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The Role of Tumor Stromal Discoidin Domain Receptor 2 (DDR2) in Breast Cancer Metastasis.

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The Role of Tumor Stromal Discoidin Domain Receptor 2 (DDR2) in Breast Cancer Metastasis.

by

Samantha Van Hove Bayer

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
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Table of Contents

List of Figures	iv
List of Tables.....	vi
Acknowledgments	vii
Abstract.....	x
Chapter 1: Introduction.....	1
1.1 Figures	19
1.2 References.....	37
Chapter 2: Stromal DDR2 is required for breast cancer lung metastasis.	50
2.1 Introduction.....	50
2.2 Results	52
2.3 Discussion.....	57
2.4 Materials and Methods	60
2.5 Figures	65
2.6 References.....	77
Chapter 3: DDR2 is critical for matrix remodeling by cancer associated fibroblasts.	80
3.1 Introduction.....	80
3.2 Results	82
3.3 Discussion.....	88
3.4 Materials and Methods	90
3.5 Figures	95
3.6 References.....	105
Chapter 4: DDR2 action promotes recruitment of Talin1 and integrin β 1 activation.	108
4.1 Introduction.....	108
4.2 Results	110
4.3 Discussion.....	114
4.4 Materials and Methods	116
4.5 Figures	118
4.6 References.....	128
Tables	131

Chapter 5: Conclusions and Future Directions 133
5.1 References..... 147

List of Figures

Chapter 1

Figure 1.1: Breast cancer progression and metastasis	19
Figure 1.2: Time to metastasis and overall survival of breast cancer subtypes	21
Figure 1.3: ECM dynamics and tumor progression.....	23
Figure 1.4: Characterization of the activation of cancer associated fibroblasts (CAFs)	25
Figure 1.5: Schematic representations of DDR1 and DDR2	27
Figure 1.6: DDR2 in the stroma is required for lung metastasis, fibrosis, and aggressive collagen phenotype	29
Figure 1.7: Integrin activation occurs in several steps	31
Figure 1.8: Focal adhesions mediate force transmission	33
Figure 1.9: Focal adhesion proteins are arranged in prescribed nanoscale layers	35

Chapter 2

Figure 2.1: FSP1-Cre targets breast tumor stromal cells.....	65
Figure 2.2: FSP1-Cre mice are normal size and FSP1-Cre deletes DDR2 in CAFs.....	67
Figure 2.3: Stromal DDR2 is required for breast cancer lung metastasis.....	69
Figure 2.4: DDR2 action in the stroma does not affect fibrosis.....	71
Figure 2.5: DDR2 affects collagen alignment, fiber width, and fiber length.....	73
Figure 2.6: Stromal DDR2 affects mechanical properties of tumors	75

Chapter 3

Figure 3.1: CAF DDR2 is required for collagen alignment but not collagen incorporation in ECM.....	95
Figure 3.2: CAF DDR2 affects cellular mechanotransduction/mechanosignaling	97
Figure 3.3: DDR2 collagen binding but not kinase activity is required for mechanotransduction and mechanosignaling.....	99
Figure 3.4: DDR2 collagen binding but not kinase activity is required for increased Intracellular tension	101
Figure 3.5: hCAF traction forces require collagen binding but not kinase activity of DDR2 ...	103

Chapter 4

Figure 4.1: DDR2 affects mechanotransduction/mechanosignaling at early time points.....	118
Figure 4.2: DDR2 does not interact with or affect expression of integrin β 1	120
Figure 4.3: DDR2 promotes integrin β 1 activation and talin1 recruitment.....	122
Figure 4.4: DDR2 affects talin1 interaction with integrin β 1	124
Figure 4.5: DDR2 action increases Rap1-GTP levels in response to collagen.....	126

List of Tables

Table 1:	Primer sequence for DDR2 mouse genotyping.....	131
Table 2:	Antibodies used for Western blot, IF, and IP.....	132

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Abstract

The Role for Discoidin Domain Receptor 2 (DDR2) in Breast Cancer Metastasis.

by

Samantha Van Hove Bayer

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Professor Gregory D. Longmore, Chair

Characteristics of breast tumor stroma, including altered collagen architecture and increased stiffness, are known to contribute to tumor invasion and metastasis. However, the cellular and molecular mechanisms by which these changes occur are not fully understood. To address this question, we used a mouse genetic model to delete Discoidin Domain Receptor 2 (DDR2) from mouse tumor stromal cells and interrogated breast cancer associated fibroblasts (CAFs) to determine the molecular events downstream of DDR2 action that may lead to changes in the tumor extracellular matrix (ECM). Our work revealed that the action of DDR2 in breast stromal cells is required for tumor lung metastasis but does not affect tumor growth or latency. Interestingly, stromal DDR2 action led to lengthened, thickened, and straightened collagen fibers while also stiffening the tumor. Tumor stiffness was found to be greatest at the invasive front of the tumor, closest to the tumor/stromal boundary; this finding was obliterated in tumor stromas without DDR2. Selectively studying CAFs *ex vivo*, we found that DDR2 promotes increased cellular contraction and traction force. Super-resolution microscopy analysis of focal adhesion complexes in CAFs revealed that DDR2 collagen binding facilitates focal adhesion maturation

and enhances integrin $\beta 1$ activation through recruitment of Talin11. We also find that DDR2 regulates Rap1 activation, suggesting a mechanism by which Talin11 is activated downstream of DDR2 collagen binding. Taken together, these results identify DDR2 as a novel mechanosensing/mechanotransducing cell surface receptor that promotes tumor invasion and metastasis by acting in tumor stromal CAFs to control ECM remodeling, in part through regulation of integrin $\beta 1$ activity via inside-out signaling.

Chapter 1: Introduction

Breast cancer

Breast cancer is the leading cause of new cancer incidence in women in the United States, and the second leading cause of cancer related death. According to the American Cancer Society, prediction models estimate that there will be approximately 250,000 new cases of invasive breast cancer diagnosed and 40,000 breast cancer related deaths for women in 2017. While mortality has decreased over the past several decades due to improvements in detection and treatment, breast cancer will still lead to the deaths of 1 in 10 women within 5 years and approximately 1 in 5 within 10 years. Additionally, incidence of breast cancer continues to rise in some populations, indicating that further research is still needed to determine how best to prevent, and, most importantly, to treat these patients (American Cancer Society, Cancer Facts & Figures, 2017).

Breast cancer, in general, is believed to arise from acquired mutations in the epithelial cells lining the mammary gland or in their progenitors. These acquired mutations must, by definition, lead to deregulation of normal cell proliferation and resistance to cell death, among other characteristics (Hanahan and Weinberg, 2011). In the epithelial lining of the normal mammary gland, there are two epithelial cell layers, one luminal and one basal. These layers arrange themselves on a basement membrane, which separates the mammary epithelial cells from the surrounding stroma. The luminal epithelial cells face the lumen of the mammary gland, express cytokeratin 8 (K8), and are secretory, while the basal epithelial cells can be appropriately described as myoepithelial cells and are more elongated, contractile, and express cytokeratin 14

(K14) (Shehata et al., 2012). Breast cancer in humans progresses through a series of increasingly complex steps from atypical ductal hyperplasia, to ductal carcinoma *in situ* (DCIS), then invasive carcinoma, which is defined by tumor cells breaking through the basement membrane and invading into the surrounding stroma, and finally metastasis to distant organs (Fig. 1.1) (Hu et al., 2008; Lerwill, 2004; Pinder, 2010).

Breast cancers can be classified by a combination of histopathological and molecular analyses, the details of which will only be briefly touched on here. Histopathological analysis of breast cancers relies heavily on hormone receptor status for classification, ie- estrogen receptor (ER +/-), progesterone receptor (PR +/-), and human epidermal growth factor receptor-2 (HER-2 +/-). A lack of these receptors is termed triple negative breast cancer (TNBC). More recently, molecular subtyping has complemented hormone receptor status classification and stratified breast cancers into four distinct subgroups; luminal A and luminal B, HER-2 overexpressing, and basal-like. Typically, luminal types are less aggressive than the HER-2 overexpressing or basal-like subtypes, with basal-like bearing the worst prognosis (Fig. 1.2). TNBCs usually fall into the basal-like subtype. Basal-like or TNBC cancers do not lend themselves to receptor specific intervention and, therefore, are generally approached with less specific and more toxic chemotherapy treatments (Cancer Genome Atlas, 2012; Sorlie et al., 2001; Sorlie et al., 2003). Overall risk for the development of breast cancer has been ascribed to many factors which can be globally described as those intrinsic to the biology patient (BRCA1/2 mutation, increased breast density, young age of menarche or high age of menopause, African-American race, etc) and those that are more social or socioeconomic (obesity, low access to care, pregnancy, breast feeding, etc) (American Cancer Society, Breast Cancer Facts & Figures, 2015-2016).

Metastasis

Metastasis of breast tumors to distant organs is the cause of most breast cancer related mortality (Lobbezoo et al., 2015), with median survival after a diagnosis of metastasis being 2 to 3 years (Cardoso et al., 2012). For tumor cells to metastasize, several things must happen. First, the tumor cells must be motile and capable of invading through the basement membrane and into an extracellular matrix comprised mostly of fibrillar collagens, primarily type I collagen, and fibronectin, among other matrix proteins. The cells invade the surrounding breast stroma either singly or as a collective, intravasate into the blood stream or lymphatics, survive in the blood stream or lymphatic system, ultimately extravasate, and seed other, non-random, organs (Fig. 1.1). This process relies on the ability of tumor cells to navigate and survive outside of their normal environment, to resist exposure to the immune system, and to start proliferating again (Nguyen et al., 2009).

The presence of tumor cells in the bloodstream (circulating tumor cells; CTCs) has been shown to be prognostic for poor outcomes (Cristofanilli et al., 2004) but can also be used to predict treatment response (Liu et al., 2009). Interestingly, CTCs have recently been shown to be more likely to result in metastases if they are circulating as collectives and also associated with cells expressing mesenchymal markers, suggesting that metastasis is not a tumor cell specific phenomenon but instead is a multicellular and multi-cell type process (Micalizzi et al., 2017; Yu et al., 2013). The totality of mutations or combinations of mutations that allow tumor cells to successfully metastasize are not entirely known, however transient acquisition of mesenchymal cell characteristics through the process of epithelial-mesenchymal transition (EMT) (Kalluri and Weinberg, 2009; Zhang et al., 2013), conditioning of a pre-metastatic niche in distant organs (Peinado et al., 2017), and the ability of a cell to undergo dormancy (Barkan et al., 2008; Lu et

al., 2011; Naumov et al., 2003) are all likely required for these relatively rare, when considering cell bulk in breast tumors, metastatic events to occur.

In women, the most common sites of breast cancer are lungs, liver, and bone (Paget, 1889; Weigelt et al., 2005). These metastases cause significant morbidity, including pathologic fractures at sites of bone metastasis, and are ultimately fatal. Breast cancer metastasis is unlikely to be cured once diagnosed, but recent improvement in treatment have prolonged patients' progression free survival periods (Chia et al., 2007). Preventing these metastases from occurring by restricting tumor cells to the primary site continues to be one of the main goals of breast cancer metastasis research.

Mammary stroma and changes during breast cancer

Normal mammary glands are surrounded and supported by a basement membrane which is set within a matrix of extracellular matrix proteins and adipose tissue. Extracellular matrix proteins were once considered a structural, but inert part of any organ or organ system, however that understanding has dramatically changed in recent years. Breast ECM is known to play roles in cell migration, differentiation, and proliferation (Engler et al., 2006; Hynes, 2009) as well as breast development (Robinson et al., 1999; Wiseman and Werb, 2002), lactation, and involution (O'Brien et al., 2010; Schedin et al., 2004). The breast ECM protein pool largely consists of fibrillar collagens, proteoglycans/glycoproteins, and fibronectin (Lu et al., 2012). These proteins are large, multidomain, and contain binding sites for receptors, such as integrins and discoidin domain receptors, which cause downstream cellular signaling. ECM proteins also have binding sites growth factors, leading to the generation of biochemical sinks or gradients that affect cell

behavior (Hynes, 2009; Oudin and Weaver, 2016). Several cell types are found in normal mammary stroma, including fibroblasts, immune cells of various types, adipocytes, and endothelial cells. Matrix composition is maintained by a balance between matrix production and remodeling. In normal tissue, fibroblasts are finely tuned to maintain tensional homeostasis; they quickly respond to changes in stiffness, such as a wound, by secreting stromal proteins and matrix degrading proteins such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) (Humphrey et al., 2014). These matrix degrading enzymes can be secreted by other cell types as well (Cathcart et al., 2015; Oudin and Weaver, 2016).

As breast cancer progresses to invasive carcinoma, tumor cells invade through the basement membrane and come into contact with stromal cells and stromal matrix proteins such as fibrillar collagens. This contact influences both the tumor cells and the stroma in a reciprocal interaction termed tumor-stromal crosstalk. For example, tumor and immune cells release growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor β 1 (TGF β) which stimulate stromal fibroblasts to proliferate and become activated, leading to increased collagen deposition in tumors (Lohr et al., 2001). In response, activated stromal fibroblasts secrete growth factors, ECM proteins, and proteases which influence tumor cell survival, invasion, and migration (Fig. 1.3) (Mueller and Fusenig, 2004). Breast cancer progression also promotes inflammation- responses from the innate immune system include increased macrophage, neutrophil, and dendritic cell infiltration, and derangements in the adaptive system can lead to altered T cell responses which favor tumor progression (Garcia-Mendoza et al., 2016; Oudin and Weaver, 2016; Quintana, 2017; Zhu et al., 2014). CAFs typically secrete chemokines and cytokines which cause an immunosuppressive, leading to reduced immune surveillance and tumor cell escape. In return, immune cells secrete activating paracrine factors which stimulate

CAFs to increase secretion of ECM proteins and ECM modifying enzymes such as MMPs which increase matrix remodeling (Kalluri, 2016).

In a disease state, the tightly controlled normal homeostasis of ECM dynamics can become markedly dysregulated in regard to protein production, matrix degradation, or overall fiber architecture. Tumors can be thought of as a chronic wound; the fibrotic or desmoplastic reaction that occurs in breast cancer is highly similar to granulation tissue found in healing wounds (Dvorak et al., 1984), however, since the wound cannot heal, some proteins typically only found in early wounds persist in cancer stroma (Mackie et al., 1987; Yeo et al., 1991). This phenomenon is not specific to breast cancer, and similar ECM derangements are found in pancreatic cancer (Jiang et al., 2016; Laklai et al., 2016) among others.

Collagen architecture in breast cancer

While many factors change in the stroma of breast tumors, one of the most dramatic is that of fibrillar collagen, especially type I. Like in other inflammatory diseases where one of the main hallmarks is fibrosis, breast cancer can result in local desmoplasia or fibrosis. In fact, this toughened tissue is often why patients are able to feel a lump in the breast which can be indicative of breast cancer. Normal breast tissue comes in a wide range of densities, from those that are primarily adipose tissue with minimal glandular components to those that are very dense in both collagen and glands. Importantly, women with mammographically dense breasts, or those in which breast density accounts for more than 50% of the tissue are at a four-fold increase in the risk of developing breast cancer of any type (Boyd et al., 2001; McCormack and dos Santos Silva, 2006). The stromal components of this mammographic density have been shown to consist

primarily of fibrillar collagens (Alowami et al., 2003; Guo et al., 2001). In addition, when women with dense breasts do get breast cancer, they are also at a greater risk of developing metastasis, independent of other factors including age, body mass index, and treatment type of initial tumor (Habel et al., 2004). In sum, these clinical correlations make the study of changes in breast stromal collagen and how they affect tumor progression extremely important, and much work has been accomplished regarding this question in recent years.

In addition to a simple increase in the amount of breast stromal collagen being significant to poor outcome (Provenzano et al., 2008a), the overall architecture of stromal collagen fibers has also been found to be extremely important. Features of collagen fibers such as increased length, increased width, and relative alignment have all been found to correlate with poor outcomes (Conklin et al., 2011; Provenzano et al., 2006). Second Harmonic Generation (SHG) analysis by two-photon microscopy of collagen fibers near the tumor-stromal boundary has allowed the development of a tumor associated collagen signature (TACS) which predicts tumor invasion (Provenzano et al., 2006) and patient survival (Conklin et al., 2011). The TACS signature is scored from 1-3 with TACS-3 being the most aggressive. TACS-1 is defined as dense collagen in the region of the tumor and TACS-2 as straightened fibers which appear to bound the tumor region. TACS-3 is indicative of collagen fibers which have been aligned perpendicular to the tumor surface (Provenzano et al., 2008a). These fibers have been shown to be sites of tumor invasion out of the primary site (Fig. 1.4) (Provenzano et al., 2008b), and the presence of any TACS-3 phenotype is prognostic for poor disease-free survival independent of other factors including tumor grade, hormone receptor status, and lymph node status (Conklin et al., 2011). These changes in collagen architecture in primary breast tumors have been attributed to an active

reorganization process (Levental et al., 2009), however the complete understanding of how these changes occur has yet to be elucidated.

Matrix stiffness and cellular mechanosignaling

As these changes in tumor stromal collagen occur and as breast tumor progresses, the overall stiffness of the tumor increases (Lopez et al., 2011). Increased stiffness in a tumor occurs through a variety of mechanisms, including an overall increase in collagen content, increased fiber thickness through crosslinking and remodeling, as well as changes in cellular contractility of stromal cells (Paszek et al., 2005). Importantly, tumor stiffness has been shown to correlate with breast cancer invasion and aggressive cancer subtypes. Using atomic force microscopy (AFM) techniques, human breast tumor stiffness was found to be highest at invasive edges of human tumors, and both basal-like and HER-2 overexpressing types were found to be stiffer overall than either Luminal A or Luminal B types (Acerbi et al., 2015).

Cells respond to mechanical forces in their environments through a process termed mechanosignaling. The process can split into two components, mechanosensing and mechanotransduction. Cells sense their physical environments through various membrane receptors, and these mechanical properties are transduced into downstream intracellular signaling events. In normal conditions, this process allows for the matrix homeostasis through responses to slackened or tightened stroma. For example, normal fibroblasts are tuned to operate at a particular stiffness. When that stiffness changes, eg- if tissue is wounded, the fibroblast produces more ECM proteins and pulls on the tissue, which, when combined with controlled proteinase activity, returns the tissue to its normal tension. When mechanosensing and mechanotransduction

goes awry in disease, this process can turn into a feed-forward loop of increasing tissue stiffness and matrix reorganization (Humphrey et al., 2014).

Because cells generally sense the stiffness of their surroundings through membrane receptors and cellular contractility, stiffened environments cause a reciprocal effect in cells that leads to increased intracellular tension (Lo et al., 2000). This effect in tumor cells and fibroblasts has been shown to be dependent on integrins linked to the cytoskeleton via focal adhesion complexes (Choquet et al., 1997) and actomyosin contractility (Matthews et al., 2006; Provenzano et al., 2008b; Zhou et al., 2017). In breast tumors, cancer associated fibroblasts (CAFs) have been shown to be the cell type responsible for the production of tumor ECM, and they also remodel and stiffen matrix (Calvo et al., 2013; Zhang et al., 2016). This suggests that CAF dependent matrix remodeling and matrix stiffening is a viable target for medical intervention in the treatment of breast cancer metastasis.

Cancer-associated fibroblasts (CAFs)

Breast cancer associated fibroblasts are a subset of mammary fibroblasts which have become permanently activated. Normally, fibroblasts become transiently activated in response to stimuli such as wounds. When stimulated, they migrate into the wound, contract and close the wound, and secrete matrix proteins that eventually form a scar (Tomasek et al., 2002). However, these temporarily activated fibroblasts revert back to a quiescent state once the wound has healed. Since cancer is a wound that does not heal, CAFs cannot and do not revert back to a quiescent state (Kalluri, 2016). Though the population of CAFs in any given tumor are heterogenous, they generally express the markers for activation, including vimentin, fibroblast

activated protein (FAP), and α -smooth muscle actin (α SMA) (Calvo et al., 2013), with the most specific being FAP (Fig. 1.5) (Jacob et al., 2012). Importantly, CAF markers have been shown to be correlated to outcome and have been successfully targeted in a mouse study of TNBC, indicating that CAFs are a good candidate for therapeutic intervention. (Paulsson and Micke, 2014; Takai et al., 2016).

Several studies have been conducted to determine the origin of CAFs, and it appears that they may derive from multiple sources. The largest source is likely from permanent activation of resident fibroblasts, however de-differentiation from epithelial cells via epithelial-mesenchymal transition (Iwano et al., 2002; Petersen et al., 2003) contributes a small portion, as do those derived from bone-marrow progenitors (Ishii et al., 2003). It is not known whether CAFs are a cancer specific phenotype or if they are the ultimate end result of any chronic wound. It is unknown how CAFs are maintained in breast tumors, but growth factor stimulation in an autocrine or paracrine manner (TGF- β and PDGF, for example) play roles (Calon et al., 2014; Elenbaas and Weinberg, 2001). It is certainly also likely that the desmoplastic and reactive stroma serves to further potentiate CAF phenotype propagation (Calvo et al., 2013; Zhang et al., 2016).

CAFs play many crucial roles in cancer progression and metastasis, both chemically and mechanically. In the same manner that tumor cells secrete paracrine factors that affect CAF activation, CAFs secrete factors which affect tumor cell migration and invasion (Fig. 1.5). In fact, CAFs can induce pre-malignant epithelial cells to become cancer when co-cultured, indicating that CAFs may also play a role in tumorigenesis (Bhowmick et al., 2004; Olumi et al., 1999). Secreted factors such as hepatocyte growth factor (HGF) (De Wever et al., 2004; Grugan et al., 2010), TGF- β (Potenta et al., 2008), and CAF-derived exosomes (Richards et al., 2017)

have all been shown to enhance tumor progression. Further, CAFs have immunomodulatory effects through numerous cytokines which have been shown to generally suppress the immune response to cancer, leading to prolonged survival and immunoescape of tumor cells (Kalluri, 2016).

Mechanically, CAFs affect tumor progression by altering the ECM through which the tumor cells travel via secretion of ECM proteins and matrix remodeling. Aberrant secretion of ECM proteins, especially fibrillar collagen, causes increased tissue fibrosis or desmoplasia, and this desmoplastic response is correlated with poor outcomes, as already discussed. Remodeling of matrix by CAFs through secretion of proteases such as matrix metalloproteinases (MMPs) and increased cellular contractility can cause matrix to become stiffened. Stiffened matrix causes breast tumor cells to become more proliferative (Paszek et al., 2005) and, in an integrin and FAK dependent manner (Provenzano et al., 2009), more invasive, suggesting that stiffened ECM allows cancer cells to more easily escape the primary site. Remodeled and aligned fibers in tumors act as highways for tumor cells (Provenzano et al., 2006), either singly or as a collective, away from a tumor, likely through a process known as durotaxis (Sunyer et al., 2016). Exactly how fibers become aligned in tumors is not yet known. Intriguingly, CAFs have also been found to lead cancer cells through matrix in CAF-remodeled tracks. Remodeling of matrix to create the tracks was dependent on both Rho-ROCK contractility and MMP secretion (Gaggioli et al., 2007).

Discoidin Domain Receptors

The Discoidin Domain Receptor (DDR) family consists of two members, DDR1 and DDR2. DDR1 exists in five isoforms due to alternative splicing, the most common of which is DDR1b, while DDR2 only has one isoform (Fig. 1.6). DDRs are single-pass transmembrane receptor tyrosine kinases (RTK) which, unlike all other RTKs, do not bind a soluble ligand but instead utilize native, triple-helical collagen as their ligand (Shrivastava et al., 1997; Vogel et al., 1997). In addition to both DDRs binding fibrillar collagens I, III, and V (Konitsiotis et al., 2008), DDR1 also binds collagen IV (Vogel et al., 1997) and DDR2 also binds collagens II and X (Leitinger and Kwan, 2006; Leitinger et al., 2004). These collagen binding preferences reflect the tissue specificity of the DDRs; DDR1 is expressed in epithelial cells where it may come into contact with basement membrane collagen IV while DDR2 is expressed in mesenchymal cells where it is mostly in contact with fibrillar collagens. However, in disease DDR2 can be aberrantly expressed by epithelial cells (Zhang et al., 2013).

