-cell junctions in normal mammary epithelial cells attached to a flexible substratum under chronic high tension conditions.
Normal EpH4 mammary epithelial cells plated on fibronectin-coated silicone rubber membranes were co-immunostained for zyxin (green) and ZO-1 (red). They were also co-stained for f-actin with rhodamine phalloidin (blue). A) Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. In high density monolayer cultures (chronic lower tension conditions) the tight junction component ZO-1 and f-actin co-localized at sites of cell-cell interaction in the apical domain only. Zyxin was punctate on the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. In sparse monolayer cultures (chronic higher tension conditions) ZO-1 and f-actin were co-localized at sites of cell-cell interaction in the apical domain only. In the basal domain zyxin was localized in what appears to be cell-ECM junctions (red arrows). In the apical domain zyxin co-localized with ZO-1 at sites of cell-cell interaction (orange arrows). B) In orthogonal slices, zyxin did not localize to sites of cell-cell interaction in the high density monolayer cultures (chronic lower tension conditions). Zyxin was localized to apical sites of cell-cell interaction (orange arrow) and basal cell-ECM adhesions (red arrow) in the sparse monolayer cultures (chronic higher tension conditions). ZO-1 was localized to apical sites of cell-cell interaction in both the high density and sparse cultures (cyan arrows). Scale bar = 10 μm.
Figure 3.10: Zyxin does not localize to apical E-cadherin-containing cell-cell junctions in pre-malignant mammary epithelial cells attached to a flexible substratum under chronic high tension conditions.
Pre-malignant ras-transformed EpRas mammary epithelial cells plated on fibronectin-coated silicone rubber membranes were co-immunostained for zyxin (green) and E-cadherin (red). They were also co-stained for f-actin with rhodamine phalloidin (blue). Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. In high density monolayer cultures (chronic lower tension conditions) the adherens junction component E-cadherin and f-actin were present at sites of cell-cell interaction in both apical and basal domains. Zyxin was punctate on both the basal and apical domains. In sparse monolayer cultures (chronic higher tension conditions) E-cadherin and f-actin were more prominent at sites of cell-cell interaction in the apical domain. In the basal domain zyxin was localized in what appears to be cell-ECM junctions and stress fibres (red arrows). In the apical domain zyxin was not localized with E-cadherin and f-actin at sites of cell-cell interaction. Scale bar = 10 μm.
Figure 3.11: Zyxin does not localize to apical ZO-1-containing cell-cell junctions in pre-malignant mammary epithelial cells attached to a flexible substratum under chronic high tension conditions.
Pre-malignant ras-transformed EpRas mammary epithelial cells plated on fibronectin-coated silicone rubber membranes were co-immunostained for zyxin (green) and ZO-1 (red). They were also co-stained for f-actin with rhodamine phalloidin (blue). A) Single confocal Z slices are shown in the apical and basal domain of cells maintained as confluent high density monolayers or as sparse, subconfluent islands. In high density monolayer cultures (chronic lower tension conditions) the tight junction component ZO-1 and f-actin co-localized at sites of cell-cell interaction in the apical domain only. Zyxin was punctate on the basal domain, presumably in small cell-ECM contacts; little zyxin was localized to the apical domain. In sparse monolayer cultures (chronic higher tension conditions) ZO-1 and f-actin were co-localized at sites of cell-cell interaction in the apical domain only. In the basal domain zyxin was localized in what appears to be cell-ECM junctions (red arrows). In the apical domain zyxin was punctate and not localized to sites of ZO-1 and f-actin co-localization at sites of cell-cell interaction. B) In orthogonal slices, zyxin did not localize to sites of cell-cell interaction in the high density monolayer cultures (chronic lower tension conditions) or sparse monolayer cultures (chronic higher tension conditions). Zyxin was basally localized to what appears to be cell-ECM adhesions (red arrow) in the sparse monolayer cultures (chronic higher tension conditions). ZO-1 was localized to apical sites of cell-cell interaction in both the high density and sparse cultures (cyan arrows). Please note that, because pre-malignant ras-transformed cells are very flat in monolayer culture, the delineation between apical and basal domains was more difficult to discern than in normal cells maintained under these conditions (compare with Fig 3.9B). Scale bar = 10 μm.
Figure 3.12: Experimental set-up to acutely increase intracellular tension using the Flexcell FX-5000 tension system.