DDRs are made up of several protein domains; an extracellular N-terminal DS domain where collagen binding occurs (Leitinger, 2003), followed by a DS-like domain, a long intracellular juxtamembrane domain, and an intracellular C-terminal kinase domain (Borza and Pozzi, 2014). Unlike for most RTKs, the DDRs exist as dimers on the cell surface in the absence of ligand, and this dimerization is mediated by the long juxtamembrane domain (Kim et al., 2014) and must bind collagen as either dimers or multimers. DDR activation kinetics are also unique in that they are extremely long, on the order of hours (Shrivastava et al., 1997; Vogel et al., 1997). It has been shown for DDR1 that receptor internalization occurs prior to receptor phosphorylation (Mihai et al., 2009), however it is not known if the same is true for DDR2.

Importantly, the binding site on collagen for the DDRs is distinct from that of integrins (Zeltz et al., 2014), indicating that DDR and integrin binding to collagen can occur simultaneously.

Though DDRs are expressed reciprocally in normal tissues, only DDR2 expression in breast cancer is correlated to decreased survival (Ren et al., 2013; Toy et al., 2015). To determine the role of DDR2 in normal tissues, mouse knockout lines were developed. DDR2 null mice are viable, however they are dwarfs and both males and females are sterile (Corsa et al., 2016; Olaso et al., 2002). The dwarf phenotype was found to be due to reduced chondrocyte proliferation, and mutations in DDR2 were later found to also be present in a subset of human dwarfism termed spondylo-meta-epiphyseal dysplasia with short limbs (SMED-SL) (Bargal et al., 2009). In addition, DDR2 null mice have delayed wound healing due to reduced fibroblast proliferation and invasion (Olaso et al., 2011). DDR2 activation has also been shown to regulate matrix metalloproteinase expression, specifically it regulates MT1-MMP or MMP14, a transmembrane metalloproteinase which activates other MMPs that degrade collagen (Majkowska et al., 2017; Olaso et al., 2002; Xu et al., 2005; Zhang et al., 2013). Taken together, these findings indicate that DDR2 plays a role in stromal cells in matrix remodeling. It was recently shown that DDR1 mediates collagen contraction, an effect that was dependent on non-muscle myosin IIA and DDR1 clustering (Coelho et al., 2017). It was not clear from this study whether DDR1 mediated collagen clustering was dependent or independent of integrin activity.

Upon collagen binding, DDR2 autophosphorylates at several tyrosine residues. Signaling downstream of DDR2 activation involves interaction with cytosolic signaling proteins carrying Src homology-2 (SH2) domains or phospho-tyrosine binding (PTB) domains (Fig. 1.5) (Valiathan et al., 2012). In the Longmore lab, DDR2 was identified as a regulator of the EMT factor Snail1. Collagen binding led to Snail1 nuclear accumulation and stabilization downstream

of Src and ERK2 phosphorylation (Zhang et al., 2013). DDR2 has also been shown to modulate integrin activation in a manner which promotes enhanced cell adhesion without affecting integrin expression levels (Xu et al., 2012). The mechanism by which DDR2 affects modulates integrin activity has not been elucidated. In addition, kinase independent functions of DDR2 have yet to be fully explored.

DDR2 in breast cancer

DDR2 has been implicated in a number of diseases including arthritis (Xu et al., 2005; Xu et al., 2010), cardiac development (Cowling et al., 2014), and several cancers including lung (Kobayashi-Watanabe et al., 2017) and breast. DDR2 is not expressed in normal breast epithelial cells, however it is aberrantly expressed in 71% of invasive ductal carcinomas biopsied . In this study, the Longmore lab showed that DDR2 activation stabilized Snail1 protein levels which promoted tumor cell migration and invasion and facilitated lung metastasis in a transplant model of breast cancer (Zhang et al., 2013). In addition, DDR2 depleted tumors had a less aggressive collagen signature (TACS 2/3) when compared to controls (Zhang et al., 2013), suggesting that DDR2 in tumor cells may either directly influence the remodeling of collagen fibers or indirectly affect the action of stromal cells in the remodeling of collagen fibers.

In an MMTV-PyMT genetic model of breast cancer in global knockout mice, DDR2 was found to be critical for metastasis without affecting tumor cell growth or latency. The Longmore lab went on to show that DDR2 in basal epithelial cells, rather than luminal epithelial cells is required for breast cancer metastasis. Further, a decrease in fibrosis at end stage and a shift to a more TACS 1/2 collagen phenotype was observed in the DDR2 knockout mice (Corsa et al.,

2016). Importantly, in a reciprocal transplant experiment, it was also shown that DDR2 is required in the host for lung metastasis (Fig. 1.7). Further, DDR2 in CAFs promoted tumor cell collective invasion, possibly in a paracrine manner (Corsa et al., 2016). It is not yet known exactly what role DDR2 plays in the various stages of metastasis, however there is evidence that DDR2 affects angiogenesis (Zhang et al., 2014) and tumor invasion, suggesting that DDR2 may play a role in both tumor cells and stromal cells at the primary site.

Integrins, focal adhesion complexes, and molecular mechanosignaling

Interestingly, DDR2 has recently been shown to affect the activation of the collagen binding integrins $\alpha1\beta1$ and $\alpha2\beta1$ (Xu et al., 2012). Integrins, in addition to being cellular adhesive receptors, have been shown to mediate signals from the extracellular environment to promote cell survival and cell migration (Hytonen and Wehrle-Haller, 2016; Kim et al., 2011). Integrins are heterodimeric transmembrane cell surface receptors which bind ECM proteins. Each integrin pair consists of one of 18 different α subunits, the most common of which is αV , and one of 8 different $\beta1$ subunits, the most common of which is $\beta1$. These α and $\beta1$ subunits combine across cell types in various iterations to form 24 different integrin pairs (Humphries et al., 2006). Each integrin pair has a distinct binding affinity for different ligands, including those for laminin, fibronectin, fibrinogen, and collagen, among others (Humphries et al., 2006). The collagen binding integrins all have the $\beta1$ subunit in common and combine with $\alpha1$, $\alpha2$, $\alpha10$, or $\alpha11$ to recognize the specific collagen peptide sequence GFOGER (Emsley et al., 2000; Knight et al., 2000). Integrin subunits consist of a large extracellular domain, a single pass transmembrane domain, and a short cytoplasmic tail (Kim et al., 2011).

Integrin activation is regulated by conformational changes of the extracellular domain. The extracellular domain can exist in a bent, inactive state, an extended but closed intermediate state, or an extended, open, and activated state. Only the activated state has high affinity for ligand (Fig. 1.8) (Luo et al., 2007). These conformational changes and activation can be induced in a bidirectional manner by transmission of force from ligand (outside-in) or through intracellular signaling culminating in talin1 binding to the $\beta 1$ subunit cytoplasmic tail (inside-out) (Kim et al., 2011; Puklin-Faucher and Sheetz, 2009). Either way, through pulling from matrix or talin1 binding and downstream contractility, integrin conformational changes are due to forces pulling the α and $\beta 1$ subunits apart and the head domains open (Puklin-Faucher et al., 2006; Puklin-Faucher and Sheetz, 2009). In addition to increases in affinity for ligand upon integrin activation, increases in avidity also occur after talin1 binding due to integrin clustering. Clustering of integrins allows for additional adhesions to the matrix, which strengthens attachments, and also creates a local increase in the concentration of actin linking proteins (ie- focal adhesion proteins) and signaling partners. The cytoplasmic tails of the integrin subunits have no inherent signaling ability and, thus, associate with signaling partners such as Src and focal adhesion kinase (FAK) to mediate downstream signaling events (Arias-Salgado et al., 2003; Jahed et al., 2014).

Traction forces to the ECM are transmitted through integrins by linkages to the actin cytoskeleton through large, dynamic protein complexes called focal adhesions (Fig. 1.9). Focal adhesion complexes are made up several proteins arranged in prescribed layers and mediate mechanotransduction, adhesion, and signaling (Liu et al., 2015; Parsons et al., 2010). The defined layers from distal to proximal are the extracellular integrin adhesion layer, an integrin signaling layer, a force transmission layer consisting primarily of talin1 and vinculin proteins,

and actin regulatory and fiber layers (Fig. 1.10) (Kanchanawong et al., 2010). It was recently shown that talin1 is the primary regulator of focal adhesion size; when the authors shortened talin1 through mutation, focal adhesion z plane length was shortened accordingly (Liu et al., 2015). This work identified the primary functional force transmission unit of a focal adhesion to be the integrin – talin1 – actin chain.

Importantly, focal adhesion complexes are the large (microns), multimeric end result of maturation from nascent focal adhesion. Focal adhesion maturation and growth occurs linearly with applied force (Hoffman et al., 2011; Puklin-Faucher and Sheetz, 2009; Roca-Cusachs et al., 2013). Further, force sensitive proteins in addition to talin1 serve to enhance integrin β 1 activation, focal adhesion maturation, and mechanotransduction. Kindlins, for example, are essential to the activation and clustering of integrins (Ye et al., 2013). They bind integrin β 1 cytoplasmic tails in a similar manner, though different location, as talin1s (Jahed et al., 2014) and have been shown to be required for normal integrin β 1 activation (Kahner et al., 2012). Further, kindlin2 knockout recapitulates integrin β 1 knockout in mice (Montanez et al., 2008). Vinculin proteins are also required for normal mechanotransduction and signaling as well as essential for focal adhesion maturation (Jahed et al., 2014).

Activation and regulation of Talin1

Talin1 is the main link between ECM binding integrins and the actin cytoskeleton, and is the critical mediator of inside out integrin activation. Integrins absolutely depend on talin1 for all of their functions, therefore, research into talin1 structure, function, and regulation has led to many insights regarding cellular mechanosensing and mechanotransduction. Talin1 is a large

protein and contains two FERM domains in the N-terminal head domain for integrin β 1 binding. The ability of talin1 to bind several integrins as well as several actin filaments and then also to dimerize promotes growth of focal adhesions and integrin activation. Talin1 exists in the cytosol as a closed protein, and is activated to an open conformation by binding to RIAM (Klapholz and Brown, 2017; Lee et al., 2009). It is also mechanoresponsive in that it stretches when bound to integrin β 1 and actin. This stretching exposes binding sites along talin1, including those for vinculin, which enhances adhesion maturation (Gingras et al., 2010). Talin1 activation appears to occur in two steps which may occur simultaneously; switching to an open conformation and recruitment to integrin adhesions. One proposed mechanism by which talin1 is activated and recruited is that protein kinase C (PKC) signaling activates Rap1-GTP. Rap1-GTP then recruits RIAM to the membrane, and RIAM binds to and recruits talin1 the membrane (Klapholz and Brown, 2017; Lee et al., 2009). RIAM prefers binding to fully folded talin1, however RIAM binding disrupts the closed conformation of talin1 (Lee et al., 2013). Therefore, recruitment of talin1 to membranes by Rap1-GTP bound RIAM and opening of talin1 conformation to allow integrin β 1 binding likely occur simultaneously. While much work has gone into establishing the necessity of talin1 in integrin activation, it is not yet known how Rap1-GTP or PKC may be activated upstream of talin1 activation.

1.1 Figures

Figure 1.1

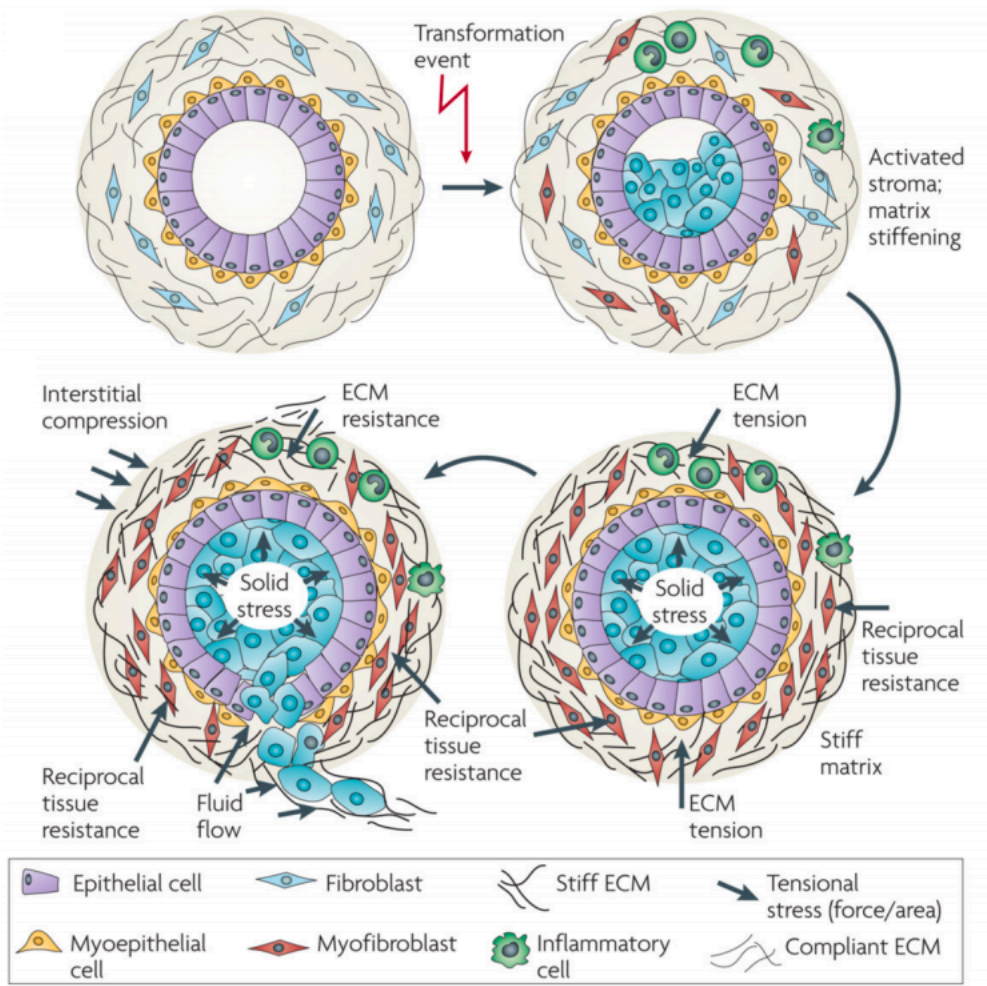


Figure 1.1: Breast cancer progression and metastasis.

Breast cancer originates in breast epithelial cells or their progenitors after a genetic alteration which releases the cells from normal growth and proliferation restriction. Hyperplasia progresses to DCIS and ultimately invades through the basement membrane into the stroma. This progression is accompanied by changes in the stroma, including stiffened matrix, activation of stromal cells, and inflammation. Downstream of these changes, cells are able to invade away from the primary site, enter the blood stream, and ultimately seed other organs. Adapted from Butcher, Alliston, and Weaver, 2009.

Figure 1.2

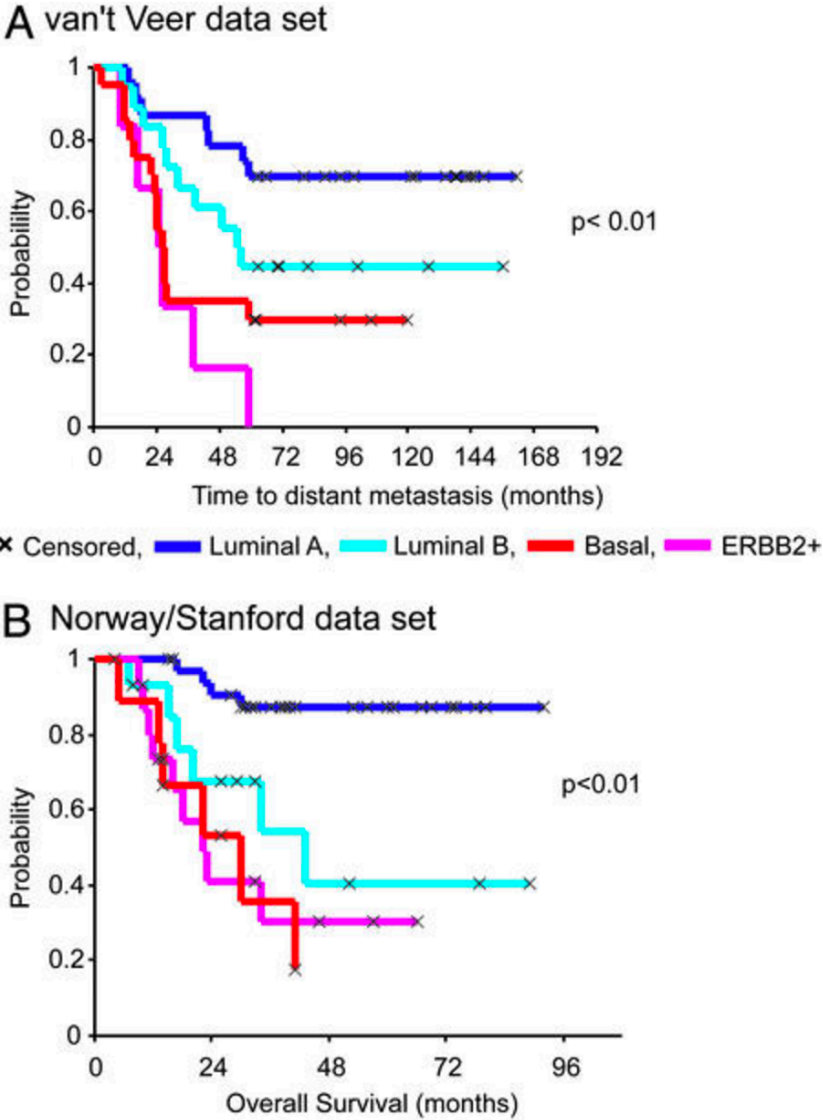
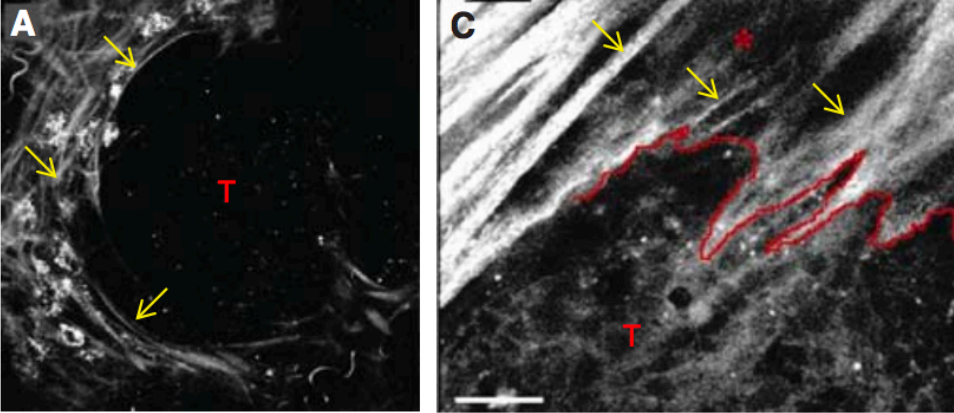


Figure 1.2: Time to metastasis and overall survival of breast cancer subtypes.

Of the four clinical breast cancer subtypes, basal types and ERBB2+ (HER2+/TNBC) progress to metastasis faster (A) and lead to worse overall survival (B) than Luminal A or B subtypes.

Adapted from Sorlie, et al., 2003.

Figure 1.3:



B TACS-2

D TACS-3

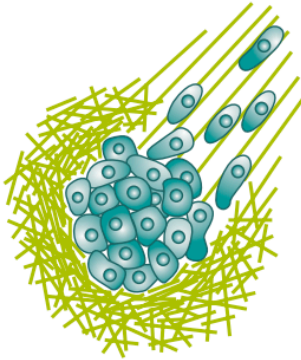
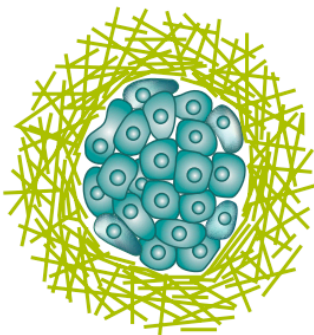


Figure 1.3: ECM dynamics and tumor progression.

As breast tumors progress, changes in ECM are effected at the same time. Overall collagen deposition increases, matrix stiffness increases, and fibers become aligned to the tumor surface. These changes are associated with local tumor invasion, aggressive breast cancer subtypes, and overall poor prognosis. (A) Image demonstrating parallel fibers in a TACS-1 phenotype near a non-invading tumor. (B) Graphical representation of A. (C) SHG image demonstrating collagen fibers which are perpendicular to the tumor surface of an invading tumor, TACS-2/3 phenotype. (D) Graphical representation of C. Adapted from Schedin and Keely, 2011.

Figure 1.4

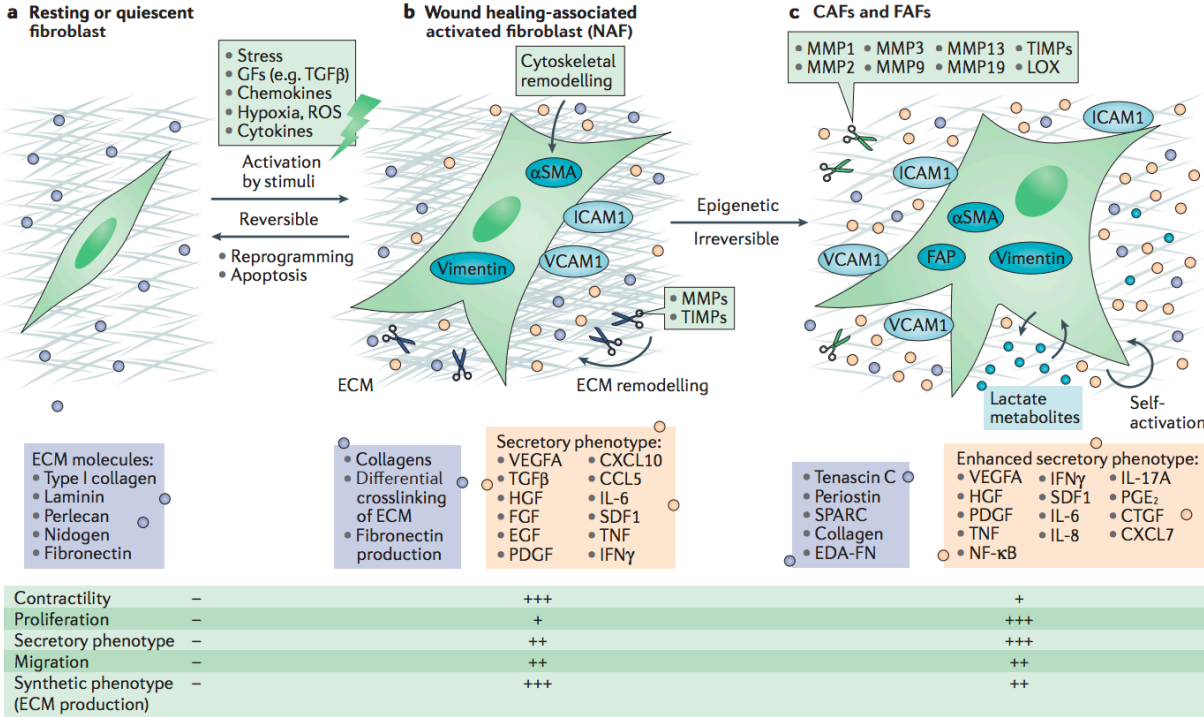


Figure 1.4: Characterization of the activation of cancer associated fibroblasts.

(A) Quiescent fibroblasts are resting, non-active cells. They exist in equilibrium with surrounding stroma and can be activated by wounds, cytokines, or other stresses.

(B) Wound-healing or temporarily activated fibroblasts increase their activity to become contractile, change cellular morphology, and increase secretion of matrix proteins, cytokines, and matrix degradation proteins to heal wounds. This state is reversible.

(C) Permanently activated fibroblast such as cancer associated fibroblasts. These cells are highly secretory, rapidly proliferate, and induce tumor progression. This state is non-reversible.

Adapted from Kalluri, 2016.