Cells were plated on top of a fibronectin-coated silicone rubber membrane in a 6-well format BioFlex plate. A single well is shown schematically in the diagram. The central region of the well is then placed atop a cylindrical rigid loading post. To acutely increase the intracellular tension on the cells attached to the substratum over the loading post a downward vacuum force is applied on the periphery of the well (i.e. where no loading post is present). This stretches the compliant silicone rubber membrane substratum over the loading post which applies pulling/stretching force in all directions (equibiaxial) on the attached cells. Modified from Figure 2 of the Flexcell Culture Plate and Loading Station Manual and used with permission from Flexcell International Corp.
Figure 3.13: Zyxin localizes to apical E-cadherin-containing cell-cell junctions in normal and not pre-malignant mammary epithelial cells attached to a flexible substratum under acute high tension conditions.
Normal EpH4 and pre-malignant ras-transformed EpRas mammary epithelial cells were plated on fibronectin-coated silicone rubber and either maintained unstretched or they were stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. They were then fixed and co-immunostained for zyxin (green) and E-cadherin (blue). Single confocal Z slices are shown in the apical and basal domains. A) In normal mammary epithelial cells, zyxin was weakly localized to apical E-cadherin containing cell-cell junctions in resting (unstretched) conditions. However, under acute high tension (stretched) conditions zyxin was prominently localized to E-cadherin containing apical cell-cell junctions (white arrows). Zyxin localization on the basal domain was punctate (presumably in small cell-ECM contacts) both in resting (unstretched) conditions and acute high tension (stretched) conditions. Zyxin intensity was quantified at E-cadherin-containing apical cell-cell junctions and a statistically significant increase was found in cells under acute high tension (stretched) conditions compared to those in resting (unstretched) conditions (see materials and methods for details on the quantification). B) In pre-malignant mammary epithelia, zyxin was not enriched in localization to E-cadherin containing apical cell-cell junctions in either resting (unstretched) or acute high tension (stretched) conditions, although there was an overall increase in the cytoplasmic zyxin signal in the latter condition. Zyxin localization to the basal domain was punctate both in resting (unstretched) conditions and under acute high tension (stretched) conditions. Zyxin intensity was quantified at E-cadherin-containing apical cell-cell junctions and no statistically significant difference was found between cells maintained under resting (unstretched) and acute high tension (stretched) conditions. Quantified data shown as mean ± standard deviation; p value obtained from unpaired Student’s t-test, n = 3; ns, not significant; *p < 0.05. Scale bar = 10 μm.
Figure 3.14: Zyxin protein expression levels do not change in normal and pre-malignant mammary epithelial cells attached to a flexible substratum under acute high tension conditions.