Figure 1.5:

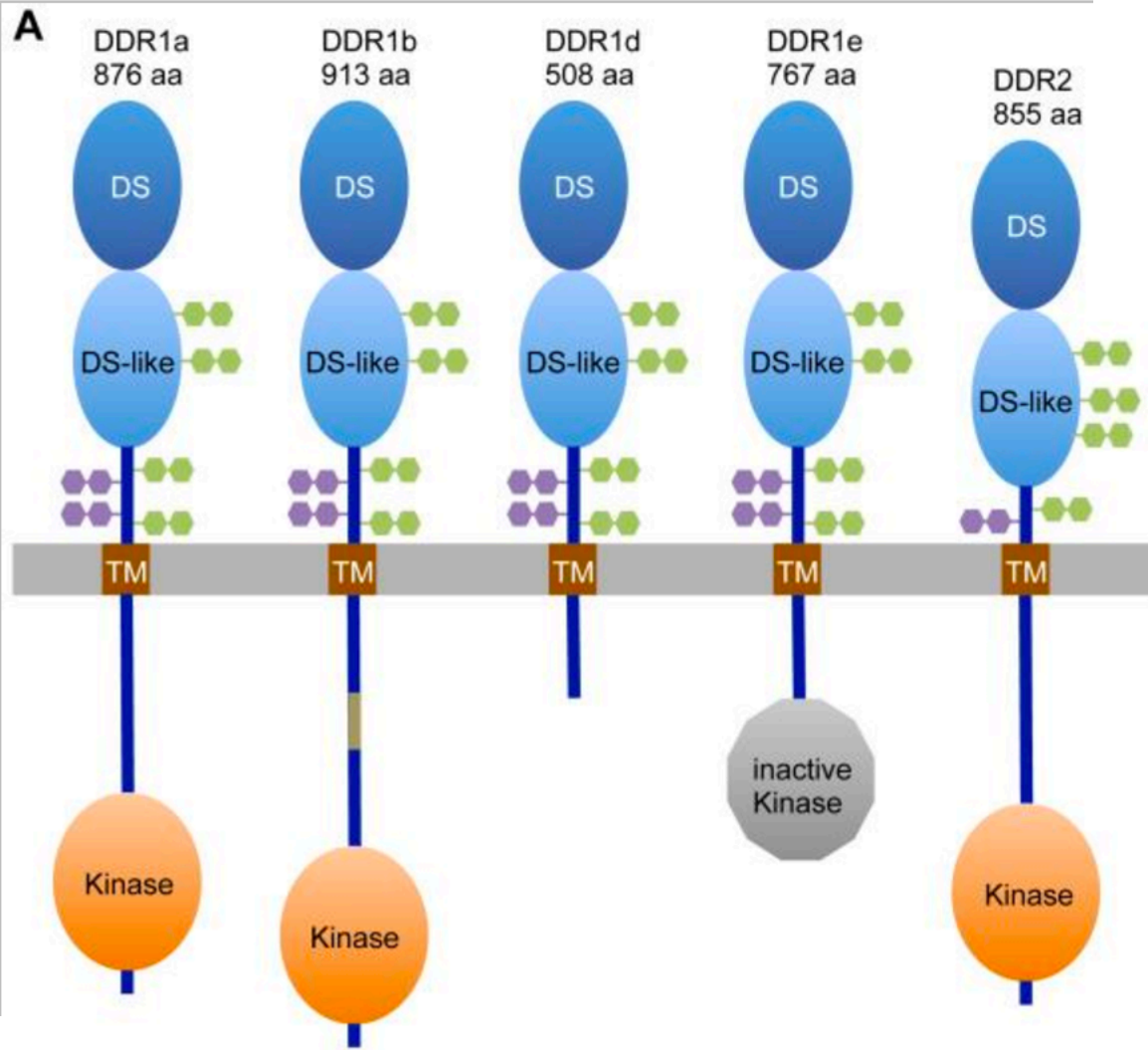


Figure 1.5: Schematic representations of DDR1 and DDR2.

Diagram depicting the five isoforms of DDR1 (DDR1b is the most common) and DDR2 proteins. DDRs are large type 1 transmembrane receptor tyrosine kinases that bind fibrillar collagen. The extracellular domain consists of a distal DS domain which mediates collagen binding and a more proximal DS-like domain. The DDRs have a large intracellular juxtamembrane domain which is required for dimerization. The cytoplasmic kinase domain is typical of RTKS. Tyrosine residues upon which the DDRs get phosphorylated as well as N- and O- glycosylation sites are indicated. Adapted from Leitinger, 2014.

Figure 1.6:

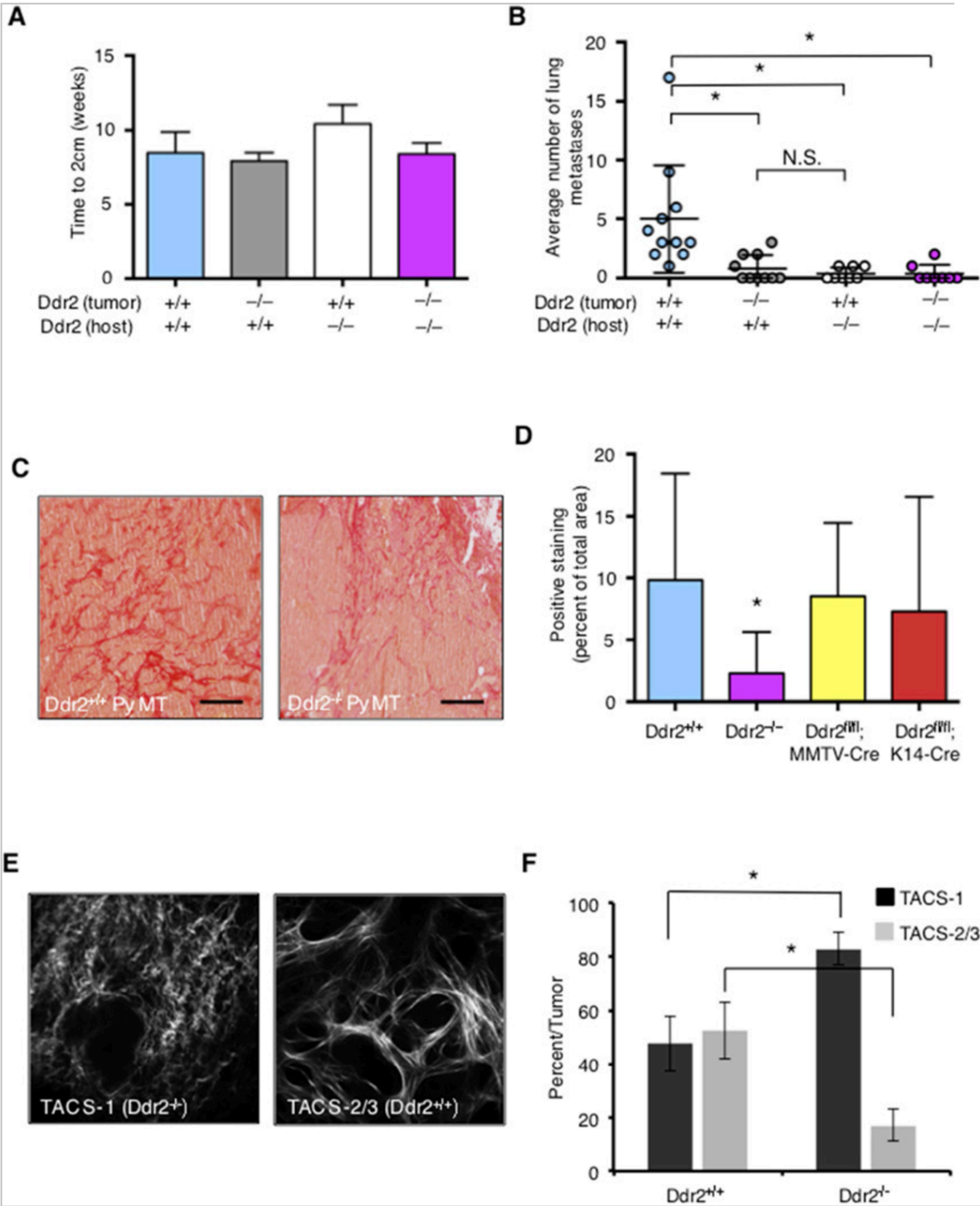


Figure 1.6: DDR2 in the stroma is required for lung metastasis, fibrosis, and aggressive collagen phenotype.

(A, B) Reciprocal transplant of WT or DDR2 null MMTV-PyMT primary tumor cells into WT or DDR2 null syngeneic host. (A) Tumor latency was unchanged. (B) DDR2 is required in both the host and stroma for breast cancer lung metastasis.

(C) Picrosirius red staining on tumor sections from WT and DDR2 null MMTV-PyMT tumors.

(D) Quantification of Picrosirius red staining from (C).

(E) SHG images of collagen organization in 10-13 week old WT and DDR2 null tumors.

(F) Quantification of TACS-1 (curly fibers) versus TACS-2/3 (straight fibers) phenotype in each group.

Adapted from Corsa, et al., 2015.

Figure 1.7

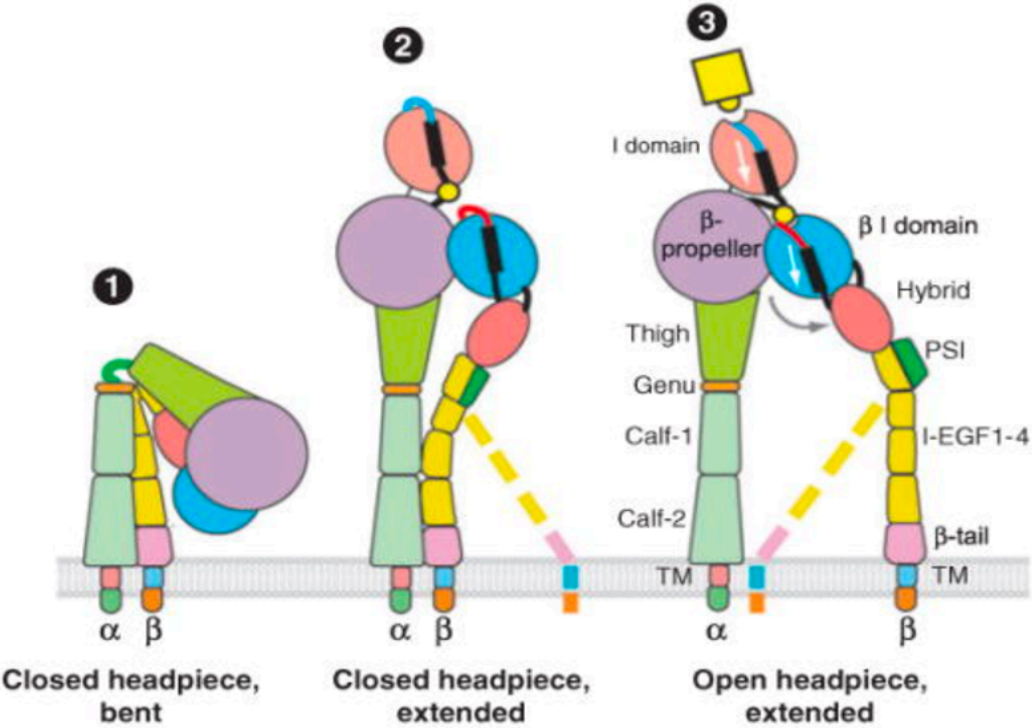


Figure 1.7: Integrin activation occurs in several steps.

Diagram depicting the three conformational states of integrins. (1) The bent, closed conformation is an inactive, low affinity state, (2) the intermediate state which is extended but closed is also inactive, and (3) the extended and open conformation which is active with high affinity for ligand. Adapted from Luo, et al., 2007.

Figure 1.8

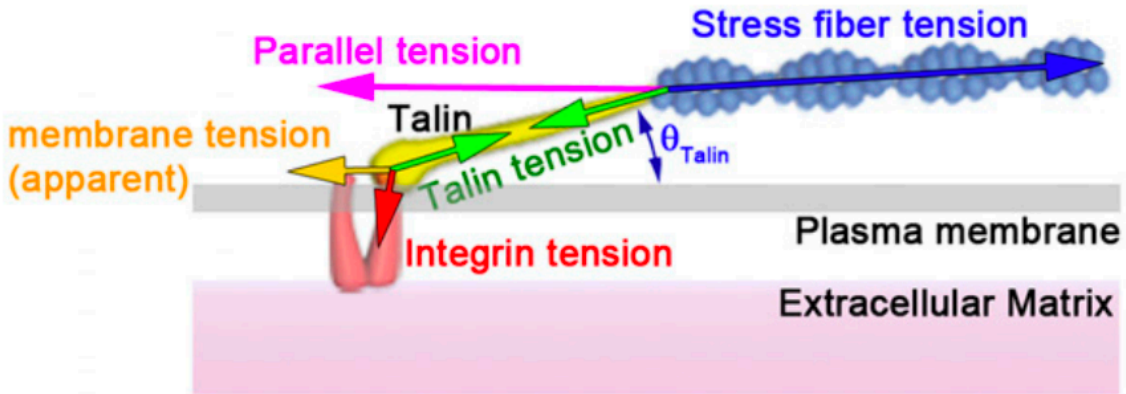


Figure 1.8: Focal adhesions mediate force transmission.

Diagram depicting the forces on a single integrin $\beta 1$ – talin1 – actin linkage. Focal adhesions grow in response to mechanical load. Integrins and talin1s are recruited to adhesions, and actomyosin contractility acts upon these adhesions to form stress fibers. Talin1 is the primary regulator of focal adhesion height. Adapted from Liu, et al., 2015.

Figure 1.8

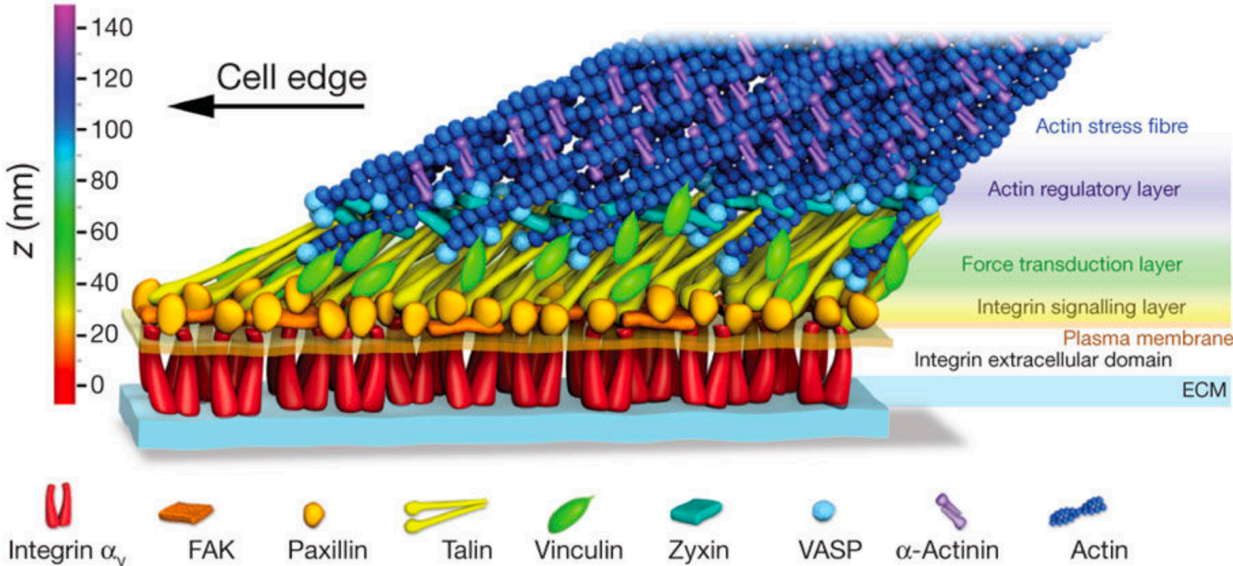


Figure 1.8: Focal adhesion proteins are arranged in prescribed nanoscale layers.

Focal adhesions are assembled in specific layers. The adhesion layer contains integrin proteins which bind extracellular matrix ligand. These integrin β chains are bound by signaling molecules near the cytoplasmic leaf of the plasma membrane, which transmit mechanical signals to the cell. Proximal to the signaling layer is the force transduction layer where talin1 and vinculin proteins are found. Talin1 and vinculin are both mechanically regulated by applied forces and serve to enhance the maturation of focal adhesions. Following the force transduction layer is that which contains actin filaments and actomyosin contractile machinery. Adapted from Kanchanawong, et al., 2010.

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Chapter 2: Stromal DDR2 is required for breast cancer lung metastasis.

2.1 Introduction

Breast cancer is the second leading cause of cancer related death in women, and, while some progress has been made, metastasis remains a significant problem for patients and clinicians. Several characteristics of breast tumor stroma are prognostic for worse outcomes, including mammographic density, or collagen content (Provenzano et al., 2008), matrix stiffness (Acerbi et al., 2015), and collagen fiber alignment (Conklin et al., 2011). Collagen alignment relative to the tumor boundary is particularly correlated with local tumor invasion; this alignment has been characterized by the development of a Tumor Associated Collagen Signature (TACS) whereby a score of TACS 1 indicates a benign stroma and a score of TACS 2/3 indicates an aggressive stroma (Conklin et al., 2011; Provenzano et al., 2006). It is not yet fully known how these changes in tumor stroma occur.

Previous work in the Longmore laboratory identified Discoidin Domain Receptor 2 (DDR2) as a regulator of the EMT transcription factor Snail1. DDR2 activation was found to lead to nuclear accumulation and subsequent stabilization of Snail1 protein in a Src/ERK2 dependent manner. The stabilization of Snail1 promoted migration and invasion of breast cancer cells and facilitated lung metastasis in a transplant model of breast cancer (Zhang et al., 2013). Importantly, the tumor associated stroma was also found to be affected by DDR2 in tumor cells; DDR2 activity promoted a TACS 2/3 aggressive phenotype, though the mechanism by which this change occurs was not elaborated.

DDR2 is a unique receptor tyrosine kinase in that it binds fibrillar collagen rather than a soluble ligand. Full activation of the receptor takes several hours, another unique aspect when compared to other RTKs (Shrivastava et al., 1997; Vogel et al., 1997). Activation of DDR2 occurs by phosphorylation of several tyrosines in the cytoplasmic tail, which recruits signaling partners bearing Src homology-2 (SH2) or phosphotyrosine binding (PTB) sites (Valiathan et al., 2012). DDR2 is not normally expressed in epithelial cells, however it has been found to be expressed in 71% of invasive ductal carcinomas (Zhang et al., 2013). High expression of DDR2 in breast tumors was also found to correlate with the triple negative breast cancer subtype and decreased overall survival when compared with low DDR2 expressing breast cancers (Toy et al., 2015). DDR2 has been shown to have roles in wound healing, angiogenesis, and cell migration and invasion.

To determine the cellular role of DDR2 in breast cancer metastasis, a conditional allele was generated in the Longmore lab. This allele in combination with β 1 actin-Cre generated global null DDR2 knockout mice (Corsa et al., 2016). DDR2 null mice are dwarfs due to defective chondrocyte maturation (Bargal et al., 2009) and are also sterile due to defects in spermatogenesis (Kano et al., 2010) and ovulation (Matsumura et al., 2009). Crossing DDR2 null mice to MMTV-PyMT generated tumor bearing mice, which, at end stage had significantly fewer lung metastases than wild type controls without affecting either tumor growth or latency. The cellular role for DDR2 was further parsed out by using MMTV-Cre to target luminal epithelial cells and K14-Cre to target basal epithelial cells. In this experiment, it was found that DDR2 in luminal epithelial cells had little effect while DDR2 in basal epithelial cells was required for metastasis (Corsa et al., 2016). In addition to lung metastases, the tumor ECM was also analyzed. Global DDR2 deletion reduced fibrosis while specific deletion in luminal or basal

epithelial cells did not. Further, a less aggressive collagen phenotype (TACS1) was more predominantly found in DDR2 global null tumors. Since stromal cells, including fibroblasts and immune cells have been implicated in fibrosis, a reciprocal transplant experiment was also performed to determine the contribution of DDR2 in the host – when wild type tumor cells were transplanted into a DDR2 null host, lung metastases were significantly reduced, indicating that DDR2 in the host is also required for metastasis (Corsa et al., 2016). The cellular and molecular mechanisms for this effect were not determined.

In this study, we sought to determine the breast tumor stromal role for DDR2 in breast cancer metastasis. We used FSP1-Cre to delete DDR2 from stromal cells in an MMTV-PyMT model of breast cancer, and, in this setting, we increased the specificity of DDR2 deletion from the entire host. We found that DDR2 is required in stromal cells for breast cancer metastasis without affecting tumor growth or latency. Further, we show that stromal DDR2 affects ECM remodeling and increases collagen fiber thickness and relative alignment.

2.2 Results

FSP1-Cre targets breast tumor stromal cells.

To test the effect of DDR2 in mammary tumor stroma, specifically cancer associated fibroblasts (CAFs), on breast cancer metastasis, we utilized the previously described conditional DDR2 flox allele (Corsa et al., 2016) in combination with the FSP1-Cre transgene in an MMTV-polyoma middle T (PyMT) model of breast cancer. We chose this model because MMTV-PyMT mice develop multifocal primary tumors which progress from mild hyperplasia to invasive

adenocarcinoma in a manner which resembles human breast cancer. Additionally, tumors develop on a predictable timescale with robust metastasis to the lungs and are hormone independent (Guy et al., 1992; Schaffhausen and Roberts, 2009).

At present, there are no promoter-Cre transgenes to selectively target CAFs, however, there are those which target CAFs as well as other cells. Therefore, we initially chose to evaluate two which have been used by other groups to target fibroblasts, FSP1-Cre and α SMA-Cre (Bhowmick et al., 2004; Cheng et al., 2005; LeBleu et al., 2013; Wu et al., 2007). Neither was expected to be absolutely efficient or specific. To determine which to choose, we utilized the ROSA-LSL-TdTomato reporter allele in early (10-11 week) MMTV-PyMT tumors and quantified the percent of TdTomato positive cells co-staining with various stromal cell markers (Fig. 2.1a). We found that TdTomato expression in FSP1-Cre mice was restricted to cells which express FAP (85%) while not expressed in K14 positive basal epithelial cells (~4%), K8 positive luminal epithelial cells (~0%), or CD31 positive endothelial cells (~0%) (Fig 2.1b). Further, the cells that express TdTomato lie in the matrix between epithelial tumor clusters and have a spindle shape morphology, indicating a cell of mesenchymal origin. In contrast, α SMA-Cre was expressed by few stromal cells overall, did not co-stain well with FAP positive cells, and was found, in some cases, to be expressed in cells within the tumor (Fig. 2.1c). Though the majority of FSP1-Cre expressing cells co-stain for CAF markers, it is also expressed in CD45 positive cells. Of the cells that express TdTomato in the FSP1-Cre; ROSA-LSL-TdTomato; MMTV-PyMT mice, only 15-20% of them were CD45 positive, however, of the CD45 positive cells, nearly 90% were TdTomato positive (Fig 2.1b).

Importantly and in contrast to DDR2 global null mice (Corsa et al., 2016; Olaso et al., 2002), FSP1-Cre; DDR2 fl/fl mice are fertile and of normal size, further indicating the relative

restriction of FSP1-Cre expression (Fig 2.2a). Additionally, genotyping of total tumor tissue and Western blot analysis of DDR2 expression in CAFs isolated from FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice showed recombination of the DDR2 locus and total deletion of DDR2 protein expression, respectively (Fig. 2.2b and Fig. 2.2c). Thus, these data indicate that FSP1-Cre is robustly expressed in stromal CAFs and is functioning to delete DDR2 in these cells.

Stromal DDR2 is required for breast cancer lung metastasis

To determine whether stromal DDR2 is required for breast cancer lung metastasis, we generated FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice and allowed them to develop tumors until end stage (2 cm maximum individual tumor size). DDR2 fl/fl; MMTV-PyMT or DDR2 fl/+; MMTV-PyMT littermates were used as wild-type controls and all mice analyzed were greater than 90% FVB strain. DDR2 deletion in the stroma did not affect overall tumor burden nor tumor latency, ie- time to end stage (~14 weeks) between groups (Fig. 2.3a, b). There was, however, a dramatic reduction in the number of lung metastases in FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice when compared to WT; MMTV-PyMT controls (Fig. 2.3c). These data indicate that stromal DDR2 expression has no effect on overall primary tumor growth but that it is necessary for breast tumor metastasis to the lung.

Stromal collagen characterization.

Because we had previously demonstrated that DDR2 global null mice have a less aggressive collagen phenotype (TACS 1 rather than TACS 2/3), we wanted to determine whether

there was a difference in extracellular matrix content or organization in the FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice. To do this, we first performed histological analysis and stained early (10-11 week) tumors with Picosirius Red or Trichrome to visualize collagen fibers (Fig. 2.4a; quantified in Fig. 2.4b). We also quantified hydroxyproline content (an amino acid specific to collagen protein) in these tumors by a colorimetric biochemical assay (Fig. 2.4c). In contrast to earlier studies in our lab performed on end stage tumors, no differences in overall fibrosis was found in FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. These data suggest that stromal DDR2 does not function to increase total fibrosis in early MMTV-PyMT tumors.

Characteristics of collagen fiber architecture, including parallel fiber orientation and increased fiber thickness, in tumor stroma is correlated to poor outcomes (Conklin et al., 2011). To determine if there was a difference in collagen architecture in FSP1-Cre; DDR2 f/f; MMTV-PyMT tumors, we specifically visualized collagen fibers by second-harmonic generation (SHG) using two-photon microscopy in early tumors (10-11 weeks). These images of early tumors were scored for TACS signature as previously described (Provenzano et al., 2006). The collagen phenotype in FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors was predominantly TACS1 (thin, curly fibers), while the collagen architecture in WT tumors was predominantly TACS 2/3 (thick, straight fibers) (Fig. 2.5a, b). Fibers in each group were further analyzed by CT-FIRE software developed at the Laboratory for Optical and Computational Imaging (LOCI) in Madison, Wisconsin. WT; MMTV-PyMT tumors were found to have significantly longer collagen fibers than FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors (Fig. 2.5c).

To get a more specific view of collagen fibers, we stained tumors with ruthenium red and tannic acid and subjected them to Focus Ion Beam Scanning Electron Microscopy (FIB-SEM). This technique allows for SEM resolution while milling through a sample to generate a 3D

reconstruction of fibers in a tumor. Similar regions of interest between samples were chosen adjacent to the tumor – stromal boundary. In wild type stroma (Fig 2.5d left), thick fibers and many cables of collagen can be seen crossing the tumor boundary, while in DDR2 global null stroma, fibers are thin, wispy, and sparse (Fig. 2.5d right). Images have been pseudocolored to indicate density of signal.

Stromal DDR2 affects mechanical properties of tumors.

Increased extracellular matrix stiffness has been shown to correlate with increased tumor aggression and poor outcomes (Acerbi et al., 2015). This increased stiffness, specifically near tumor boundaries causes increased tumor cell invasion away from the primary tumor and tumor progression (Butcher et al., 2009). High intratumor tension or stiffness can be due to increased collagen deposition (Provenzano et al., 2008) and/or increased collagen crosslinking (Schedin and Keely, 2011).

To determine if the change in TACS phenotype in FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors caused a change in tumor stiffness, we used atomic force microscopy (AFM) to interrogate flash-frozen, unfixed tumors from 10-11 week old mice. In order to avoid bias of a nano-scale tip on an AFM cantilever, we used cantilevers with a 5 um borosilicate glass sphere affixed to the tip (NovaScan). This allowed for a more general representation of the stiffness in any area in the tumor. Nuclei were stained with propidium iodide and force maps (50 um x 50 um) of indentations were taken either in the tumor core or at the tumor – stromal boundary (Fig. 2.6a). Results indicate that WT tumors are stiffer overall (Young's Elastic Modulus (E) of 0.440 kPa) than FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors, which had an elastic modulus of about

0.300 kPa (Fig. 2.6b, c). These stiffness measurements on flash-frozen WT; MMTV-PyMT tumors agree with other groups' findings (Lopez et al., 2011). Further, when regions of the tumor were segregated, data indicate that, like reported human tumors (Acerbi et al., 2015), WT; MMTV-PyMT tumors are stiffer at the tumor – stromal boundary ($E = 0.452$ kPa) than in the tumor core ($E = 0.323$ kPa) (Fig. 2.6d). In contrast, FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors are of similar stiffness throughout (Edge; $E = 0.239$ kPa and core; $E = 0.314$ kPa) (Fig. 2.6e). Results are quantified in Figure 2.6f. Together, these results indicate that DDR2 in the mammary tumor stroma affects the mechanical properties of tumors and promotes the formation of a gradient of increasing stiffness from the tumor core to the tumor – stromal boundary.

2.3 Discussion

Breast cancer metastasis is the leading cause of cancer related deaths in women in the United States, regardless of breast cancer subtype at diagnosis (American Cancer Society). The metastatic cascade begins when tumor cells escape from the primary site, are able to intravasate, survive in the blood stream as CTCs, extravasate, and seed distant organs (Butcher et al., 2009). Understanding these various steps may uncover new ways to intercede and prevent metastasis in the future. The Longmore lab has shown that DDR2 in tumor cells has marked effects on breast cancer metastasis, promoting not only cell invasion but also stromal changes which promote cancer progression (Corsa et al., 2016). In that study, DDR2 was shown to be most important in K14 positive basal epithelial cells, and, while the non-breast cancer compartment was explored in a reciprocal transplant model utilizing DDR2 global null mice, the role for DDR2 in stromal cells was not established.

In this study, we show that DDR2 action is required in stromal cells for breast cancer lung metastasis, and further show that there is no effect of stromal cell DDR2 on tumor growth or latency (Fig. 2.3). We use the stromal specific FSP1-Cre in combination with the DDR2 conditional flox allele previously generated in the lab to target stromal cells in an MMTV-PyMT model of breast cancer. FSP1-Cre is expressed primarily in cancer associated fibroblasts (CAFs) as demonstrated by co-expression of ROSA-LSL-TdTomato (driven by FSP1-Cre) and FAP staining in cells that lie within breast tumor matrix (Fig. 2.1). In contrast to DDR2 global null mice, FSP1-Cre; DDR2 fl/fl mice are normal size and fertile, further supporting the restricted expression of FSP1-Cre and indicating that DDR2 in FSP1-Cre expressing cells is not required for skeletal growth, spermatogenesis, or ovulation (Fig. 2.2). Some of the cells which express FSP1-Cre are CD45 positive, indicating that a subset of cells of hematopoietic origin is also partially targeted in this model. CAF populations are extremely heterogeneous, and groups have shown that bone marrow derived CAFs and CAF precursors can and do express CD45 (McDonald et al., 2015), so it is possible that the co-expressing FSP1-Cre/CD45⁺ cells are CAFs. It is more likely, however, that these cells are macrophages (Osterreicher et al., 2011). More work needs to be done in the future to determine the role of DDR2 in immune cells on breast cancer lung metastasis.

Accumulation of collagen in breast stroma has been shown to be prognostic for poor outcomes. Despite the fact that a decrease in fibrosis was seen in previous studies of DDR2 global null mice, no change in fibrosis or total collagen content as assessed by hydroxyproline assay was found in FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors (Fig. 2.4). Tumor – stromal crosstalk and CAF – immune crosstalk are well established phenomena that can promote or inhibit the progression of cancer (Kalluri, 2016). While it is widely accepted that stromal

fibroblasts produce the majority of ECM proteins, they are influenced by paracrine factors from surrounding stromal cells and tumor cells (Papageorgis and Stylianopoulos, 2015). It is possible that tumor – stromal or CAF – immune crosstalk in the DDR2 global null mouse is impaired in a manner that depends on DDR2 action, which leads to reduced overall fibrosis in that model but not in FSP1-Cre; DDR2 fl/fl mice. This would suggest that DDR2 is not required on CAFs to respond to signals which increase ECM protein production but may be required in other cells to induce factors which promote fibrosis.

The action of DDR2 in stromal cells was essential for the formation of an aligned, aggressive (TACS-2/3) collagen matrix in an MMTV-PyMT genetic model of breast cancer. Second harmonic generation analysis of tumors showed that FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice primarily have curly, thin fibers rather than straight, thick fibers (Fig 2.5a,b). Further, DDR2 in stromal cells contributes to the formation of an increasing stiffness gradient leading away from the primary site. AFM data demonstrate that FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors are equally compliant throughout tumor and stromal regions while WT; MMTV-PyMT tumors demonstrate increasing stiffness as one moves from the tumor core to the tumor – stromal boundary (Fig. 2.6f). Since cells tend to migrate up a stiffness gradient by durotaxis (Lo et al., 2000), it is possible that tumor cells do not metastasize in this model because they are unlikely to migrate away from the primary tumor due to the absence of a stiffness gradient. Characteristics of tumor matrix collagen fibers have been shown to lead to increased local invasion as well as to be prognostic for poor outcomes (Provenzano et al., 2006; Provenzano et al., 2009; Provenzano et al., 2008). Whether the increase in stiffness *in vivo* is solely due to increases in collagen fiber characteristics such as increased width and alignment or if cellular characteristics such as increased contractility contribute remains to be seen. We show changes in

tumor stromal alignment and stiffness without a change in total collagen content or fibrosis, suggesting a new model by which stromal remodeling separately from collagen deposition increases breast tumor stiffness and promotes cancer progression.

In conclusion, we have identified a subset of stromal cells, likely CAFs, in which DDR2 is required to form stiffened, aligned collagen matrix without affecting overall collagen deposition. This change in tumor stroma is correlated to a significant decrease in lung metastases and suggests that both a stiffness gradient and thick, aligned fibers facilitate tumor cell escape from the primary site.

2.4 Materials and Methods

Mouse genetic tumor studies

The conditional DDR2 flox allele was generated as previously described (Corsa et al., 2016). This allele was crossed to FSP1-Cre and MMTV-PyMT mice to generate FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice. Wild type littermates were used as controls. We received the FVB/n FSP1-Cre mice as a generous gift from the Werb laboratory (San Francisco, CA). Tumor bearing mice were monitored weekly until end stage (2 cm single tumor). The mice were then euthanized and tumors and lungs collected. All mice analyzed were >90% FVB/n strain.

Immunofluorescence

Tumors were dissected away from the skin and then cut into <1cm pieces to allow efficient fixation. Tumors were fixed overnight in 10% neutral buffered formalin and then equilibrated in 30% sucrose overnight at 4 degrees. Equilibrated tissues were embedded in OCT and

cryosectioned at 5-10 um per section. Sections were post-fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.1% Triton X-100 for 5 min, and blocked in 5% goat serum for 1 hour at room temperature with washes in 1X PBS in between each step. Primary antibodies were incubated overnight at 4 degrees. Sections were then washed twice with 1X PBS and secondary antibody added for 1 hour at room temperature. Sections were then washed four times in 1X PBS, mounted in VectaShield with DAPI (VWR, 101098-044), and sealed with nail polish. Images were taken on an inverted Nikon epifluorescence microscope. Brightness and contrast adjustment as well as co-staining quantification was done manually in ImageJ.

Lung metastasis analysis

Lungs were fixed overnight in 10% neutral buffered formalin and then embedded in paraffin. Three 5 um sections were taken 200 um apart per lung and stained with hematoxylin and eosin. Metastases were counted in all lobes and documented as average number of total lung metastases.

Western blotting

Cells were lysed in 1X RIPA buffer supplemented with 1mM PMSF, 1mM sodium vanadate, 1mM sodium fluoride, and 10 ug/ml each aprotinin and leupeptin. Lysates were sonicated twice for 30 seconds and centrifuged at 14,000 RPM, 10 min. Cleared lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and blocked for 1 hour at room temperature in 5% non-fat dry milk, 1X TBS-0.5% Tween. Membranes were incubated in primary antibody overnight at 4 degrees with gentle agitation, washed twice with TBS-0.5% Tween, and incubated with anti-mouse or anti-rabbit HRP secondary antibody for one hour at room temperature.

Membranes were then washed four times with TBS-0.5% Tween and developed with ECL (Pierce, 32106).

Mouse Genotyping

Mouse tails were cut with a clean razor blade (~2 mm) and bleeding stopped with styptic powder. DNA was extracted and PCR run using KAPA Biosystems HotStart PCR (KK5621).

Hydroxyproline quantification

Non-necrotic tumor tissues were dissected away from the skin and non-tumor tissue removed. The samples were dried overnight in a lyophilizer and then hydrolyzed in 6N HCl (Thermo Fisher Scientific P24308) at 103-106 degrees Celsius for 48 hours. Samples were then re-dried in a lyophilizer and resuspended in water. Total protein and hydroxyproline were quantified separately. Total protein amount was assayed by adding 100ul of working solution (245mg ninhydrin (Sigma 151173), 9ml ethylene glycol, 4.8m 4N sodium acetate, 0.3ml SnCl₂ (100mg/1mL ethylene glycol)) to 5uL of resuspended hydrolyzed protein, baked at 85 degrees Celsius for 10 minutes, and then read at 575nm on a plate reader. Standard curve generated using Pickering #0125056H. Hydroxyproline was assayed by adding to 50 ul of sample, 100 ul of chloramine T at room temperature for 20 min, then adding 100ul Erlich's solution at 65 degrees Celsius for 20 min. The plate was then read at 550nm on a plate reader. Standard hydroxyproline resuspended at 1mg/ml (Sigma H54409). Results are reported as fraction hydroxyproline per mg total protein.

Second Harmonic Generation and TACS score

Tumors from 10-12 week old mice were dissected and fixed in 10% neutral buffered formalin overnight at room temperature. They were then embedded in paraffin and sectioned in 5-10um sections. In some cases, sections were stained with H&E, picosirius red, or trichrome stain prior to SHG imaging. Prior staining has no effect on SHG signal. Images were acquired on a Zeiss LSM 880 Airyscan confocal microscope using an inverted, motorized Zeiss Axio Observer Z1 frame. Two-photon images were collected at 880nm, using non-descanned detectors set to 440nm for SHG. Three to four z-stacks were acquired (step size 2 um) per tumor. The z-stacks were compressed and TACSsignature was scored by three blinded reviewers as TACS-1 (curly fibers) or TACS-2/3 (straight fibers) as previously described (Corsa et al., 2016; Provenzano et al., 2006).

FIB-SEM

Mice were perfused with pre-warmed, 37-degree, Ringer's solution (155mM NaCl, 3mM KCl, 2mM CaCl₂, 1mM MgCl₂, 3mM NaH₂PO₄, 5mM HEPES, pH 7.4, 10mM glucose) for 2 min and then for 5 min with pre-warmed, 37-degree fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.05% ruthenium red, 0.2% tannic acid in 0.15M cacodylate). Tumors were then dissected out and placed in fixative for 15min at 37 degrees, then 4 degrees overnight. Samples were embedded in resin and scanned by FIB-SEM.

Atomic Force Microscopy

Non-necrotic 10-12 week tumors were gently dissected away from the skin and flash frozen in OCT. Tumors were sectioned at 20 um per section. Just prior to AFM, tissues were quickly thawed in 1XPBS at room temperature and then maintained in 1X PBS supplemented with

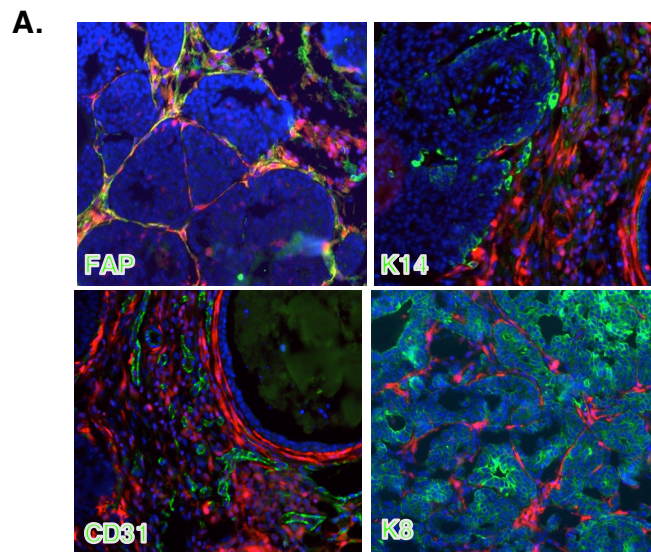
protease inhibitor cocktail (Roche Diagnostics, 11836170001) and propidium iodide (20ug/ml). 5-6 force maps were taken of at least two tumors from three mice per group. AFM was performed as described (Acerbi et al., 2015). All indentations were taken on an MFP-3D-BIO AFM (Asylum Research) mounted on an Olympus X711 inverted fluorescent microscope in an TMC acoustic noise enclosure. We used silicon nitride cantilever tips with a 5 um borosilicate glass sphere affixed to the tip with a spring constant of 0.06 N/m (Novascan, Boone, IA). The cantilever was calibrated with thermal oscillation prior to each experiment. Indentations were taken at 20 um/second loading rate with a maximum force of 5 nN, and force maps were generated using the FMAP function on IGOR software (Asylum Research). The Hertz method was used to calculate elasticity and Poisson's ratio of 0.5 was used to calculate Young's elastic modulus.

Statistical analysis

All p-values were calculated using Student's unpaired, two-tailed T-Tests. p-values are noted in figure legends.

2.5 Figures

Figure 2.1

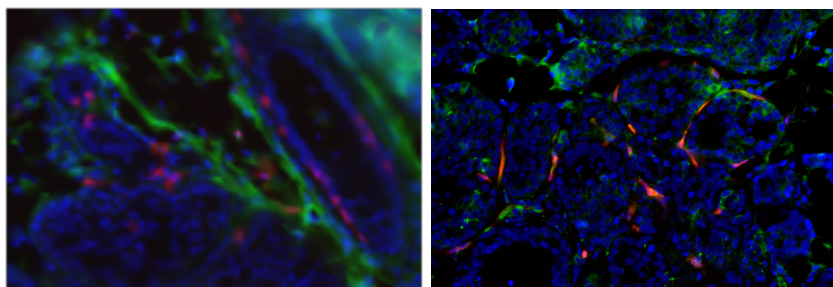


FSP1-Cre; ROSA-LSL-TdTomato

B.

Cell identity marker	Merge / total GFP	Merge / total TdTomato
FAP (activated fibroblast)	75-80%	80-85%
K14 (basal epithelial)	<4%	~0%
K8 (luminal epithelial)	~0%	~0%
CD31 (endothelial)	~0%	~0%
CD45 (immune)	90%	15-20%

C.



α SMA-Cre; ROSA-LSL-TdTomato

FAP
65

Figure 2.1: FSP1-Cre targets breast tumor stromal cells.

(A) Representative immunofluorescence images showing co-staining of cell lineage markers with FSP1-Cre; ROSA-LSL-TdTomato. (B) Quantification of co-staining; represented as co-stained cells/total number of lineage marker positive cells or co-stained cells/total number of TdTomato positive cells. (C) Representative images of α SMA-Cre; ROSA-LSL-TdTomato tumors co-stained with FAP. Images demonstrate little TdTomato expression overall, intratumor staining and staining in normal glands (left) and little co-staining with FAP (left and right).

Figure 2.2

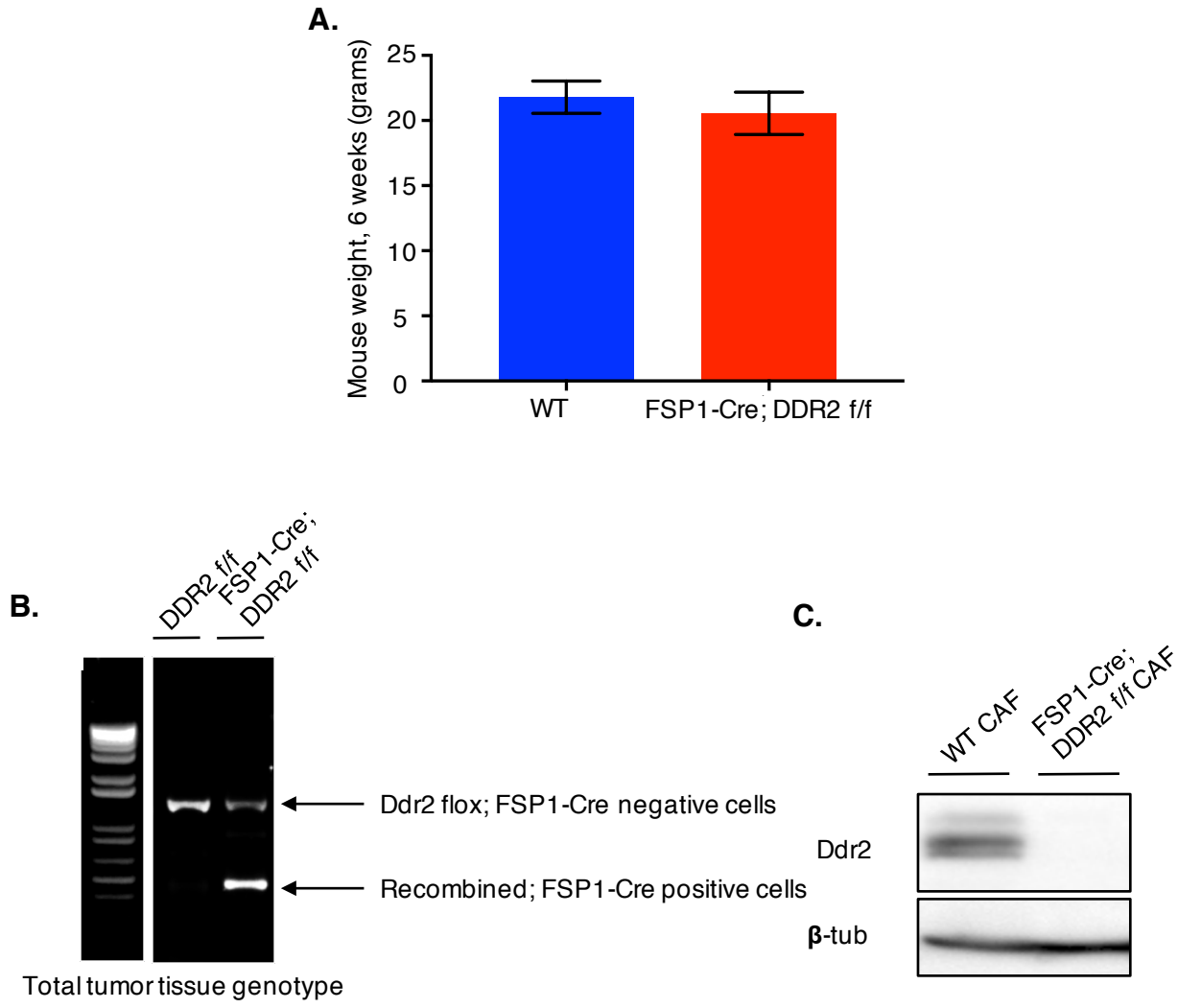


Figure 2.2: FSP1-Cre mice are normal size and FSP1-Cre deletes DDR2 in CAFs.

(A) Bar graph showing equivalent body weight (g) of WT and FSP1-Cre mice. (B) Total tumor genotyping showing DDR2 locus recombination (C) Western blot demonstrating DDR2 knockout in FSP1-Cre; DDR2 fl/fl CAFs.