Normal EpH4 and pre-malignant ras-transformed EpRas mammary epithelial cells were plated on fibronectin-coated silicone rubber and maintained either unstretched or they were stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. Cells were then lysed and the proteins from whole cell lysates (10 µg per lane) were then analyzed by Western blotting with antibodies against zyxin and GAPDH. The expected molecular weight of zyxin is 83 kDa, while that of GAPDH is 37 kDa. Zyxin protein levels remained similar between cells in resting (unstretched) conditions and those under acute high tension (stretched) conditions in both normal and pre-malignant mammary epithelial cells. GAPDH was used as a loading control.
Figure 3.15: Zyxin localizes to apical junctional actin and this is associated with the formation of apical actin stress fibres in normal, but not pre-malignant ras-transformed, cells that are subjected to an acute increase in intracellular tension.
Normal EpH4 and pre-malignant ras-transformed EpRas mammary epithelial cells were plated on fibronectin-coated silicone rubber and either maintained unstretched or they were stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. Cells were then fixed and immunostained for zyxin (green) and co-stained for f-actin using rhodamine phalloidin (red). Single confocal Z slices are shown in the apical and basal domains of the cells. A) In normal mammary epithelial cells zyxin was weakly localized with apical junctional actin under resting (unstretched) conditions. In contrast, under acute high tension (stretched) conditions, zyxin did prominently localize to sites of junctional actin as can be seen in these merged images of the co-staining (white arrows). Zyxin staining was punctate (presumably in small cell-ECM contacts) in the basal domains of normal cells maintained in both the resting (unstretched) conditions and acute high tension (stretched) conditions. B) In images of the f-actin staining alone, the non-junctional apical f-actin of normal cells under resting (unstretched) conditions was found in very small puncta that I concluded were microvillus-like structures. Under acute high tension (stretched) conditions these non-junctional puncta disappeared and apical stress fibre-like structures appeared (blue arrows). Actin structures on the basal surface of normal cells were similar in both the resting (unstretched) and acute high tension (stretched) conditions. C) In pre-malignant mammary epithelial cells, zyxin was punctate and cytoplasmic and it was not enriched in localization to sites of apical junctional actin in both the resting (unstretched) and acute high tension (stretched) conditions in images of the dual staining. Zyxin localization was also punctate and non-junctional in the basal domain within cells both in resting (unstretched) conditions and under acute high tension (stretched) conditions. D) In images of the f-actin staining alone, the non-junctional apical actin of pre-malignant ras-transformed cells was organized in small puncta that were present in both the resting (unstretched) and acute high tension (stretched) conditions.
Furthermore, there was little or no evidence of apical stress fibre formation in the acute high tension (stretched) condition. Actin structures on the basal surface of pre-malignant ras-transformed cells were also similar in both the resting (unstretched) and acute high tension (stretched) conditions. Scale bar = 10 μm.
Figure 3.16: Phosphorylated myosin light chain 2 (pMLC2) localizes to apical junctional actin and this is associated with the formation of apical actin stress fibres in normal, but pMLC2 was weakly localized to apical junctional actin with no formation of apical actin stress fibres in pre-malignant ras-transformed, cells that are subjected to an acute increase in intracellular tension.
Normal EpH4 and pre-malignant ras-transformed EpRas mammary epithelial cells were plated on fibronectin-coated silicone rubber and either maintained unstretched or they were stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. Cells were then fixed immunostained for pMLC2 (S19) (green) and co-stained for f-actin using rhodamine phalloidin (red). Single confocal Z slices are shown in the apical and basal domains of the cells. A) In normal mammary epithelial cells pMLC2 was very weakly localized to apical cell junctions in resting (unstretched) conditions. In contrast, under acute high tension (stretched) conditions, pMLC2 strongly localized to apical cell junctions (white arrows). pMLC2 staining was punctate and non-junctional in the basal domains of normal cells maintained in both the resting (unstretched) conditions and acute high tension (stretched) conditions. B) The non-junctional apical f-actin of normal cells under resting (unstretched) conditions was found in very small puncta that I concluded were microvillus-like structures. Under acute high tension (stretched) conditions these non-junctional puncta disappeared and apical stress fibres appeared (blue arrows). Actin structures on the basal surface of normal cells were similar in both the resting (unstretched) and acute high tension (stretched) conditions. C) In pre-malignant mammary epithelial cells, pMLC2 was punctate and cytoplasmic and it was weakly localized to sites of cell junctions in the apical and basal domains in both the resting (unstretched) and acute high tension (stretched) conditions. D) The non-junctional apical actin of pre-malignant ras-transformed cells was organized in small puncta that were present in both the resting (unstretched) and acute high tension (stretched) conditions. Furthermore, there was little or no evidence of apical stress fibre formation in the acute high tension (stretched) condition. Actin structures on the basal surface of pre-malignant ras-transformed cells were also similar in both the resting (unstretched) and acute high tension (stretched) conditions. Scale bar = 10 μm.
Figure 3.17: The acute high tension-induced localization of phosphorylated myosin light chain 2 (pMLC2) to apical cell junctions and apical stress fibre formation in normal mammary epithelial cells requires ROCK signaling.
Normal EpH4 and pre-malignant ras-transformed (EpRas) mammary epithelial cells plated on fibronectin-coated silicone rubber were pre-treated for 45 minutes with 10 μM of the ROCK inhibitor Y-27632 and then either maintained at a resting, unstretched state or stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. Cells were then fixed and immunostained for pMLC2 (green) and they were co-stained for f-actin using rhodamine phalloidin (red). Single confocal Z slices are shown in the apical domains of the cells only. A) In untreated normal cells, an acute increase in intracellular tension (stretched) caused pMLC2 to localize to apical cell junctions (orange arrows) and this was blocked by the treatment with Y-27632. B) In untreated normal cells, apical actin-containing puncta disappeared and apical stress fibres appeared when intracellular tension was acutely increased (stretched condition, cyan arrows). This ability of increased tension to initiate the loss of apical puncta and the formation of apical stress fibres was blocked by treatment with Y-27632. C) In pre-malignant mammary epithelia cells, either untreated or treated with Y-27632, pMLC2 was weakly localized to apical cell junctions in resting (unstretched) or acute high tension (stretched) conditions. D) In pre-malignant mammary epithelia, both untreated and treated with Y-27632, apical actin remained in punctate microvillus-like structures in both resting (unstretched) and acute high intracellular tension (stretched) conditions and no apical stress fibres formed. Scale bar = 10 μm.
Figure 3.18: The acute high tension-induced localization of zyxin to apical cell junctions in normal mammary epithelial cells requires ROCK signaling.
Normal EpH4 and pre-malignant ras-transformed (EpRas) mammary epithelial cells plated on fibronectin-coated silicone rubber were pre-treated for 45 minutes with 10 μM of the ROCK inhibitor Y-27632 and then either maintained at a resting, unstretched state or stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. Cells were then fixed and co-immunostained for zyxin (green) and E-cadherin (blue). They were also co-stained for f-actin using rhodamine phalloidin (red). Single confocal Z slices are shown in the apical domains of the cells only. 