Figure 2.3

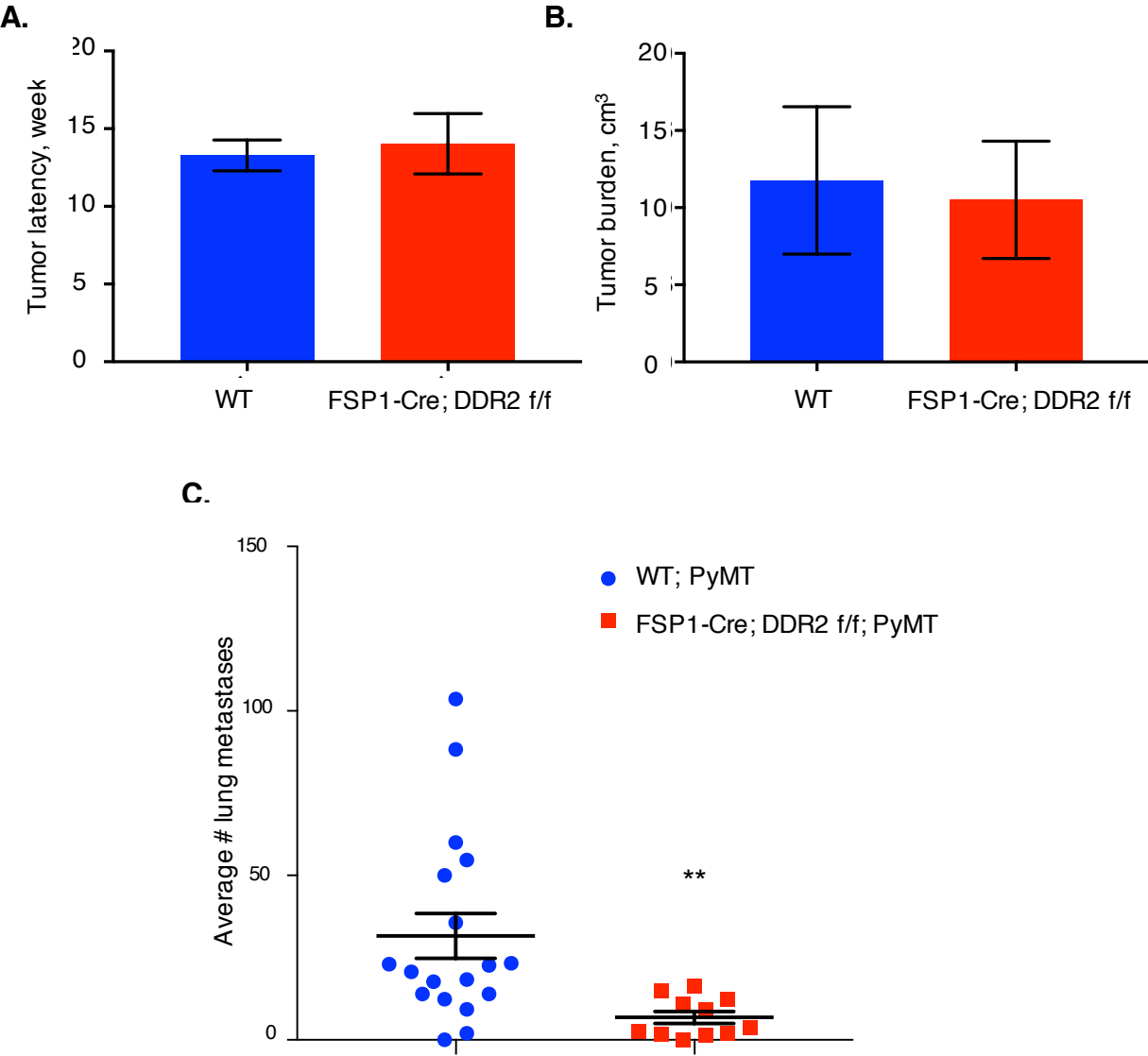
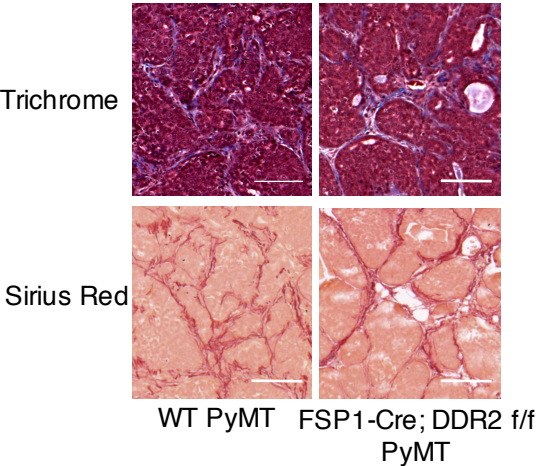


Figure 2.3: Stromal DDR2 is required for breast cancer lung metastasis.

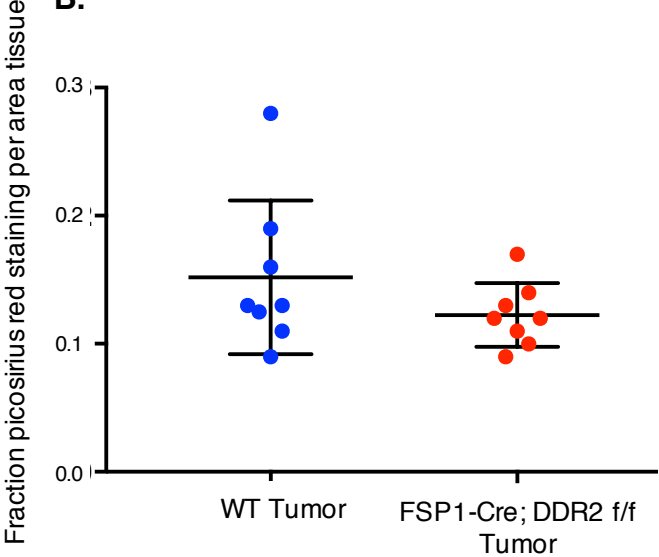
(A) Primary tumor growth rate in WT; MMTV-PyMT versus FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice as represented by time to end stage (single tumor >2cm). n = 10-18 per group. (B) Total primary tumor burden in WT; MMTV-PyMT versus FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice. Represented as the total volume of all primary tumors per mouse. n = 10-18 per group. (C) Number of lung metastases in WT; PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice, represented at average number of lung metastases per whole lung. n = 10-18 per group. ** p = 0.0095. All mice were >90% FVB/n strain.

Figure 2.4

A.



B.



C.

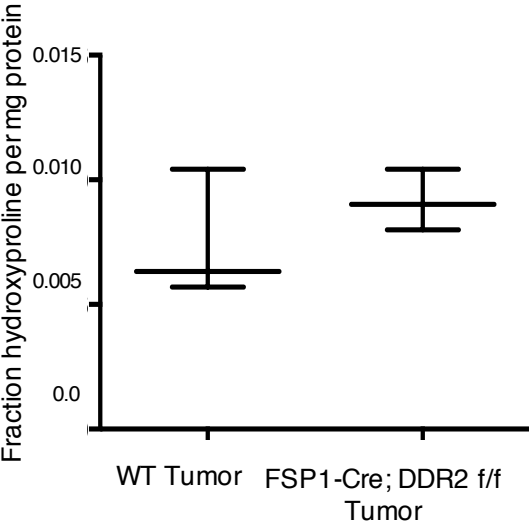


Figure 2.4: DDR2 action in the stroma does not affect fibrosis.

(A) Representative images of trichrome staining (top) and picosirius red staining (bottom) in 10-12 week WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. (B)

Quantification of area of picosirius red staining per area of tumor. n = 8 per group. (C)

Hydroxyproline quantification in 10-12 week WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl;

MMTV-PyMT tumors. Represented at fraction of hydroxyproline per mg of total protein. n = 3 per group.

Figure 2.5

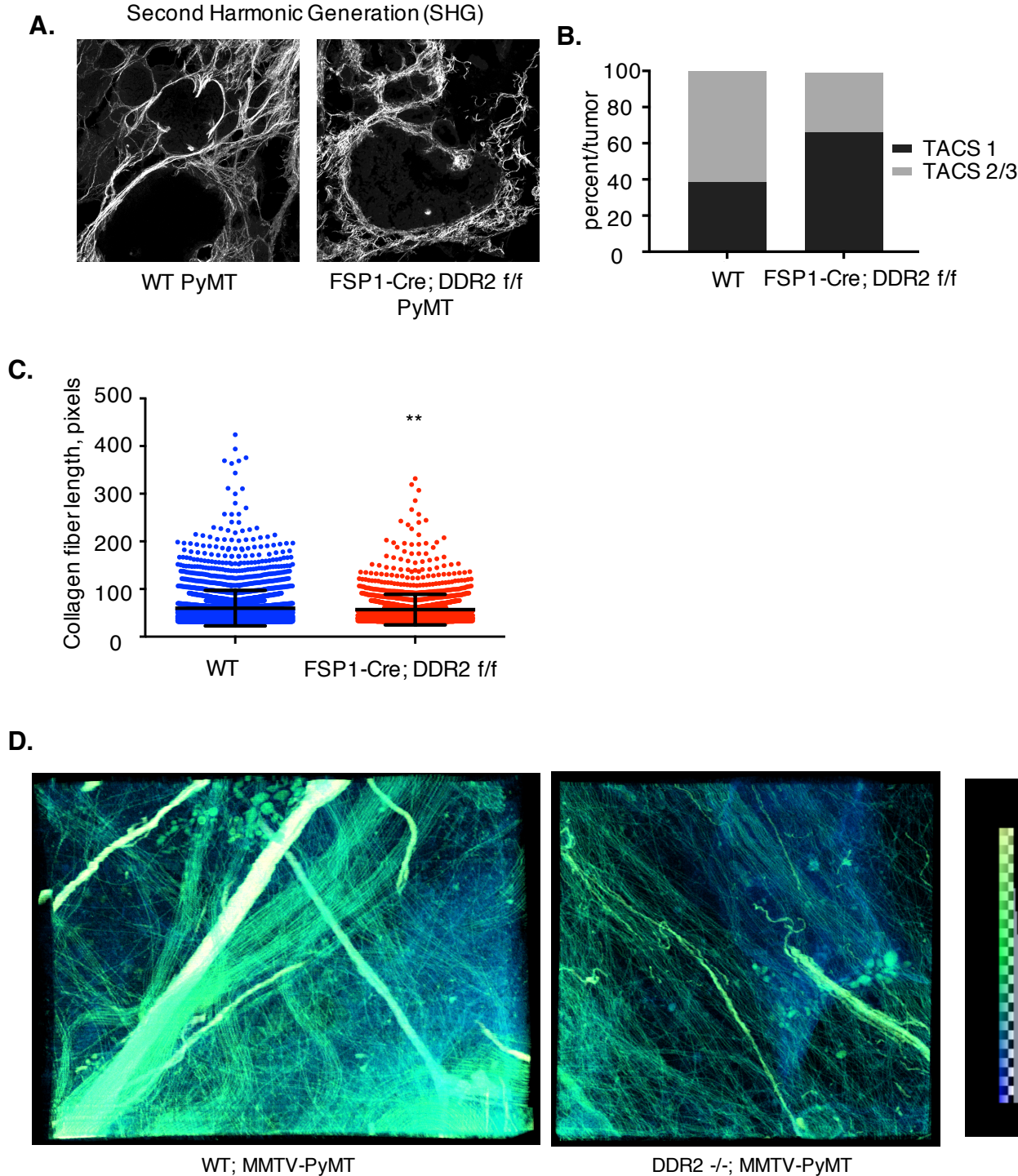


Figure 2.5: DDR2 affects collagen alignment, fiber width, and fiber length

(A) Representative second harmonic images (SHG) of 10-12 week WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. (B) Quantification of TACS-1 vs TACS 2/3 phenotype in (A). n = 6 per group. (C) Quantification of collagen fiber length in WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors (same images as in (A)) as calculated using CT-FIRE software (LOCI, Madison, WI). ** p = 0.003 (D) FIB-SEM reconstruction of collagen fibers from WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT. Images are pseudocolored to indicate intensity of signal. In each image, the viewer is looking directly at the tumor – stromal boundary from the stromal side.

Figure 2.6

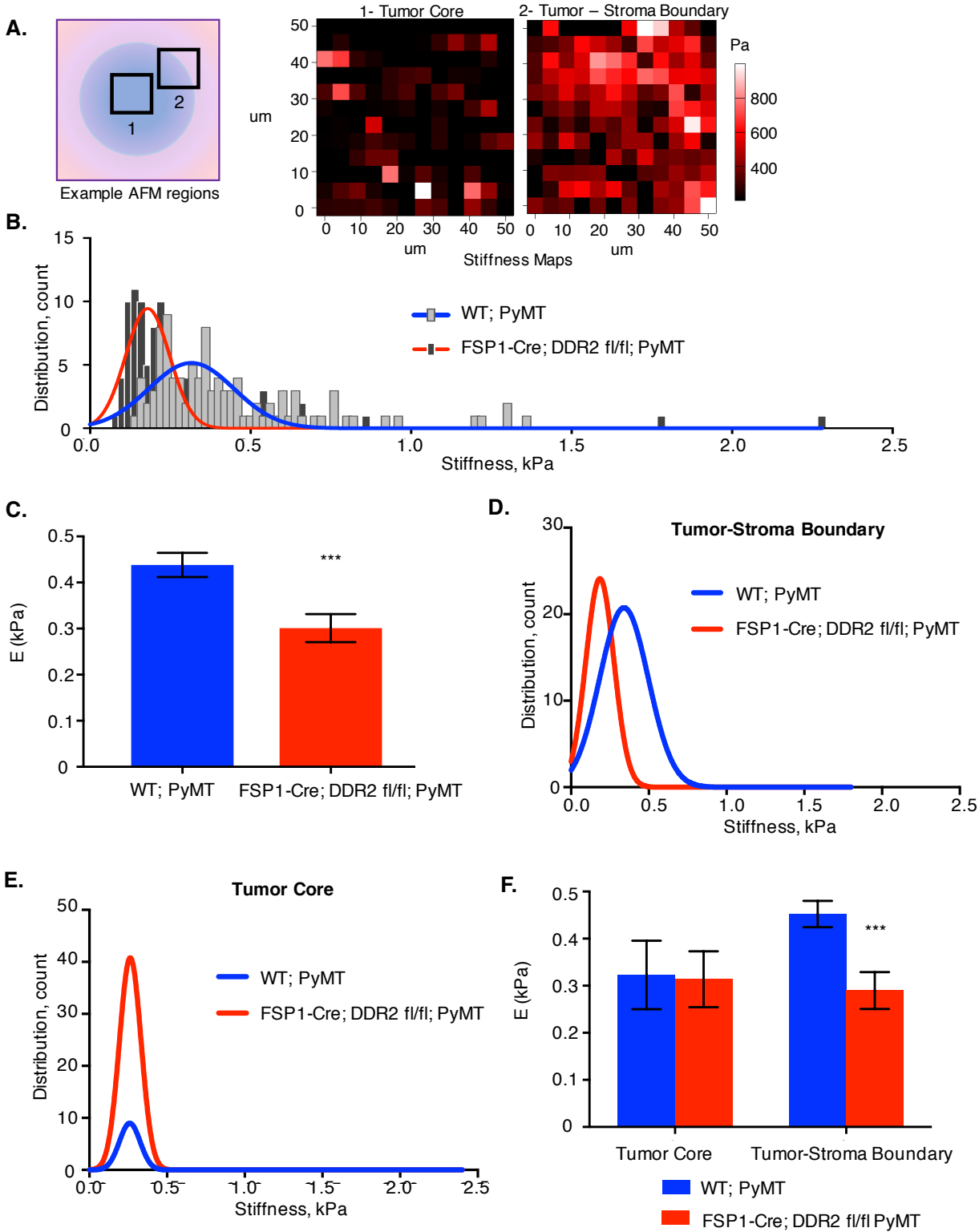


Figure 2.6: Stromal DDR2 affects mechanical properties of tumors.

(A) Schematic representation (left) and representative stiffness maps from WT; MMTV-PyMT tumor- either tumor core (left) or tumor – stromal boundary (right). (B) Compiled tumor stiffness measurements for WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice. Data are represented as histograms fit with a Gaussian curve. (C) Average stiffness of WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. *** $p = 0.0008$ (D) Stiffness of the tumor – stromal boundary of WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. (E) Stiffness of the tumor core of WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. (F) Quantification of (D) and (E). $n = 6$ tumors per group, data plotted are the top 100 best fitting curves per group. *** $p = 0.0003$

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Chapter 3: DDR2 is critical for matrix remodeling by cancer associated fibroblasts.

3.1 Introduction

Breast tumors are made up of a complex milieu of cells and matrix components, including ECM proteins, soluble paracrine factors, and enzymes (Joyce and Pollard, 2009). This mixture can promote cancer progression in a number of ways- Non-cancerous cell types, such as cancer associated fibroblasts, have been shown to promote tumor aggression and metastasis through secretion of paracrine factors and changes in the extracellular matrix (Kalluri, 2016; Potenta et al., 2008). Increased stromal collagen deposition, alignment of collagen fibers, and fiber thickening have all been associated with local tumor invasion and poor prognosis (Conklin et al., 2011; Levental et al., 2009; Provenzano et al., 2006; Provenzano et al., 2008). Tissue stiffness, especially at leading edges of invading tumor cells, has been shown to predict aggressive breast cancer subtypes (Acerbi et al., 2015; Lopez et al., 2011) and is known to reciprocally increase intracellular tension, leading to increased cellular contractility and improved invasion (Oudin and Weaver, 2016; Paszek et al., 2005; Provenzano et al., 2009). It is not yet fully known which factors or cell types are responsible for altering the breast stroma to one which promotes tumor aggression.

The Longmore lab has demonstrated that the collagen binding receptor tyrosine kinase DDR2 is necessary in both the breast basal epithelial cells and the host for breast cancer lung metastasis (Corsa et al., 2016). Further, using the stromal specific FSP1-Cre, we have more

precisely demonstrated that DDR2 in stromal cells is required for breast cancer lung metastasis (see; chapter 2). In all cases, DDR2 expression had no effect on tumor growth or tumor latency, suggesting that the reduction in lung metastases is due to a defect in escape from the primary site, survival in the periphery, or re-growth in distant organs. Unpublished work in the lab has shown that in a tail vein injection model, there are no differences in lung colonization between DDR2 null mice and wild type when injected with wild type tumor cells, suggesting that DDR2 in the lung is not necessary for extravasation, seeding, and outgrowth of tumor cells. In addition, no differences in circulating tumor cells (CTCs) were found in DDR2 null mice or in a transplant model, suggesting that there is no defect in intravasation or survival in the bloodstream related to DDR2 action (Corsa et al., 2016). In sum, these data suggest that the primary means by which breast cancer metastasis is reduced in this model lies in the ability of the tumor to locally invade away from the primary site. It was shown that both DDR2 null basal epithelial cells and DDR2 null CAFs regulate collective invasion of tumor organoids, though whether this effect was through paracrine secretion, direct contact between cells types, or due to changes in the surrounding ECM was not established (Corsa et al., 2016).

Analysis of the tumor stroma in these models have collectively demonstrated a defect in collagen architecture related to DDR2 action. DDR2 $-/-$; MMTV-PyMT (Corsa et al., 2016) and FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors (see; chapter 2) have a predominantly TACS-1 collagen phenotype. The collagen fibers near the tumor – stromal boundary are thin and curly rather than thick and straightened. Further, FIB-SEM analysis of tumor ECM collagen fibers shows reduced thickness of fibers as well as reduced total collagen amount in 10-11 week old DDR2 global null; MMTV-PyMT tumors which is in agreement with published data (see; chapter 2 and (Corsa et al., 2016)). In DDR2 $-/-$; MMTV-PyMT mice, but not in MMTV-Cre;

DDR2 fl/fl; MMTV-PyMT or K14-Cre; DDR2 fl/fl; MMTV-PyMT mice, reduced overall fibrosis was seen in end stage tumors (Corsa et al., 2016). This suggested that DDR2 in the host enhanced collagen deposition in breast cancer, however no difference in fibrosis or collagen content was seen in FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors. Further work is required to determine the cause of reduced total fibrosis in the DDR2 global null model.

In previously published work from the Longmore lab, we demonstrated that DDR2 is necessary in CAFs for the production of an aligned matrix *ex vivo*, suggesting that the defect in collagen alignment *in vivo* is due to defective CAF function (Corsa et al., 2016). In this study, we sought to determine whether DDR2 in CAFs is required matrix remodeling. We show that DDR2 re-expression can rescue the collagen alignment defect and confirm that DDR2 is not required for incorporation of collagen into matrix. We further show that DDR2 action affects cellular contractility and the generation of intracellular tension in CAFs.

3.2 Results

CAF DDR2 is required for collagen alignment but not collagen incorporation into ECM.

Extracellular matrix maintenance depends upon a balance of matrix production and matrix remodeling, which includes both degradation and fiber rearrangement. In cancer, normal homeostasis goes awry and leads to increased collagen deposition and acquisition of an aggressive collagen fiber phenotype (Lopez et al., 2011; Provenzano et al., 2006; Provenzano et al., 2008). Since we had previously seen a change in breast stromal collagen signature (TACS) in stromal knockout FSP1-Cre; DDR2 fl/fl; MMTV-PyMT mice and since fibroblasts are primarily responsible for matrix production and remodeling, we wanted to test the effect of DDR2 on

breast CAFs (see; chapter 2). Breast CAFs were isolated from 10-12 week old WT; MMTV-PyMT or FSP1-Cre; DDR2 fl/fl; MMTV-PyMT tumors and immortalized by escape from senescence (greater than 20 passages). Resultant cell lines were tested for the presence of typical CAF markers, including α -SMA, N-cadherin and FSP1, and the absence of epithelial markers such as E-cadherin (Fig 3.1a). Cells were also shown have a fibroblast-like cellular morphology by immunofluorescence (Fig 3.1b).

To determine whether DDR2 affected CAF matrix assembly, CAFs were plated to hyperconfluency and supplemented with ascorbic acid for 7 days. Ascorbic acid was replenished every other day. After 7 days, CAFs were extracted from the extracellular matrix by alkaline detergent extraction. Resultant cell-free ECMs were stained for collagen 1 α 1 and imaged by confocal microscopy. Results indicate that DDR2 is required for the formation of an aligned collagen matrix (Fig. 3.1c) and that this phenotype is specific to DDR2. FSP1-Cre; DDR2 fl/fl CAFs rescued with DDR2-myc transduction (Fig. 3.1d) were able to make an aligned matrix (Fig. 3.1c, right). Some of these similar data have been previously published in other reports from the Longmore lab (Corsa et al., 2016).

Because changes in ECM structure can be due to matrix protein production, remodeling, or both, we wanted to test whether DDR2 in CAFs had an effect on the amount of collagen which was incorporated into *ex vivo* produced ECMs. In *in vivo* models, no change in collagen content was apparent, though collagen architecture was altered in the presence of stromal DDR2 (Figs. 2.4, 2.5). To test collagen incorporation into *ex vivo* ECMs, cell-free ECM was hydrolyzed to constituent amino acids and the amount of hydroxyproline was measured by colorimetric assay. Results show no difference in hydroxyproline content between WT and FSP1-Cre; DDR2 fl/fl CAFs (Fig. 3.1e). Taken together, these results suggest that DDR2 action in CAFs is

required to remodel matrix to an aligned collagen phenotype but is not required for the incorporation of collagen into ECM.

CAF DDR2 affects cellular mechanotransduction/mechanosignaling.

It is not entirely known how collagen fibers become aligned in an ECM, however one proposed mechanism involves cells pulling on fibers and thereby reorienting them (Sawhney and Howard, 2002). Since ECMs from FSP1-Cre; DDR2 fl/fl CAFs were unable to make an aligned matrix, we tested whether their cellular contractility was affected. To do so, WT or FSP1-Cre; DDR2 fl/fl CAFs were embedded into 1mg/ml collagen gels and allowed to contract that gel over three days. At day 3, the collagen gels were imaged and percent gel area remaining was quantified. WT CAFs were able to contract a collagen gel to less than 5% of the original gel area, while FSP1-Cre; DDR2 fl/fl CAFs were not (Fig. 3.2a). This result was also seen when human CAFs (hCAF), WT or shDDR2, were embedded in collagen gels (Fig. 3.2b). shDDR2 knockdown is shown in Figure 3.2c. These results indicate that DDR2 is required in CAFs for robust cellular contractility, a hallmark of activated fibroblasts.

Mechanical signals are transmitted to cells through contacts with their extracellular microenvironment. These contacts are made up of transmembrane receptors for matrix proteins and the coordinated complex of proteins in focal adhesion complexes that link these receptors to the actin cytoskeleton (Liu et al., 2015; Wozniak et al., 2004). When extracellular matrix stiffness increases, a reciprocal increase in intracellular tension is observed. One indication of increased intracellular tension is focal adhesion size; as the cell pulls harder on stiff matrix, focal adhesions enlarge to be able to transmit that force (Riveline et al., 2001). We observed focal

adhesions in WT and FSP1-Cre; DDR2 fl/fl CAFs by staining for vinculin. Cells were plated on collagen 1 or fibronectin coated coverslips and allowed to adhere and spread out overnight. Focal adhesions were imaged by confocal microscopy and area of vinculin staining quantified. As can be seen in figure 3.2d, the area of vinculin staining in FSP1-Cre; DDR2 fl/fl CAFs is significantly smaller (3.2 um^2) than that of WT CAFs (4.4 um^2), indicating an overall decrease in focal adhesion size. Further, this effect was only seen when cells were plated on collagen 1, no difference in vinculin staining was seen when plated on fibronectin (Fig. 3.2e, f). Reduced focal adhesion size was also found when hCAF were shDDR2 depleted (Fig. 3.3c). These results indicate that DDR2 action in CAFs is required for cellular contractility and suggests that DDR2 may play a role in focal adhesion assembly or maturation, further suggesting that DDR2 action in CAFs is required for mechanotransduction or mechanosignaling.

DDR2 collagen binding but not kinase activity may be required for mechanotransduction or mechanosignaling.

DDR2 is a unique receptor tyrosine kinase in that it binds fibrillar collagen rather than a soluble ligand. It also has extremely long activation kinetics, on the order of hours (Shrivastava et al., 1997; Vogel et al., 1997). DDR2 kinase activity can be abrogated by a mutation in the kinase domain (K608E) (Zhang et al., 2013) and collagen binding can be disrupted by a mutation in the DS domain (W52A) (Carafoli et al., 2009; Ichikawa et al., 2007). The W52A mutant retains kinase activity, however it cannot be activated. We confirmed these phenotypes by expressing DDR2 WT, K608E, and W52A in HEK 293T cells in the presence or absence of

collagen 1 overnight. As can be seen by both phospho-DDR2 and IP-pTyrosine blot, only WT DDR2 is phosphorylated in the presence of collagen 1 (Fig. 3.3b).

To test whether DDR2 kinase activity or DDR2 collagen binding can rescue the focal adhesion size defect found in DDR2 shRNA depleted hCAFs, we rescued DDR2 expression with YFP-tagged DDR2-WT, DDR2-K608E, or DDR2-W52A by viral transduction. Cells were then plated on collagen 1-coated coverslips overnight and stained for vinculin (Fig. 3.3a). Only cells expressing similar levels of DDR2-YFP as quantified by fluorescence intensity were included in our analysis (Fig. 3.4c). Results indicate that DDR2 collagen binding but not kinase activity is required to rescue focal adhesion size. hCAF shDDR2 resWT and hCAF shDDR2 resK608E cells had similar focal adhesion size as hCAF shSCR controls, while hCAF shDDR2 resW52A results were unchanged from hCAF shDDR2 cells (Fig. 3.3c).

While focal adhesion size can be indicative of intracellular tension, we also wanted to see if there was a difference in mechanosignaling to actomyosin contractility machinery. Cellular contractility is dependent on adhering to a substrate and actomyosin machinery; cells cannot contract in the presence of myosin inhibitors, for example (Calvo et al., 2013). Cells also cannot form mature focal adhesions without the ability to pull on the ECM (Humphrey et al., 2014; Zhou et al., 2017). Myosin is activated in a Rho/ROCK dependent manner whereby Rho GTPase activates ROCK which in turn activates Myosin Light Chain Kinase (MLCK) and inhibits MLC Phosphatase. MLCK phosphorylates Myosin Light Chain (MLC), leading to cell contraction and stress fiber formation (Riching and Keely, 2015). To determine whether reduced focal adhesion size was indicative of reduced intracellular tension, we stained hCAFs for pMLC levels. Cells were imaged by confocal microscopy and the amount of pMLC, corrected for cell size, was quantified. Results indicate that focal adhesion size correlates with total cellular pMLC levels.

hCAF shDDR2 cells had less total pMLC than controls, and this phenotype was rescued by DDR2-WT and DDR2-K608E but not DDR2-W52A (Fig. 3.4a, b). Again, only cells with a similar amount of DDR2-YFP expression were considered in the analysis (Fig. 3.4c). These results suggest that DDR2 collagen binding but not kinase activity is required for CAF mechanotransduction or mechanosignaling.

DDR2 collagen binding but not kinase activity is required for cellular traction force.

The ability to generate intracellular tension allows a cell to respond to its extracellular microenvironment and generate traction forces upon it. Traction force, or pulling, on fibers can be one method by which cells remodel matrix (Sawhney and Howard, 2002). To test whether cellular traction force is defective in DDR2 depleted hCAFs, we plated cells on soft (792 Pa) collagen 1-coated hydrogels embedded with fluorescent beads and allowed them to adhere and spread out overnight (Fig. 3.5a). The next day, initial images of the tense state were acquired, cells were then trypsinized to release tension in the gel, and re-imaged. By comparing the position of the fluorescent beads before and after trypsinization, bead displacements can be measured, and, in combination with known characteristics of the hydrogels, local traction forces can be calculated. Results of this experiment indicate that DDR2 is required for the generation of traction forces, and that collagen binding but not kinase activity is necessary (Fig. 3.5b). The top row shows a heat map of the bead displacement field, the middle row shows the same images overlaid with bright field images of the cells, and with forces calculated in the bottom row (Fig. 3.5b). These data, taken together with focal adhesion size and pMLC level data, suggest that

DDR2 collagen binding positively affects the mechanical properties of CAFs and is sufficient to promote increased traction forces and intracellular tension.

3.3 Discussion

Matrix remodeling is a fundamental process in both normal matrix homeostasis and cancer. We have shown in other studies that CAFs isolated from FSP1-Cre; DDR2 fl/fl; MMTV-PyMT are unable to make an aligned collagen matrix, however the cellular or molecular mechanism for how this change occurs was not established (Corsa et al., 2016). In this study, we show that DDR2 re-expression in FSP1-Cre; DDR2 fl/fl CAFs can rescue the collagen alignment defect found in FSP1-Cre; DDR2 fl/fl CAFs, however, similar to *in vivo* experiments (chapter 2), this defect does not appear to be due to the ability of CAFs to incorporate collagen into the matrix (Fig. 3.1). This may be explained by the possibility that collagen alignment is mostly due to matrix modeling or re-modeling and not by production of collagen. Further work is needed to determine if enhancing a CAF's ability to remodel, ie- by increasing contractility, for example, can rescue the collagen alignment defect in FSP1-Cre; DDR2 fl/fl CAFs.

In this study, we have shown that DDR2 action affects the mechanotransducing and mechanosignaling properties of CAFs. The ability of a cell to sense, transmit, and respond to mechanical information about its extracellular matrix environment is critical for effective cellular adaptation and survival (Hytonen and Wehrle-Haller, 2016; Matthews et al., 2006; Provenzano et al., 2009; Provenzano and Keely, 2011). In breast cancer, tissues stiffen as cancer progresses and this increased stiffness causes a reciprocal increase in intracellular tension and contractility in tumor and stromal cells (Lopez et al., 2011). Here we show that DDR2 action promotes marked

contraction of collagen gels, growth of focal adhesions in response to mechanical load, and increased intracellular tension (Fig. 3.2-4). These data support the idea that DDR2 is a novel mechanosensing or mechanotransducing cell surface receptor and critical for matrix remodeling. Many groups have shown that cells pull on collagen fibers and that these forces are transmitted over long distances (Sawhney and Howard, 2002). It is likely that small traction forces on collagen fibers from cells causes alignment of those fibers. Here we show that shDDR2 depleted human CAFs have very little ability to generate individual cell traction forces (Fig. 3.5). If a cell cannot contract or pull on its surroundings, a cell cannot remodel collagen fibers.

Here we demonstrate a novel kinase independent function of DDR2 in CAFs. DDR2 binds and is activated by fibrillar collagens. Full activation of the receptor takes several hours and leads to phosphorylation of several tyrosine residues in the cytoplasmic tail. Both kinase activity and the ability to bind collagen are necessary for DDR2 signaling functions (Shrivastava et al., 1997; Vogel et al., 1997). Most studies to date have focused on the kinase dependent functions of DDR2, though some recent reports and unpublished data from the Longmore lab indicate that DDR2 may have kinase-independent functions (Xu et al., 2012). In this study, kinase dead DDR2 (K608E) is able to fully rescue growth of focal adhesion, intracellular phospho-MLC levels, and traction forces in shDDR2 depleted human CAFs. In sum, these data suggest that canonical signaling pathways downstream of DDR2 activation are unlikely to play a direct role in matrix remodeling. Collagen binding, however, is required. It is possible that DDR2 collagen binding promotes DDR2 clustering, which creates a local increase in the concentration of kinase independent signaling partners. It is also possible that DDR2 collagen binding allows it to act as a co-receptor for other transmembrane signaling molecules. Further, since integrins are the collagen receptors which are typically thought of in cell adhesion,

migration, and contraction, it is possible that DDR2 is directly or indirectly affecting integrin activity (Xu et al., 2012).

In conclusion, the work presented here may explain, in part, how CAFs remodel collagen matrix to an aligned phenotype in breast tumor stroma. DDR2 appears to be a novel mechanosensing/mechanotransducing transmembrane receptor which influences a cell's ability to contract and generate force, and, thus, move collagen fibers.

3.4 Materials and Methods

Isolation of CAFs

MMTV-PyMT tumors were dissected, minced, and minced pieces transferred to ~20 mL of digestion media per tumor (DMEM, 1% fbs, 0.2% Collagenase A (Roche), 0.2% trypsin (Gibco 27250-018), 50 µg/mL gentamycin, 5 µg/mL insulin) and rocked at 37 degrees for 30–45 minutes. The digested tissue was then washed twice with serum free media and treated with DNase for 5 min at room temperature. Tissue was resuspended in ice-cold serum free media and serially centrifuged four times. Single cell fractions were collected and plated for 25–30 minutes in DMEM, 10% fbs at 37 degrees Celsius, 5% CO₂, 20% O₂. CAFs will be adhered to the plate while other cells will not. The supernatant and non-adherent cells were removed and CAFs were maintained in DMEM, 10% fbs at 37 degrees Celsius, 5% CO₂, 20% O₂ for 20+ passages, splitting 1–2 times per week. The immortalized primary cell lines were then submitted to FACS with PDGFR α antibodies.

***ex vivo* ECM synthesis and analysis**

Human CAFs were plated to confluence on 12mm glass coverslips in DMEM supplemented with 10% FBS and 50 µg/ml ascorbic acid, and media was changed daily for 7 days. Cells were extracted on day 7 (25 mmol/L Tris-HCl, pH 7.4; 150 mmol/L sodium chloride; 0.5% Triton X-100; and 20 mmol/L ammonia hydroxide) for 3–5 minutes. Cellular debris was carefully washed away with 1X PBS. Resultant cell free ECMs were fixed in 4% paraformaldehyde for 15 minutes at room temperature and then blocked with 5% FBS in 1X PBS. ECMs were then incubated in mouse anti-fibronectin antibody (diluted 1:100, BD Biosciences) overnight at 4 degrees, washed twice, and then incubated in goat anti-mouse AlexaFluor 488 secondary (diluted 1:500, Life Technologies), washed four times, mounted in Vectashield (VWR, 101098-044), and sealed with nail polish. Immunofluorescence was analyzed on a confocal microscope (LSM 700; Carl Zeiss, Jena, Germany) at room temperature with Zen 2009 software. ImageJ was used to adjust brightness and contrast.

Hydroxyproline quantification

Cell-free *ex vivo* ECMs were collected and dried overnight in a lyophilizer and then hydrolyzed in 6N HCl (Thermo Fisher Scientific P24308) at 103-106 degrees Celsius for 48 hours. Samples were then re-dried in a lyophilizer and resuspended in water. Total protein and hydroxyproline were quantified separately. Total protein amount was assayed by adding 100ul of working solution (245mg ninhydrin (Sigma 151173), 9ml ethylene glycol, 4.8m 4N sodium acetate, 0.3ml SnCl₂ (100mg/1mL ethylene glycol)) to 5uL of resuspended hydrolyzed protein, baked at 85 degrees Celsius for 10 minutes, and then read at 575nm on a plate reader. Standard curve generated using Pickering #0125056H. Hydroxyproline was assayed by adding to 50 ul of sample, 100 ul of chloramine T at room temperature for 20 min, then adding 100ul Erlich's solution at 65 degrees Celsius for 20 min. The plate was then read at 550nm on a plate reader.

Standard hydroxyproline resuspended at 1mg/ml (Sigma H54409). Results are reported as fraction hydroxyproline per mg total protein.

Western blotting

Cells were lysed in 1X RIPA buffer supplemented with 1mM PMSF, 1mM sodium vanadate, 1mM sodium fluoride, and 10 ug/ml each aprotinin and leupeptin. Lysates were sonicated twice for 30 seconds and centrifuged at 14,000 RPM, 10 min. Cleared lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and blocked for 1 hour at room temperature in 5% non-fat dry milk, 1X TBS-0.5% Tween. Membranes were incubated in primary antibody overnight at 4 degrees with gentle agitation, washed twice with TBS-0.5% Tween, and incubated with anti-mouse or anti-rabbit HRP secondary antibody for one hour at room temperature. Membranes were then washed four times with TBS-0.5% Tween and developed with ECL (Pierce, 32106).

Gel contraction

2×10^5 CAFs were embedded in 100 ul of 1mg/ml collagen 1 gel (Rat tail collagen, Corning CB354249) which was then spread with a pipet tip into the well of glass bottom 12mm Mattek dishes. The gel was allowed to solidify at 37 degrees Celsius for 20 min after which 2 mL of DMEM + 10% fbs was added and gels were gently detached with a pipet tip. Gels were imaged after 3 days, and percent contraction was calculated relative to initial gel area by tracing in ImageJ.

Immunofluorescence on cells

For collagen or fibronectin coating, 50 ug/mL collagen or fibronectin in water was spread on 12mm glass coverslips (no. 1.5, high precision) and allowed to dry at room temperature

overnight. The next day, the coverslips were blocked with 1% BSA in PBS for 1 hour at room temperature and sterilized under UV for 20 min. Cells were plated sparsely (1×10^4 per coverslip) and allowed to adhere and spread for indicated times. Cells were then fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized in 0.1% Triton X-100 in PBS for 5 minutes at room temperature, washed with PBS, and blocked with 5% normal goat serum in PBS. Primary antibodies were added and incubated at 4 degrees Celsius overnight. Coverslips were washed and secondary fluorescent antibody added for 1 hour at room temperature. Coverslips were washed again and mounted in Prolong Diamond mounting medium. After curing for 24 hours, cells were imaged by confocal microscopy on NIS-Elements software (Nikon A1Rsi, inverted). Z-stacks were taken with a step size of 0.2 μm with a 40X objective. Z-stacks were flattened by maximum intensity projection, and focal adhesions were quantified in ImageJ by subtracting the background, thresholding to the same level for all samples, and running particle analysis. p-MLC images were taken in the same manner, but levels were quantified by tracing cell outlines and measure integrated density, corrected for background and cell area.

Traction force

Glass coverslips were activated with 3-APTMS for 5 min and fixed in 0.5% glutaraldehyde for 30 minutes at room temperature. Hydrophobic coverslips were made by treatment with Sigmacote. Soft (792 Pa) polyacrylamide hydrogels were made by polymerizing (final concentrations of 5% acrylamide and 0.1% bis-acrylamide with 0.5% dark red fluorescent beads, 0.2 μm (Thermo Fisher Scientific F8807)) gel in a sandwich between the functionalized and hydrophobic coverslips. Gels were allowed to polymerize for 30 minutes at room temperature, the sandwich separated and washed. The surface of the gel was functionalized with 0.5mg/mL

sulfo-SANPAH in 50mM HEPES, pH 8.2 under UV light for 10 min. Gels were extensively washed and then incubated with 50 ug/mL collagen 1 in 50 mM HEPES, pH 8.2 overnight at 4 degrees Celsius. The next day, gels were washed and equilibrated in DMEM. Cells were plated sparsely and allowed to adhere and spread overnight. During microscopy, cells were kept at 37 degrees and under 5% CO₂ in an incubated plate holder. Images were taken before and after trypsinization, and bead displacements calculated with a Matlab program.

Statistical Analysis

All p-values were calculated using Student's unpaired, two-tailed T-Tests. p-values are noted in figure legends.

3.5 Figures

Figure 3.1

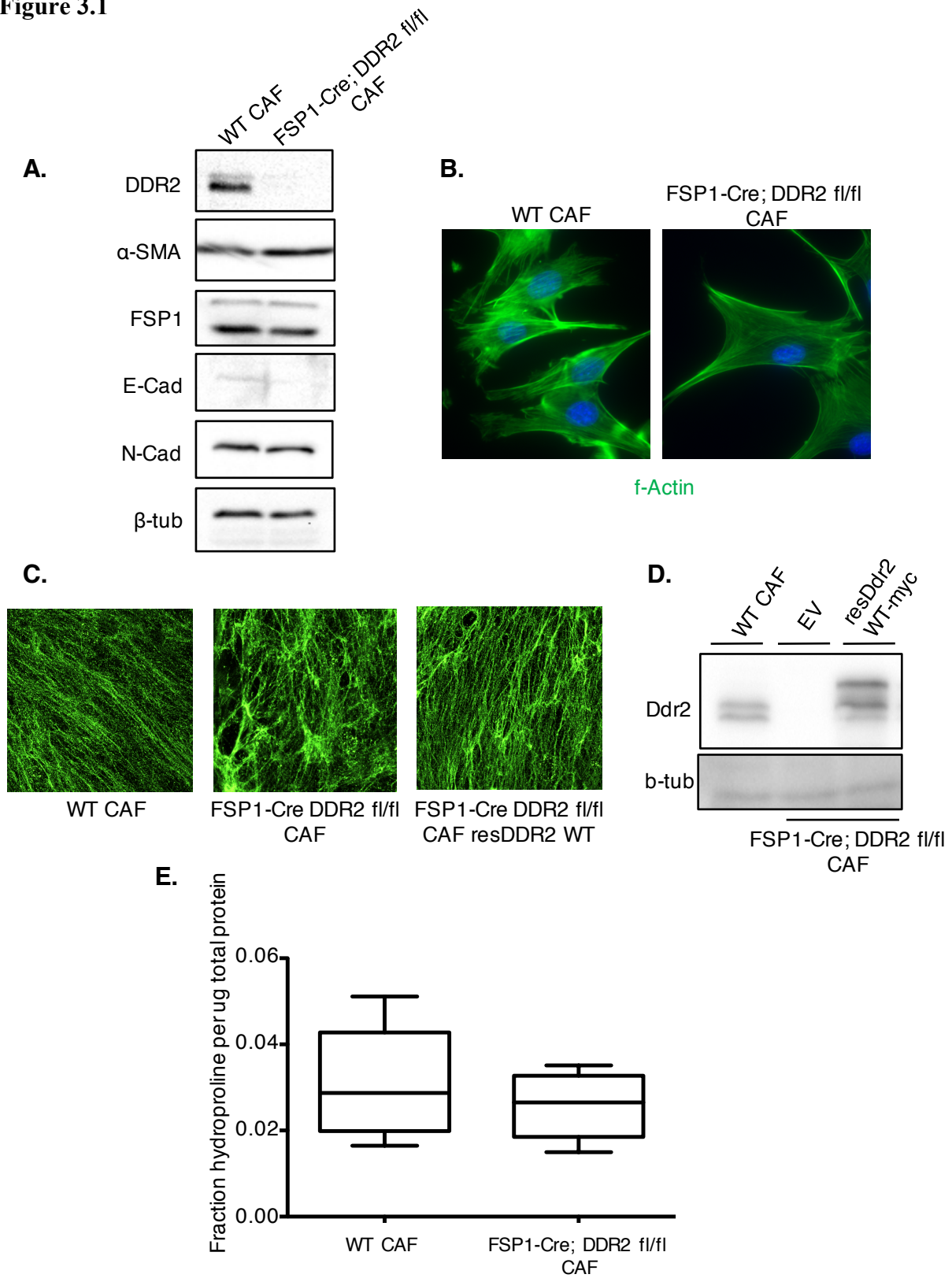


Figure 3.1: CAF DDR2 is required for collagen alignment but not collagen incorporation into ECM.

(A) Western blots of WT and FSP1-Cre; DDR2 fl/fl CAFs showing expression of mesenchymal and CAF markers. (B) Representative images of WT and FSP1-Cre; DDR2 fl/fl CAFs stained with Phalloidin-568 which has been pseudocolored green. (C) *ex vivo* produced ECMs from WT, FSP1-Cre; DDR2 fl/fl, or FSP1-Cre; DDR2 fl/fl resDDR2-WT CAFs. Cells are extracted and resultant cell-free ECMs stained for collagen 1. (D) Western blot showing relative Ddr2 expression in WT, FSP1-Cre; DDR2 fl/fl, and FSP1-Cre; DDR2 fl/f; resDDR2-WT CAFs. (E) Hydroxyproline quantification of *ex vivo* produced cell-free ECMs. n = 6-8 per group.

Figure 3.2

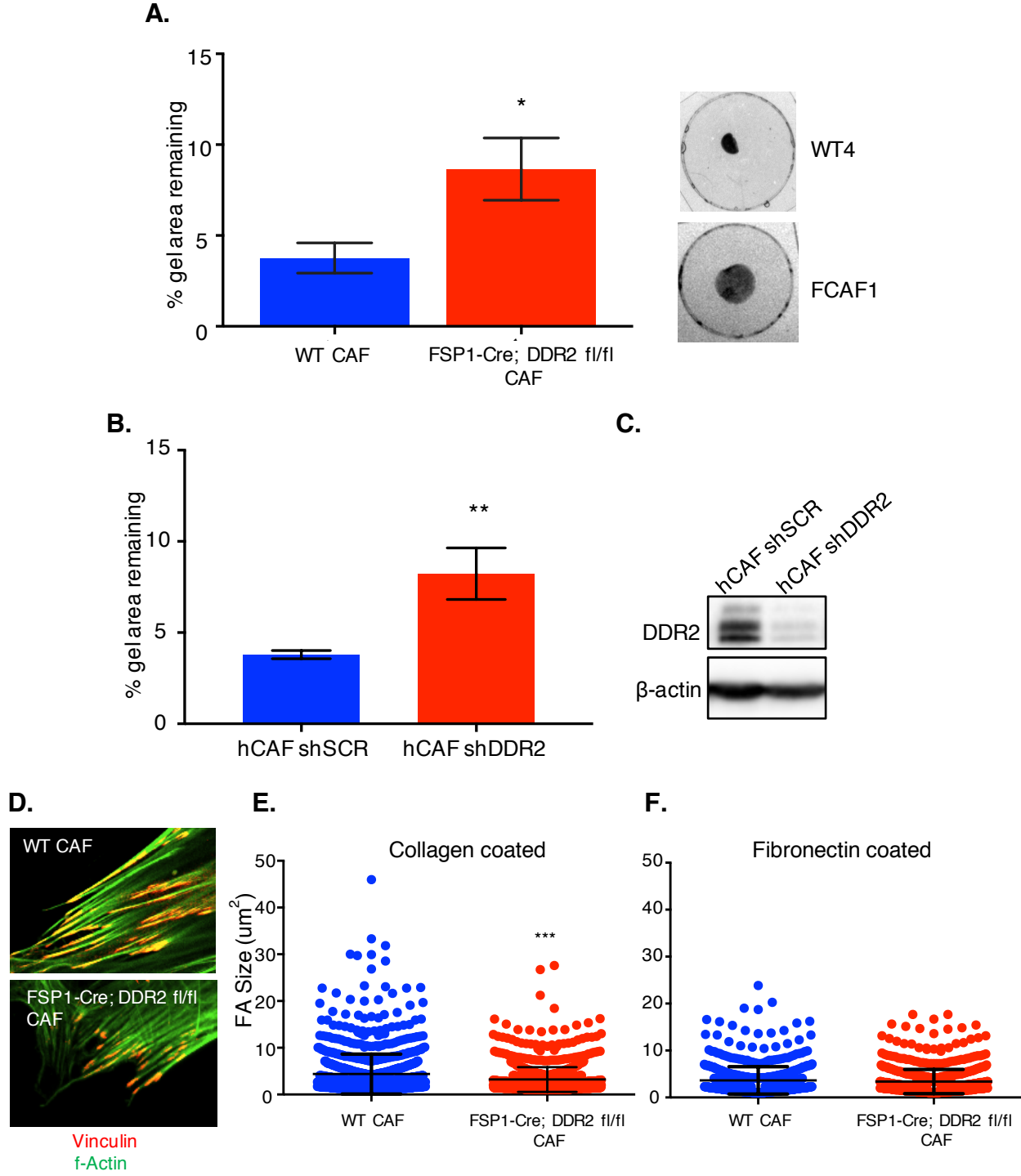


Figure 3.2: CAF DDR2 affects cellular mechanotransduction/mechanosignaling.