A) In untreated normal cells, an acute increase in intracellular tension (stretched) caused zyxin to localize to apical E-cadherin containing cell-cell junctions (orange arrows) and this was blocked by the treatment with Y-27632. 

B) In untreated normal cells, apical actin-containing puncta disappeared and apical stress fibres appeared when intracellular tension was acutely increased (stretched condition, cyan arrows). This ability of increased tension to initiate the loss of apical puncta and the formation of apical stress fibres was blocked by treatment with Y-27632. 

C) In pre-malignant mammary epithelia cells, either untreated or treated with Y-27632, zyxin was not localized to apical E-cadherin containing cell-cell junctions in resting (unstretched) or acute high tension (stretched) conditions. 

D) In pre-malignant mammary epithelia, both untreated and treated with Y-27632, apical actin remained in punctate microvillus-like structures in both resting (unstretched) and acute high intracellular tension (stretched) conditions and no apical stress fibres formed. Scale bar = 10 μm.
Figure 3.19: Pharmacologic inhibition of MAPK and PI3K signalling in normal and pre-malignant mammary epithelial cells using PD98059 and LY294002.
Normal EpH4 and pre-malignant ras-transformed (EpRas) mammary epithelial cells were plated on fibronectin-coated tissue culture plastic and were then treated for 1 hour with the MAPK inhibitor PD98059 (PD; 40 μM), the PI3K inhibitor LY294002 (LY; 20 μM), insulin (INS; 5 μg/mL), a combination of PD and LY, or a combination of all three compounds. The cells were then lysed and proteins from whole cell lysates were separated by SDS-PAGE and subjected to Western blotting. The blots were probed with phosphorylation-specific antibodies against ERK1/2 (MAPK signalling) and Akt (PI3K signalling) and their respective total proteins. Expected molecular weights of ERK1/2 are 44 kDa and 42 kDa respectively, while the expected molecular weight of Akt is 60 kDa. p = phosphorylated form; t = total protein. A) Compared to normal mammary epithelial cells, MAPK signalling by ERK1/2 phosphorylation at threonine 202 and tyrosine 204 (T202/Y204) was increased in pre-malignant ras-transformed mammary epithelial cells (green arrows), as expected. Treatment with the MEK1/2 inhibitor PD alone or in combination with other treatments reduced the phosphorylation of ERK1/2 in both normal and pre-malignant cells. B) Compared to normal mammary epithelial cells, PI3K signalling by Akt phosphorylation at serine 473 was increased in pre-malignant ras-transformed mammary epithelial cells (green arrows), as expected. Treatment with the PI3K inhibitor LY, either alone or in combination with other treatments reduced the phosphorylation of Akt in both normal and pre-malignant cells. Treatment with INS alone increased the phosphorylation of Akt in both normal and pre-malignant cells.
Figure 3.20: The ability of an acute increase in intracellular tension to localize zyxin to apical E-cadherin-containing cell junctions is not rescued by the short term pharmacologic inhibition of MAPK and/or PI3K in pre-malignant mammary epithelial cells.
Pre-malignant ras-transformed (EpRas) mammary epithelial cells were pre-treated for 1 hour with 40 μM PD98059 (PD), 20 μM LY294002 (LY) or a combination of both PD and LY and were then maintained at a resting, unstretched state or they were stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. In A and B, whole cell lysates were subjected to Western blotting and the blots were probed with phosphorylation-specific antibodies against ERK1/2 (MAPK signalling) and Akt (PI3K signalling) as well as antibodies against their respective total proteins (US = unstretched; S = stretched; p = phosphorylated form; t = total protein). In C, D and E, cells were fixed and co-immunostained for zyxin (green) and E-cadherin (blue). Single confocal Z slices are shown in the apical cell domains. A) ERK1/2 phosphorylation at T202/Y204 was not increased when the cells were subjected to an acute increase in intracellular tension (S). However, ERK1/2 phosphorylation was greatly reduced by PD treatment alone and by dual treatment with both PD and LY (PD+LY). B) Akt phosphorylation at S473 was not increased when the cells were subjected to an acute increase in intracellular tension (S). However, Akt phosphorylation was greatly reduced by LY treatment alone and by a dual treatment with both PD and LY (PD+LY).

Cells treated with PD (C), LY (D) and PD and LY together (E) did not localize zyxin to apical E-cadherin-containing cell junctions when intracellular tension was acutely increased (stretched). Scale bar = 10 μm.
Chapter 4: Discussion

I have determined that there is a tensional response at cell-cell junctions in normal mammary epithelial cells. Specifically, a chronic increase in intracellular tension caused zyxin and pMLC2 to localize to apical cell-cell junctions under higher tension in normal cells. In addition, acutely increasing intracellular tension in normal cells induced zyxin and pMLC2 localization to cell-cell junctions and the formation of apical actin fibres with a concurrent loss of what appeared to be apical microvilli. In contrast, pre-malignant ras oncogene-transformed mammary epithelial cells did not respond to increased tension in this manner. Therefore, pre-malignancy, at least in the context of ras oncogene-mediated transformation, compromises tensional homeostasis at cell-cell junctions in mammary epithelial cells.

Researchers have developed a variety of methods to increase intracellular tension at cell-cell junctions (Borghi et al., 2012; le Duc et al., 2010). In the case of zyxin relocalization, this was previously done by plating epithelial cells under high traction force conditions or by initiating an epithelial to mesenchymal transformation (EMT) by applying growth factors such as TGF-β1 (Crawford and Beckerle, 1991; Mori et al., 2009; Sperry et al., 2010). In the latter case, driving cells to become mesenchymal will cause them to become more contractile, which increases intracellular tension (Bhowmick et al., 2001) that induces the formation of zyxin-dependent apical actin fibres (Mori et al., 2009). However, it is important to note mesenchymal transformation completely changes an epithelial cell’s differentiation program. The changes observed include a reduction in apico-basal polarity, an increase in front-rear polarity, global morphological alterations and, ultimately, a loss in cell-cell junctions themselves (reviewed in Kalluri and Weinberg, 2009). Therefore, it is very difficult to examine the effects of increased intracellular tension in isolation using the EMT paradigm. As such, I characterized a method of
modulating intracellular tension at cell-cell junctions in the absence motogenic factor-induced morphological effects or overt EMT by applying vacuum force to confluent monolayers of cells resting on a flexible rubber substratum. In so doing, I have shown that tension-dependent zyxin relocalization to cell-cell junctions and the formation of apical actin bundles that resemble stress fibres can occur independently of the global change in differentiation programs that are induced by motogenic factors that induce an EMT.

The formation of apical actin stress fibres during epithelial sheet formation in keratinocytes is dependent on the Rho-ROCK contractility pathway (Vaezi et al., 2002). In addition to a requirement for zyxin in the formation of apical stress fibres under certain conditions (Mori et al., 2009), tuberous sclerosis protein 1 (TSC1), a suppressor of mammalian target of rapamycin complex 1 (mTORC1), has been shown to promote the formation of tight junction-associated apical actin fibres (Ohsawa et al., 2013). From a mechanistic point of view, it appears that TSC1 induces the formation of these apical actin fibres by activating the Rho-ROCK pathway and reducing Rac1 activity in apico-basal polarized cells (Ohsawa et al., 2013). My results indicate that tension-induced apical stress fibre formation is also Rho-ROCK dependent, which suggests that TSC1 may be involved upstream in this process as well.

Proteins and complexes that modify actin fibre structure and bundling may also play a role in the tension-dependent formation of apical stress fibres I observed here. For example, the ‘epithelial protein lost in neoplasm’ (EPLIN) stabilizes apical actin bundles in a different context (Abe and Takeichi, 2008) but may be important in tension as well. In addition, the formation of apical stress fibres may involve Mena/VASP proteins given that they can be recruited by zyxin to sites of tension-induced actin-filament damage (Nguyen et al., 2010). Furthermore, actin bundling proteins such as α-actinin may be involved as their localization to actin stress fibres is
regulated by increases in actomyosin contractility (Hotulainen and Lappalainen, 2006; Kreis and Birchmeier, 1980). Therefore, future experiments designed to investigate whether TSC1, EPLIN and/or actin bundling proteins play a role in tension-dependent apical actin fibre formation in normal mammary epithelial cells, and whether the functions of these proteins are misregulated to functional effect in ras-transformed pre-malignant cells, are worth considering.

ROCK-mediated actomyosin contractility is known to be involved in the tensional response at cell-cell junctions (le Duc et al., 2010; Leerberg et al., 2014). In normal mammary epithelial cells, I found that the contractility marker pMLC2 relocalized to cell-cell junctions in response to an increase in intracellular tension and that this response was abolished by the ROCK inhibitor Y-27632. As such it will next be important to verify RhoA activation under conditions of increased tension in these cells, both biochemically and using optical FRET-based sensors to determine if the activation occurs specifically at apical cell-cell junctions. Furthermore, the localization and activities of RhoGAPs and RhoGEFs should be studied as we do not know whether the tension-mediated increase in contractility occurs due to a localized reduction in RhoGAP activity or an increase in RhoGEF activity. Interestingly, evidence for both has been observed where differences in contractility are initiated under conditions where tension has not been directly modulated. For example, under high contractility conditions the protein centralspindlin can recruit RhoGEFs to adherens junctions while, conversely, the DDR1-Par3/Par6 complex inhibits RhoE activity and decreases contractility at adherens junctions via RhoGAP activation (Hidalgo-Carcedo et al., 2011; Ratheesh et al., 2012).