(A) Collagen gel contraction of WT and FSP1-Cre; DDR2 fl/fl CAFs. Data are presented as percent gel remaining. n = 6 per group. * p = 0.28 (B) Collagen gel contraction of human CAF shSCR and shDDR2. Data are presented as percent gel remaining. n = 3 per group. ** p = 0.006 (C) Western blot showing relative knockdown of DDR2 in hCAF shSCR and shDDR2 cells. (D) Representative confocal image of focal adhesions in WT and FSP1-Cre; DDR2 fl/fl CAFs. (E) Quantification of focal adhesion size on collagen coated coverslips. *** p < 0.0001 (F) Quantification of focal adhesion size on fibronectin coated coverslips.

Figure 3.3

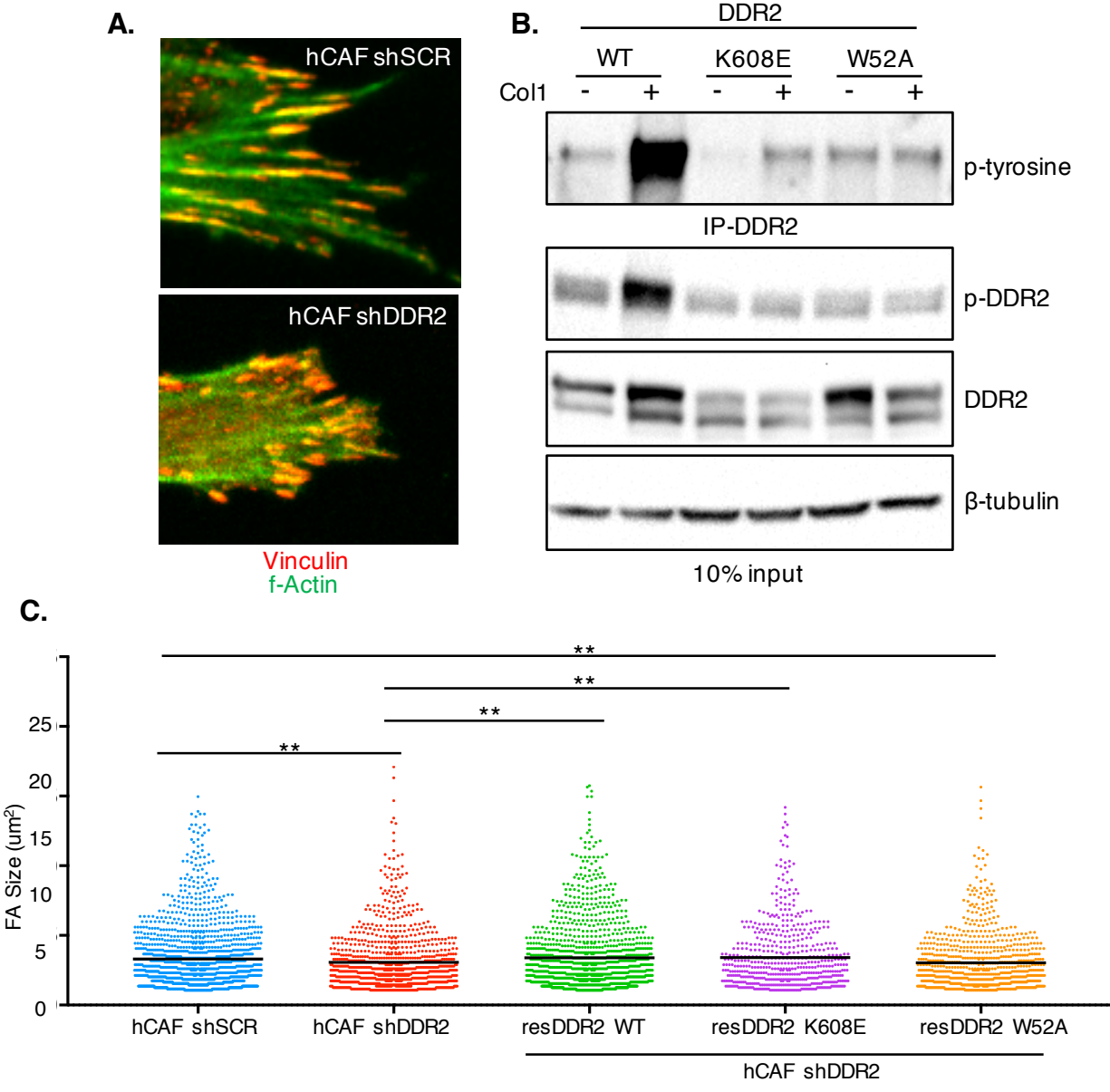


Figure 3.3: DDR2 collagen binding but not kinase activity is required for mechanotransduction or mechanosignaling.

(A) Representative confocal images of focal adhesions in hCAF shSCR and hCAF shDDR2 cells. (B) IP-pTyr and Western blot showing DDR2 phosphorylation status after collagen stimulation for YFP-tagged DDR2 WT, DDR2 K608E, and DDR2 W52A rescue constructs. (C) Quantification of focal adhesion size on collagen coated coverslips for hCAF shSCR, hCAF shDDR2, and shDDR2 plus DDR2-WT, DDR2-K608E, or DDR2-W52A. ** $p < 0.01$ for each.

Figure 3.4

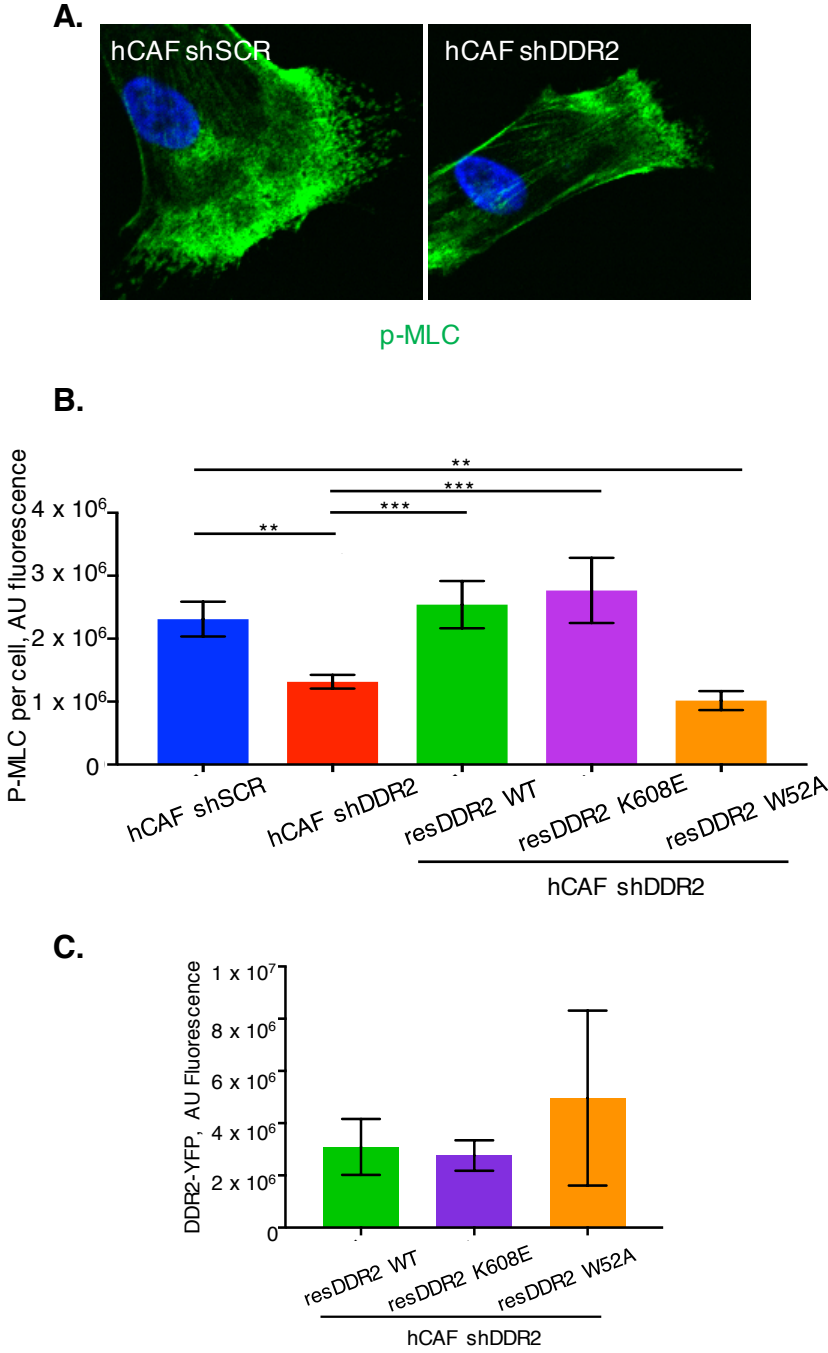


Figure 3.4: DDR2 collagen binding but not kinase activity is required for increased intracellular tension.

(A) Representative confocal images of hCAF shSCR or hCAF shDDR2 cells stained for p-MLC.

(B) Quantification of p-MLC levels in hCAF shSCR, hCAF shDDR2 or shDDR2 plus DDR2-

WT, DDR2-K608E, or DDR2-W52A. ** $p < 0.01$ for each. (C) Quantification of DDR2-YFP

expression levels in DDR2-WT, DDR2-K608E, and DDR2-W52A mutant rescue cells analyzed.

Figure 3.5

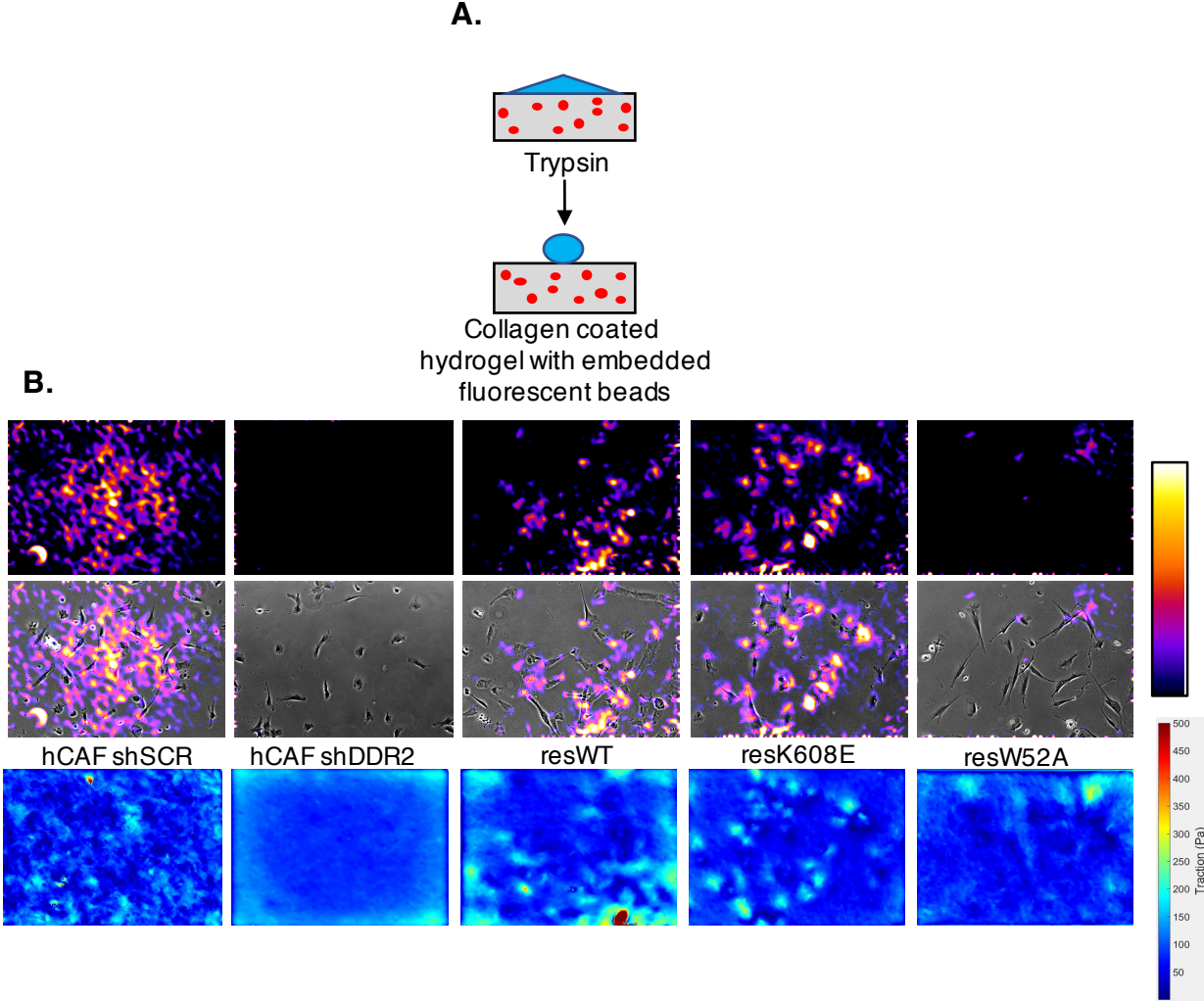


Figure 3.5: hCAF traction forces require collagen binding but not kinase activity of DDR2.

(A) Schematic diagram of traction force protocol. (B) Top row- heat map of bead displacement field; middle row- heat map overlaid with bright field image of cells; bottom row- field of calculated forces.

3.6 References

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Chapter 4: DDR2 action promotes recruitment of Talin1 and integrin β 1 activation.

4.1 Introduction

Transmitting mechanical information from the extracellular environment to intracellular signaling is essential for cell survival, migration, and differentiation (Hytonen and Wehrle-Haller, 2016). Critical to these events are cell adhesion receptors such as integrins and intracellular structural proteins and signaling partners (Humphrey et al., 2014). Fibroblasts, in particular, are especially tuned to respond to changes in extracellular signals like tension and can remodel matrix by pulling on tissues, such as in wound healing or cancer (Kalluri, 2016). Changes in extracellular matrix architecture through remodeling can cause increased stiffness of tissues (Schedin and Keely, 2011), a phenomenon that has been shown to enhance cellular invasion through a process known as durotaxis (Lo et al., 2000; Pelham and Wang, 1997) and correlates with breast tumor progression and aggressive breast cancer subtypes (Acerbi et al., 2015; Butcher et al., 2009). Increased substrate stiffness causes a reciprocal increase in intracellular tension in cells. This tension is transmitted from the actin cytoskeleton to adhesions through actomyosin contractility and focal adhesion complexes (Liu et al., 2015). Focal adhesions grow larger and stronger under mechanical load, thereby enabling a cell to pull harder on its substrate (Riveline et al., 2001).

Collagen receptor crosstalk is likely to play a role in regulating cell behavior in response to extracellular signals, but little work has gone into understanding the crosstalk between the

discoidin domain receptors and integrins. Discoidin domain receptors and integrins bind distinct collagen motifs (Leitinger et al., 2004; Xu et al., 2012), and it is known that DDR1 and DDR2 activation can occur independent of integrins (Vogel et al., 2000; Zhang et al., 2013). The reverse has not been fully explored, though it has been shown that DDR2 binding to DDR2 specific ligand positively influences cell adhesion by enhancing the activity of integrins $\alpha1\beta1$ and $\alpha2\beta1$. This increase in integrin activity was not due to a change in the amount of integrin $\beta1$ expressed on the cell surface, indicating that DDR2 activity is sufficient to induce a conformational change in integrins (Xu et al., 2012). The mechanism for how DDR2 activity promotes integrin activation was not defined.

Integrins can be activated bidirectionally, either through contacts with ECM ligands (outside-in) or through intracellular signaling events that ultimately promote talin1 recruitment to integrin $\beta1$ cytoplasmic tails and subsequent integrin conformational change and activation (inside-out) (Kim et al., 2011). This increase in integrin affinity for ligand is complemented by increases in avidity. As integrins are activated at adhesions, more are recruited and activated via talin1 and kindlin proteins, serving to strengthen and grow the focal adhesion complex (Kahner et al., 2012). The pathways upstream of talin1 recruitment have not been fully elucidated though much work has been accomplished. Understanding these critical signaling mechanisms for mechanotransmission will be important in determining how tumor stroma is remodeled and how cancer cells metastasize.

In this study, we sought to understand the mechanism by which DDR2 promoted larger focal adhesions, increased intracellular tension, and greater cellular contractility in breast CAFs (see; chapter 3). We show that differences in focal adhesion size exist at early timepoints after plating, suggesting a defect in mechanotransduction or mechanosignaling in the absence of

DDR2. We show that integrin $\beta 1$ activation is reduced without affecting the amount of total integrin $\beta 1$ in cell protrusions and, further, show that DDR2 is required for talin1 recruitment and integrin $\beta 1$ activation. This effect appears to be downstream of increases in Rap1-GTP levels in CAFs in response to DDR2 action.

4.2 Results

DDR2 action affects early mechanotransmission.

As cells spread out and migrate, they form small nascent focal adhesions at the leading edges of lamellipodia. Cell surface adhesion molecules, ie- integrins, make contact with substrate and are activated either by inside out or outside in signaling (Riveline et al., 2001). These signaling events occur within seconds to minutes, and, since earlier studies were done on cells plated overnight, we wanted to determine whether DDR2 in CAFs affected early mechanotransmission events as well. WT or shDDR2 depleted human CAFs were plated on collagen coated coverslips for 30 or 60 min. Cells were then fixed, stained for vinculin, and imaged by confocal microscopy. Results indicate that DDR2 is required at early timepoints to increase focal adhesion size (Fig. 4.1a). DDR2 depleted CAFs have both a delay in increasing focal adhesion size and a lower maximum size, as evidenced by data in Figure 4.1a and presented in chapter 3. The focal adhesions of DDR2 depleted cells do not increase in size under mechanical load in the same manner as WT DDR2 CAFs. These results indicate that DDR2 is required for cells to adequately respond to extracellular mechanical signals.

DDR2 does not co-localize with or affect the expression of integrin β 1 in cell protrusions.

Cellular force generation and mechanotransduction on collagen coated coverslips is largely dependent on the collagen binding integrins (Xu et al., 2012). There are four collagen binding integrins in mammals, all of which have the β 1 subunit in common. Further, focal adhesion formation and maturation depend on integrin β 1 expression and activity. DDR2 has not been shown to be a strong adhesive molecule, though cells will adhere but not spread on DDR2 specific peptide (Xu et al., 2012). Because we observed a defect in focal adhesion size on collagen and because DDR2 has been shown by other groups to promote integrin β 1 activation, we wanted to test whether integrin β 1 levels were affected in shDDR2 depleted CAFs.

To this end, we sparsely plated shSCR, shDDR2 CAFs on collagen coated coverslips for 15 min. Cells were then fixed and stained for total integrin β 1 and imaged by n-SIM super-resolution microscopy. We chose super-resolution microscopy in order to get a more detailed, higher resolution image of focal adhesion proteins. Quantification of total fluorescence of integrin β 1 was restricted to regions of interest in cell protrusions and normalized to area. Importantly, results indicate that there is no difference in the amount of integrin β 1 in cell protrusions (Fig. 4.2b), a result that is in agreement with published reports (Xu et al., 2012).

Because integrin β 1 activity is influenced by its binding partners, we wanted to determine if DDR2 was interacting with integrin β 1. In a similar manner to above, we plated hCAF shDDR2 cells expressing rescue DDR2 WT-YFP cDNA on collagen coated plates for 15 min. Cells were then fixed, stained for integrin β 1 and talin1, and imaged by n-SIM super-resolution microscopy. Co-localization was determined using overlaid images and Pearson's coefficient calculation; the well-established talin1 – integrin β 1 interaction was used as a positive control.

As can be seen in Figure 4.2a, talin1 and integrin β 1 co-localize in cell protrusions (Pearson's coefficient = 0.37) while DDR2-YFP and integrin β 1 do not (Pearson's coefficient = 0.19). Importantly, DDR2-YFP also does not co-localize with talin1 (Pearson's coefficient = 0.17). These data indicate that differences in transmission of mechanical signals to focal adhesions in CAFs is not due to dysregulation of integrin β 1 expression or protrusion localization.

DDR2 action promotes integrin β 1 activation and talin1 recruitment.

Integrins are activated in a series of conformational changes in both the extracellular and transmembrane domains from a bent, closed, inactive, low affinity state to an extended but closed inactive state to an extended, open, active, and high affinity state (Kim et al., 2011). These conformation changes are generally achieved through application of force at the distal or proximal ends of the protein. Talin1 binding to integrin β 1 cytoplasmic tails activates and links integrin β 1 to the actin cytoskeleton, creating a mechanical bridge whereby actomyosin contractility can act upon integrin adhesions (Klapholz and Brown, 2017).

To determine if DDR2 affects integrin β 1 activation or talin1 recruitment, we plated hCAF shSCR or shDDR2 on collagen coated coverslips for 15min. The cells were then fixed and stained with antibody against the extended conformation of integrin β 1 (9EG7 clone), indicating partial or full activation, and talin1. Samples were imaged by n-SIM super-resolution microscopy and amount of activated integrin β 1 or talin1 quantified. Fluorescence intensity was measured in equivalent cell protrusions and normalized to area. Results indicate that DDR2 action is required in CAFs for the activation of integrin β 1 (Fig. 4.3a, top, quantified in Fig. 4.3b). Remarkably, talin1 recruitment to cell protrusions was dramatically inhibited in DDR2 depleted CAFs (Fig.

4.3a, bottom, quantified in Fig. 4.3c). Extracellular signal in the talin1 channel is autofluorescence from local accumulations in the collagen coating.

Since talin1 binding to integrin β 1 cytoplasmic tails is a key final step in the full activation of integrins, we also assessed whether there was a difference in the co-localization of talin1 and activated integrin β 1 by super-resolution imaging. As can be seen in Figure 4.4, talin1 association with active integrin β 1 was dramatically reduced in the absence of DDR2 (quantified in Fig. 4.4b). To confirm this phenotype, we plated hCAF shSCR or hCAF shDDR2 cells on collagen coated plates, lysed the cells, immunoprecipitated talin1, and blotted for integrin β 1. Results demonstrate that upon collagen stimulation, a sharp rise in talin1: integrin β 1 association is found in hCAF shSCR but not in hCAF shDDR2 cells (Fig. 4.4c). This is in spite of the fact that talin1 IP was equivalent between groups, as was total talin1 and integrin β 1 expression. These data indicate that while DDR2 action has no effect on talin1 or integrin β 1 protein levels, it has a dramatic effect on the ability of talin1 to interact with integrin β 1. In sum, this result identifies a novel pathway by which DDR2, likely in a kinase independent manner, stimulates integrin β 1 activity by leading to the recruitment of talin1 to adhesions.

DDR2 action increases Rap1-GTP in CAFs.

Talin1 is recruited to the membrane and therefore to integrin β 1 cytoplasmic tails by association with RIAM. RIAM is activated by bound Rap1-GTP, which is upregulated in response to many factors, including local PIP₂ and G α 13 levels (Lee et al., 2009; Yang et al., 2014). Because we saw difference in talin1 recruitment and subsequent loss of integrin β 1 activation in shDDR2 depleted CAFs, we asked whether there was a change in Rap1-GTP levels.

Cells were serum starved and plated on collagen coated plates for 15min. Cells were lysed and Rap1-GTP immunoprecipitated with Ral-GDS-RBD agarose beads (EMD Millipore). Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF, and blotted for Rap1. Results indicate that Rap1-GTP levels increase 1.5 fold in WT CAFs when stimulated by collagen for 15min, but not in shDDR2 depleted CAFs (Fig. 4.6a). Calculations are normalized to both β -tubulin and total Rap1 amounts in each sample. These results indicate that DDR2 action facilitates Rap1-GTP activity in CAFs and suggests a mechanism by which DDR2 action promotes talin1 recruitment through increased Rap1-GTP activity.