Another inhibitor of actomyosin contractility, blebbistatin, which reduces the affinity of NMII for actin, abolished the tensional response of normal mammary epithelial cells but did not alter the compromised tensional response of pre-malignant mammary epithelial cells in my
experiments in terms of the zyxin relocalization and apical stress fibre formation (Kovács et al., 2004). This was expected. However, blebbistatin also generated a confounding result as it appeared to increase the recruitment of pMLC2 to cell-cell junctions under high tension conditions in normal mammary epithelial cells and this increase in recruitment did not occur in pre-malignant mammary epithelial cells (see appendix A, Fig A.1). Blebbistatin inhibits actomyosin contractility independently of MLC2 phosphorylation. Thus, under certain conditions upstream kinases can still phosphorylate MLC2 in a blebbistatin-inhibited NMII complex (Kovács et al., 2004; Shutova et al., 2012). In addition, the circumferential apical actin belt structure associated with apical cell-cell junctions is altered by blebbistatin (Ayollo et al., 2009). Therefore, the increased localization of pMLC2 to apical cell-cell junctions that I observed after blebbistatin treatment under conditions of high tension may have been caused by a combination of upstream kinase activity and alterations in actin structure.

Zyxin phosphorylation at serine 142 (S142; S144 in mouse) has been associated with its function and subcellular localization (Call et al., 2011; Hoffman et al., 2012; Suresh Babu et al., 2012). For example, MAPK-mediated S142 phosphorylation increases the localization of zyxin to adherens junction where it stabilizes them (Call et al., 2011; Hoffman et al., 2012). Although I did not investigate the phosphorylation of S142 on zyxin in these studies, it is worth investigating in the future, particularly as it may be altered between the normal and ras-transformed cells given that the latter have chronically upregulated MAPK signalling. Alternatively, since zyxin relocalizes to sites of actin stress fibre damage (Smith et al., 2010), it is conceivable that zyxin may localize to actin damage at the circumferential belt, possibly from activation of contractility in normal cells under high intracellular tension. As the LIM domains of zyxin are required for its localization to stress fibres (Hoffman et al., 2012), an epitope-tagged
mutant zyxin without LIM domains could be expressed in an effort to determine if it is the formation of apical stress fibres in normal cells that is responsible for the high tension response. If this turns out to be the case, the lack of zyxin localization to apical cell junctions in premalignant cells might then occur because of a disruption in the ability of these cells to form junction-apical stress fibres under high tension conditions. The cell-cell junctional localization of tension-sensitive proteins other than zyxin such as vinculin or p130Cas would also be important to investigate in the future to elucidate whether this may be a zyxin-specific alteration in tensional response (Leerberg et al., 2014; Sawada et al., 2006).

Ras oncogene-mediated transformation of normal mammary epithelial cells makes them sensitive to TGF-β1-mediated EMT and this sensitivity is dependent on the ability of mutated Ras to constitutively activate both the MAPK and PI3K pathways (Janda et al., 2002b). Therefore, I acutely inhibited these pathways pharmacologically, either alone or together, in an effort to determine if their constitutive activation contributed to the loss of the tensional response in the premalignant ras-transformed mammary epithelial cells. This was not the case. However, as constitutive MAPK and PI3K signaling can initiate stable epigenetic changes within cells (Dunn and Davie, 2005; Dyson et al., 2005; Zuo et al., 2011), it is possible that their acute pharmacologic inhibition did not reverse a change to a tension response caused by the stable ras-transformation. Thus, to determine the role of pathway activation in the loss of the tensional response with certainty, mammary epithelial cells that have been transformed with effector domain mutants that only activate either the MAPK or PI3K pathways exclusively (Janda et al., 2002a, 2002b) could be examined.

While ras-transformation alone does not lead to the dissolution of E-cadherin-mediated adherens junctions or tight junctions, it does induce N-cadherin expression which is not normally
expressed in mammary epithelial cells (Janda et al., 2002a; and see appendix A, Fig A.2). The mechanical significance of the concurrent presence of both E-cadherin and N-cadherin at cell-cell junctions is not well known. However, in cancerous cells, the upregulation of N-cadherin has been traditionally associated with a mesenchymally-transformed phenotype where its presence contributes to cell motility and invasion when E-cadherin expression is suppressed (Maeda et al., 2005). Interestingly, the ectopic subsequent E-cadherin expression in mesenchymal, N-cadherin expressing cell lines does not reduce invasion (Nieman et al., 1999). Furthermore, N-cadherin and E-cadherin can bind to each other, but only homotypic adhesions between subtypes of cadherins are mechanically sensitive (le Duc et al., 2010; Tabdili et al., 2012). Therefore, it is conceivable that the presence of non-mechanically responsive E- and N-cadherin heterotypic binding along junctional domains could result in a reduction of the tensional response in the ras-transformed mammary epithelial cells. Whether N-cadherin does play such a role in disrupting the tensional response could be assessed with certainty by removing the N-cadherin from the ras-transformed mammary epithelial cells and by force expressing N-cadherin in the normal mammary cells. In summary, a model of the system with selected future directions has been summarized in a schematic (Fig. 4.1).

The cellular tensegrity model proposed by Donald Ingber and colleagues postulates that whole tissues act in a mechanically coordinated manner due to the integration of cytoskeletal elements across cell junctions (Ingber, 2003). Clearly, this is the case for the normal mammary epithelial cell monolayers that I used here. Interestingly, however, while the ras-transformed cells continued to form cell junctions it appears that they lost a considerable degree of mechanical coordination, at least as it pertains to the ability to respond to changes in intracellular tension. This is a novel finding in the context of pre-malignant change and it could prove to be
functionally important as it may prime the still junctionally-coupled cells for junctional breakdown in response to mesenchymally transforming motogenic agents such as TGF-ß1. It is also conceivable that losing mechanical coordination may lead to local differences in force-sensing which could cause geographically-restricted groups of cells to protrude into the surrounding stroma without undergoing an overt EMT as occurs when cells become ‘collectively’ invasive (reviewed in Friedl and Alexander, 2011). More broadly, this may mean that while normal tissues initiate coordinated mechanical responses to changes in tension in an effort to maintain tissue integrity and function, pre-malignant tissues may have lost this mechanical coordination which then contributes to tissue disruption and ultimately, local invasion. Further investigation of this possibility will require future tension modulating experiments to be carried out in a 3D context where histiotypic changes to normal and pre-malignant mammary epithelial cell-generated alveolar and ductular structures can be examined in detail when intracellular tension is altered.

4.1 Closing remarks

In this study, I compared the mechanical response of cell-cell junctions in normal and pre-malignant mammary epithelial cells when intracellular tension was increased both chronically and acutely. I found that the homeostatic mechanical response of pre-malignant cells is compromised compared to normal cells. Therefore, I tentatively conclude that even though cell-cell junctions are still present in pre-malignant ras-transformed mammary epithelial cells these junctions are functionally different than those present in normal mammary epithelial cells. Given that these differences could contribute to a later progression to the metastatic state, it will be of future interest to determine whether they arise universally in different epithelial cell types transformed by different means.
Figure 4.1: Model of compromised tensional homeostasis at cell-cell junctions in pre-malignant mammary epithelia and future directions.
Normal mammary epithelial cells respond to an increase in intracellular tension with the localization of zyxin and pMLC2 to cell-cell junctions and the formation of apical actin stress fibres, all responses that require the RhoA-ROCK pathway. The localization of zyxin to cell-cell junctions is presumed to occur due to the tension-induced damage in junction-associated actin, but it has yet to be confirmed. However, ras-transformed pre-malignant mammary epithelia do not have any of the examined responses to an increase in intracellular tension that are present in normal mammary epithelia. This alteration in the tensional homeostasis at cell-cell junctions in ras-transformed pre-malignant mammary epithelial cells could be associated with the junctional localization of other tension-sensitive proteins such as vinculin and p130Cas that can be examined in the future. It could also be due to altered localization and activity of RhoA and modulators of RhoA such as RhoGEFs and RhoGAPs which can be studied in future experiments. Unlike normal mammary epithelial cells, ras-transformed pre-malignant mammary epithelia also have concurrent junctional E-cadherin and N-cadherin expression that could alter tensional response at the cell-cell junctions and this would be worthwhile to examine at a later time by removing N-cadherin from the pre-malignant cells. Finally, short term pharmacological inhibition of upregulated MAPK and PI3K signalling in pre-malignant ras-transformed cells does not restore tensional response. Therefore in the future, to examine the individual contribution of MAPK and PI3K signalling to altered tensional homeostasis at cell-cell junctions in pre-malignant cells, mutant forms of constitutively-active ras with mutant effector domains defective in signalling to each pathway individually will be introduced into cells and their tensional responses will be monitored.
References


Appendices

Appendix A

A.1 Mechanical response under acute high intracellular tension conditions in normal EpH4 mammary epithelial cells is inhibited with the NMII inhibitor blebbistatin

As an alternative to ROCK inhibitor Y-27632, I used blebbistatin, an inhibitor of NMII function to examine the role of contractility in the mechanical response at cell-cell junctions under acute high tension conditions. The mechanical response was analyzed by immunocytochemistry. When intracellular tension was acutely increased in normal mammary epithelia, zyxin did not relocalize to cell-cell junctions and apical stress fibres did not form (Fig A.1A and B). However, pMLC2 localization to cell-cell junctions was increased after stretching cells treated with blebbistatin. In pre-malignant cells, there was no difference between blebbistatin treated and untreated conditions after acutely increasing intracellular tension (Fig A.1C and D).
Figure A.1: Blebbistatin inhibits the localization of zyxin to apical E-cadherin-containing cell junctions and the formation of apical actin stress fibres induced by an acute increase in intracellular tension in normal mammary epithelial cells.
Normal EpH4 and pre-malignant ras-transformed (EpRas) mammary epithelial cells plated on fibronectin-coated silicone rubber substrata were pre-treated for 30 minutes with 50 μM of the actomyosin contractility inhibitor blebbistatin and maintained either unstretched or they were stretched for 5 minutes at 10% elongation to acutely increase intracellular tension. Cells were then fixed and were then co-immunostained for zyxin (green) and E-cadherin (blue) in A & C, and for pMLC2 (green) in B & D. They were also co-stained for f-actin with rhodamine phalloidin (red). Single confocal Z slices of the apical domain of the cells are shown. A) In blebbistatin-treated normal mammary epithelial cells zyxin did not localize to apical E-cadherin containing cell-cell junctions under acute high tension (stretched) conditions. B) In blebbistatin treated normal mammary epithelia, an acute increase in intracellular tension (stretched) caused pMLC2 localization to apical cell-junctional actin. In these cells, apical actin remained as punctate microvillus-like structures after an acute increase in intracellular tension (stretched). C) In blebbistatin treated pre-malignant mammary epithelia, zyxin did not localize to apical E-cadherin mediated cell-cell junctions under acute high tension (stretched) conditions. D) pMLC2 did not localize to cell junctional actin under acute high tension (stretched) conditions in blebbistatin treated pre-malignant mammary epithelia. In these cells, apical actin was organized into punctate microvillus-like structures both in resting (unstretched) conditions and under acute high tension (stretched) conditions. Scale bar = 10 μm.
A.2 Pre-malignant EpRas mammary epithelial cells express N-cadherin

To verify previous reports that pre-malignant ras-transformed EpRas mammary epithelial cells express N-cadherin, I plated EpH4 normal mammary epithelial and EpRas pre-malignant mammary epithelia onto coverslips for immunocytochemistry and plastic tissue culture dishes for western blotting. EpRas pre-malignant cells exhibited junctional staining of N-cadherin, which was absent in EpH4 normal mammary epithelia (Fig A.2A). To confirm that this was not a property of EMT, we also stained for E-cadherin. We found that E-cadherin was junctional in both EpRas pre-malignant mammary epithelia and EpH4 normal mammary epithelia (Fig A.2A). Western blot analysis confirmed the expression of N-cadherin in EpRas pre-malignant mammary epithelial cells but it was not expressed in EpH4 normal mammary epithelia (Fig A.2B). N-cadherin antibody used was from Abcam (ab18203); rabbit anti-N-cadherin; western blotting concentration 1:1000, immunostaining concentration 1:100.
Normal EpH4 and pre-malignant ras-transformed (EpRas) mammary epithelia were plated on fibronectin coated coverslips or tissue culture plates as confluent high density monolayers. In A, cells were fixed and co-immunostained for E-cadherin (red) and N-cadherin (green). In B, cells were lysed and proteins were separated by SDS-PAGE and then subjected to Western blotting with antibodies against N-cadherin. The expected molecular weight of N-cadherin is 150 kDa. A) Normal mammary epithelial cells and pre-malignant mammary epithelia both contained

Figure A.2: N-cadherin is expressed in EpRas pre-malignant mammary epithelial cells
junctional E-cadherin. In contrast, only the pre-malignant cells contained N-cadherin and it was overwhelmingly junctional. Scale bar = 10 μm. B) Western blotting confirmed that normal mammary epithelial cells did not contain N-cadherin while pre-malignant mammary epithelial cells did. GAPDH was used as a loading control.