4.3 Discussion

We have shown that DDR2 action in CAFs promotes the activation of integrin β 1 without affecting the total amount of integrin β 1 localized to cell protrusions or in total cell lysate. We have further demonstrated that DDR2 facilitates talin1 recruitment and binding to integrin β 1 cytoplasmic tails, likely downstream of increased Rap1-GTP levels. Importantly, DDR2 does not interact with integrin β 1 or talin1, suggesting that regulation of this interaction is downstream of a signaling event rather than mislocalization or other sequestration problem. Because we performed these experiments at time points much shorter than DDR2 activation times, it is likely that DDR2 activates Rap1-GTP in a kinase independent manner, though we do not yet know with certainty. Future studies will utilize DDR2 mutants to parse out the relative contribution of collagen binding and kinase activity in this setting. It is also possible that DDR2 clustering is required for signal propagation. Other groups have shown that DDR1 mediates collagen contraction in a manner that is dependent on clustering and interaction with non-muscle myosin IIA (NMAII), however the authors state that DDR1 kinase activity is required for their

phenotype (Coelho et al., 2017). It is possible that DDR2 acts in a similar, if kinase independent, manner, however we do not yet know if DDR2 interacts with NMAII.

Integrin activation is bidirectional, and we have identified a novel mechanism by which DDR2 action regulates integrin $\beta 1$ in an inside – out manner. This result is important because it suggests that the phenotypes we see *in vivo* may be due to reduced integrin $\beta 1$ activity. Integrin activity in cancers have been associated with cell survival, proliferation, and invasion (Keely et al., 1998; Levental et al., 2009). Targeting integrin activity as a therapy is not feasible, but if there were a way to target integrins indirectly, ie- through inhibition of DDR2, we would expect to see not only increased specificity, but decreased toxicity. Further, Rap1-GTP acts on RIAM which recruits and activates talin1 (Lee et al., 2009; Yang et al., 2014). We do not yet know what the effect is of DDR2 on RIAM activity, nor do we know the pathway between DDR2 and Rap1-GTP activity. Future work could focus on identifying more pieces of this pathway. Initial ideas would be to test whether DDR2 to Rap1-GTP is a new, independent pathway or if it feeds into already described pathways including PIP₂, PKC, and G α 13 (Martel et al., 2001; Schiemer et al., 2016).

In summary, we have identified DDR2 as a novel mechanotransducing/mechanosignaling collagen binding RTK. While more work remains to tease out the full mechanism, it is clear that DDR2 action promotes talin1 recruitment and subsequent integrin $\beta 1$ activation in CAFs, likely in a kinase independent manner.

4.4 Materials and Methods

Immunofluorescence and microscopy

For collagen coating, 50 ug/mL collagen in water was spread on 12mm glass coverslips (no. 1.5, high precision) and allowed to dry at room temperature overnight. The next day, the coverslips were blocked with 1% BSA in PBS for 1 hour at room temperature and sterilized under UV for 20 min. Cells were serum starved and removed from tissue culture plates non-enzymatically. They were then plated sparsely (1×10^4 per coverslip) and allowed to adhere and spread for indicated times. Cells were then fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized in 0.1% Triton X-100 in PBS for 5 minutes at room temperature, washed with PBS, and blocked with 5% normal goat serum in PBS. Primary antibodies were added and incubated at 4 degrees Celsius overnight. Coverslips were washed and secondary fluorescent antibody added for 1 hour at room temperature. Coverslips were washed again and mounted in Prolong Diamond mounting medium. Coverslips were allowed to cure for 24 hours. For focal adhesion quantification, cells were imaged by confocal microscopy on NIS-Elements software (Nikon A1Rsi, inverted). Z-stacks were taken with a step size of 0.2 um with a 40X objective. Z-stacks were flattened by maximum intensity projection, and focal adhesions were quantified in ImageJ by subtracting the background, thresholding to the same level for all samples, and running particle analysis. For n-SIM super-resolution microscopy, images were taken with NIS-Elements software on a Nikon Ti-E microscope with a high NA 100X objective. Fluorescence was captured with an Andor Zyla 4.2 Megapixel sCMOS camera. Z-stacks were taken for all images with a step size of 0.15um. n-SIM images were reconstructed in NIS-Elements, and fluorescence intensity co-localization quantification was done in ImageJ.

Co-Immunoprecipitation

Tissue culture dishes were coated with 50 ug/mL collagen in water and allowed to dry overnight at room temperature. The next day, dishes were blocked with 1% BSA for 1 hour at room temperature and then sterilized under UV light for 20min. Cells were serum starved overnight and then removed from plates non-enzymatically. Cells were allowed to adhere for 1 hour and then lysed in 20mM Tris, pH 7.5, 1% Triton X-100, 0.1% SDS, 150 mM CaCl₂ supplemented with 1mM PMSF, 1mM sodium vanadate, 1mM sodium fluoride, and 10 ug/ml each aprotinin and leupeptin. Equal amounts of protein were pre-cleared with Protein G sepharose beads and then incubated with talin1 antibody overnight with gentle agitation. Protein G sepharose beads were added for 1 hour at 4 degrees, then beads were washed four times with CoIP buffer, resuspended in 2X Laemmli sample buffer, boiled, and separated by SDS-PAGE.

Rap1-GTP assay

For Rap1-GTP immunoprecipitation, we followed the manufacturer's instructions (EMD Millipore 17-321). Briefly, cells were serum starved overnight and then plated on collagen coated tissue culture dishes for 15 min. They were lysed in the supplied lysis buffer supplement with 1mM PMSF, 1mM sodium vanadate, 1mM sodium fluoride, and 10 ug/ml each aprotinin and leupeptin. Samples were sheared with passages through 27G needle, spun, and supernatants incubated with Ral-GDS-RBD agarose beads at 4 degrees for 45 minutes. Beads were washed and resuspended in 2X Laemmli buffer, boiled, and separated by SDS-PAGE.

Statistical Analysis

All p-values were calculated using Student's unpaired, two-tailed T-Tests. p-values are noted in figure legends.

4.5 Figures

Figure 4.1

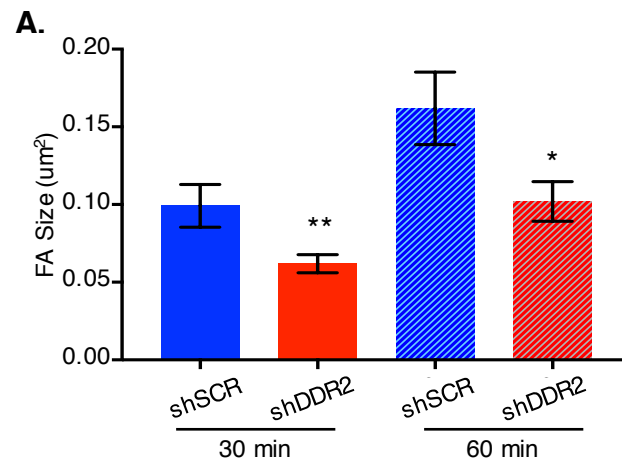


Figure 4.1: DDR2 affects mechanotransduction/mechanosignaling at early timepoints.

(A) Quantification of focal adhesion size at 30 and 60 min for hCAF shSCR and hCAF shDDR2.

** $p = 0.003$ and * $p = 0.015$.

Figure 4.2

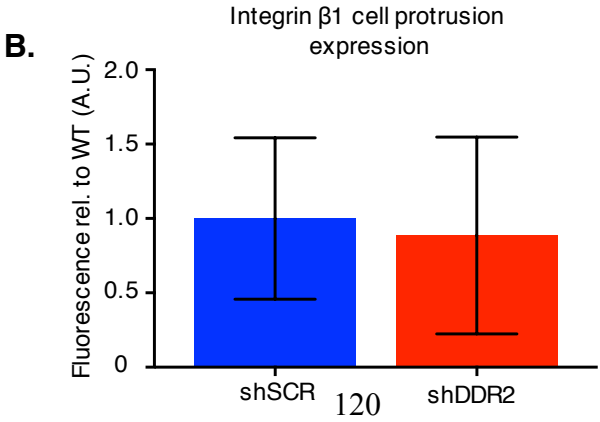
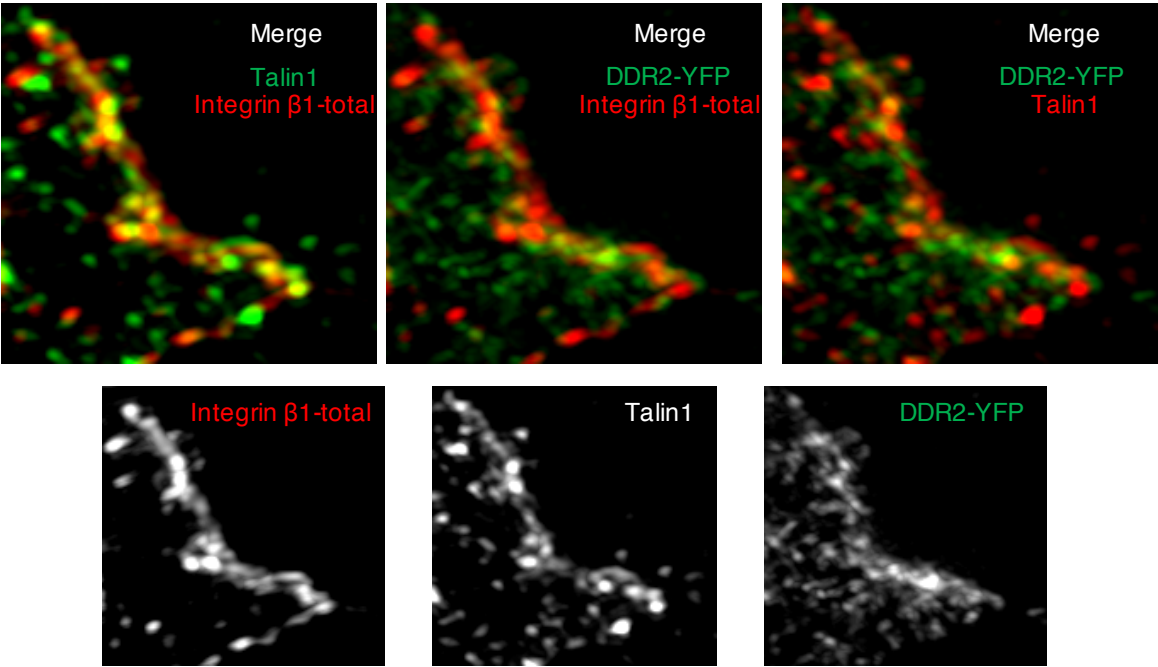
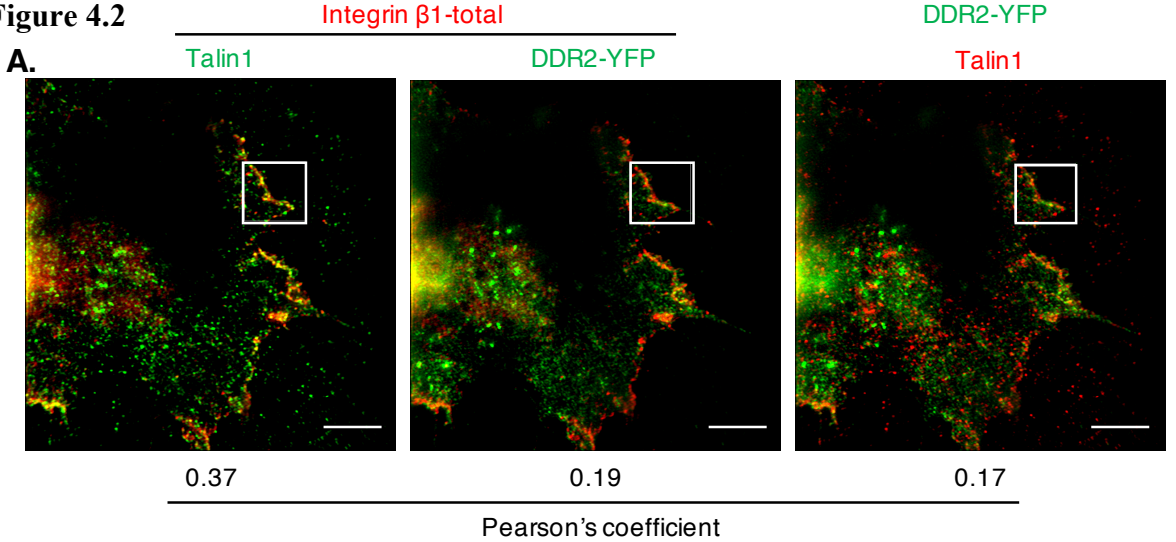


Figure 4.2: DDR2 does not interact with or affect expression of integrin β 1.

(A) Top; Representative super-resolution images of DDR2-YFP, integrin β 1, and talin1. Scale bar = 5 μ m. Middle; Zoomed in view of white box in top row. Bottom; Gray scale super-resolution image of individual protein expression. Co-localization quantified by Pearson's coefficient. (B) Quantification of integrin β 1 fluorescence in cell protrusions of hCAF shSCR and hCAF shDDR2, normalized by area.

Figure 4.3

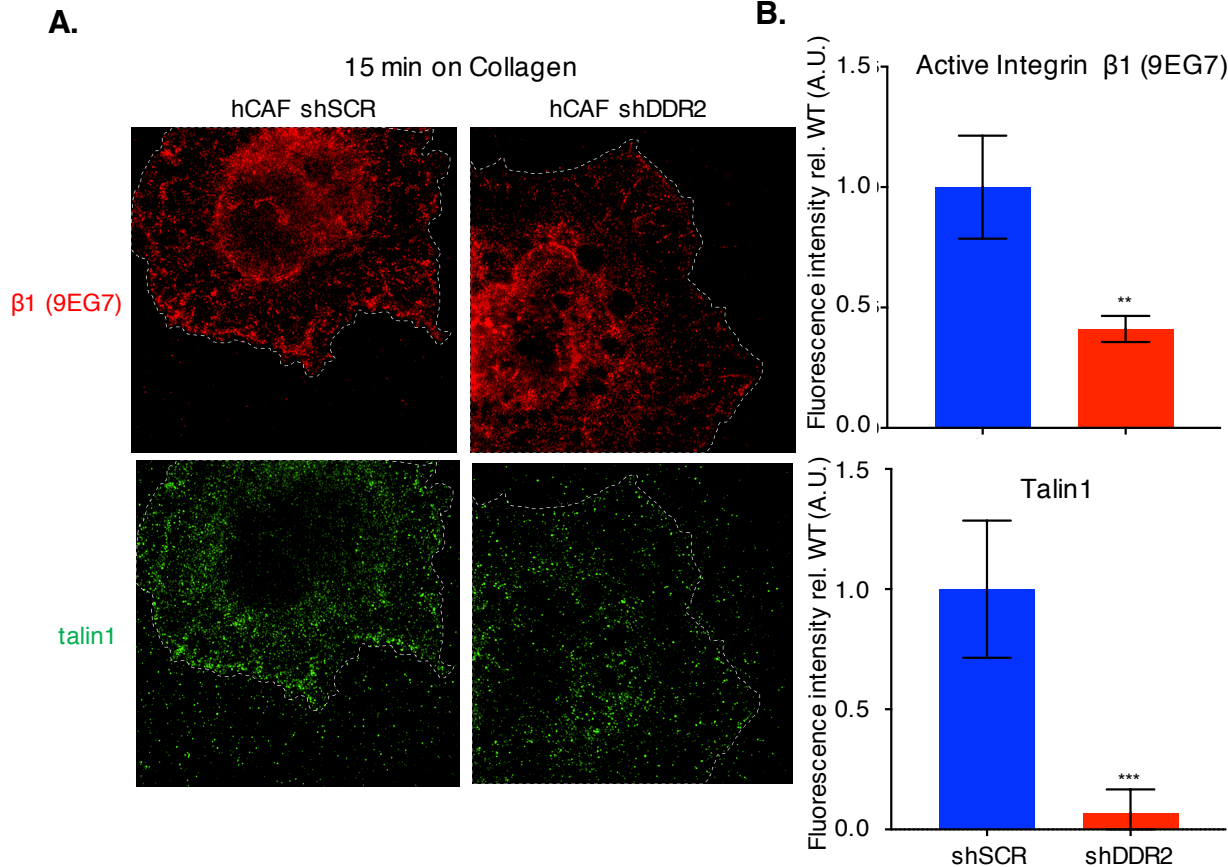


Figure 4.3: DDR2 promotes integrin β 1 activation and talin1 recruitment.

(A) Representative super-resolution images of hCAF shSCR or hCAF shDDR2 showing integrin β 1 (9EG7) (top) or talin1 expression (bottom) when plated for 15min on collagen coated coverslips. Cell boundary outlined in white dotted line. (B) Quantification of integrin β 1 (9EG7) or talin1 cell protrusion expression. ** $p = 0.009$, *** $p = 0.0003$.

Figure 4.4

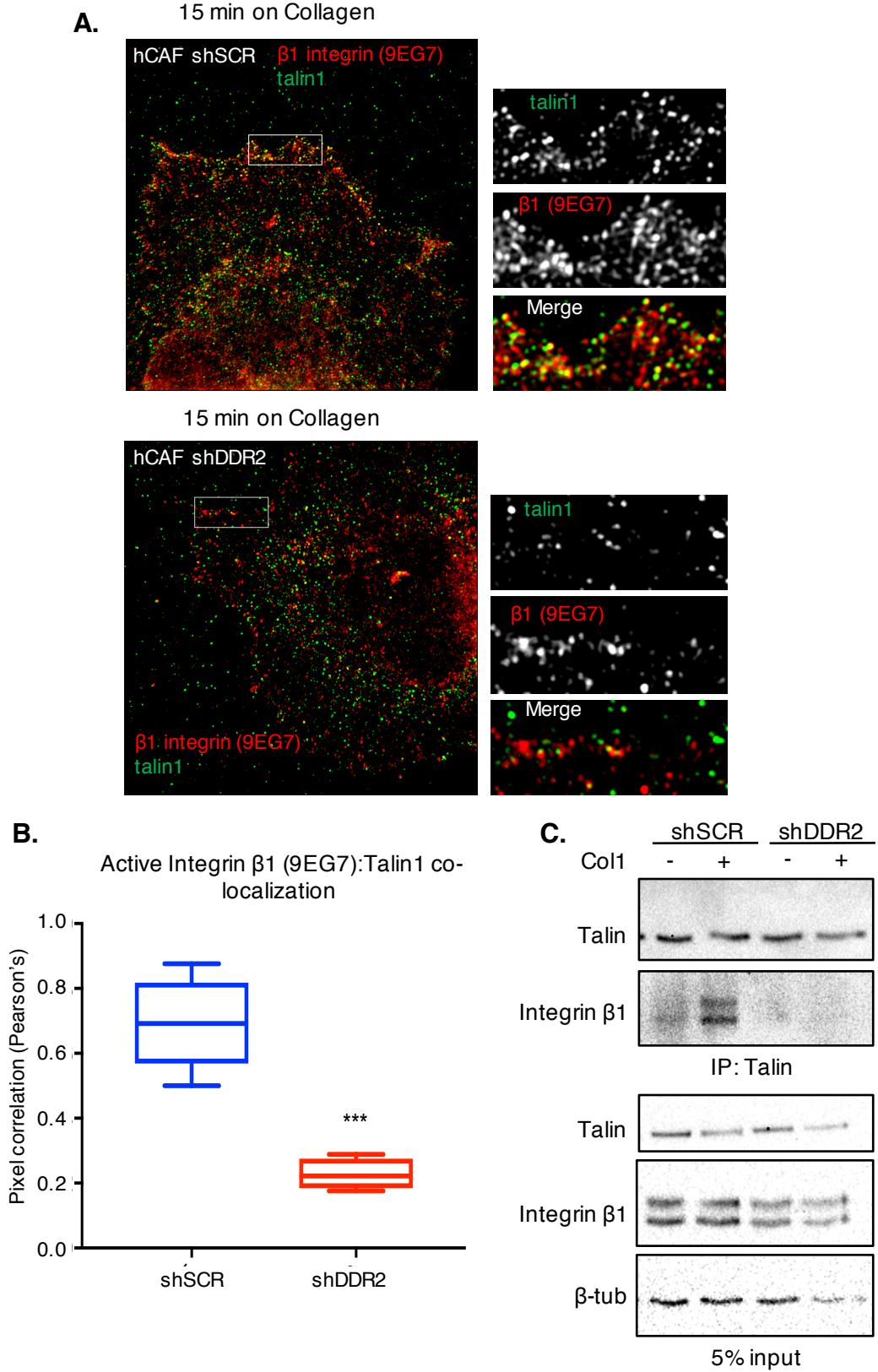


Figure 4.4: DDR2 affects talin1 interaction with integrin β 1.

(A) Representative super-resolution images of hCAF shSCR and shDDR2 showing integrin β 1 (9EG7) and talin1 co-localization. Inset- close up of boxed region. (B) Quantification of co-localization by Pearson's coefficient. *** $p = 0.0004$. (C) Coimmunoprecipitation of talin1 and integrin β 1 and input of hCAF shSCR and shDDR2 cells plated on collagen coated plates or control for 1 hour.

Figure 4.5

A.

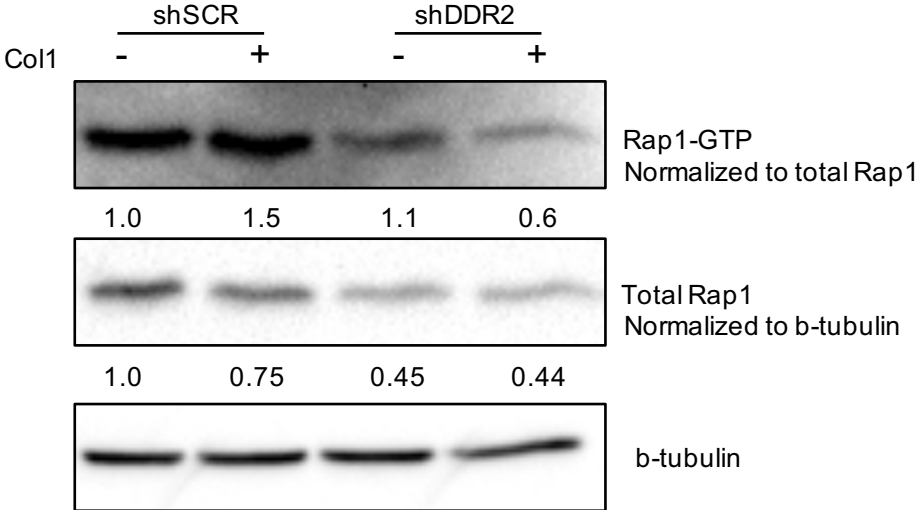


Figure 4.5: DDR2 action increases Rap1-GTP levels in response to collagen.

(A) Representative Western blots showing Rap1-GTP immunoprecipitation and input controls for hCAF shSCR and shDDR2 plated on collagen or control for 15 min. Quantified by densitometry in ImageJ.

4.6 References

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Tables

Table 1: Primer sequences for DDR2 mouse genotyping.

Name	Sequence (5' – 3')
FRT 5' Fwd	CTGTGTCTCTGGCTCAAAGTGTC
Targeted exon Rv	CCTTCCCAAGGCAGACCATTC
PyMT Fwd	GGAAGCAAGTACTTCACAAGGG
PyMT Rv	GGAAAGTCACTAGGAGCAGGG
Cre Fwd	GCATTACCGGTTCGATGCAACGAGTGATGAG
Cre Rv	GAGTGAACGAACCTGGTCGAAATCAGTGCG
ROSA-LSL-TdTomato Fwd	GAGGGCCGCCACCACCTGTTCTGTACGG
ROSA-LSL-TdTomato Rv	ATGATACAAAGGCATTAAAGCAGCGTATCC
ROSA-WT Fwd	GGGGAGTGTTGCAATACCTTTCTGGGAGTTC
ROSA-WT Rv	AAAACCGAAAATCTGTGGGAAGTCTTGTC

Table 2: Antibodies used for Western blot, IF, and IP

Antibody	Source	Application	Concentration
A-SMA (1A4)	Sigma A2547	WB	1:10,000
β -actin	Sigma A5441	WB	1:10,000
β -tubulin	Sigma T4026	WB	1:2000
CD31	Abcam ab28364	IF	1:100
CD45	BD Biosciences 550539	IF	1:100
Collagen 1a1	EMD Millipore AB765P	IF	1:200
DDR2	CST 12133	WB	1:1000
FAP	EMD Millipore ABT11	IF	1:100
Integrin β 1	BD Biosciences 552828	IF	1:50
Integrin β 1	CST 4706	WB	1:500
Integrin β 1 (9EG7)	BD Biosciences 553715	IF	1:50
K14	Covance PRB-155P	IF	1:100
K8	DSHB TROMA-1	IF	1:100
p-MLC (Ser19)	CST 3671	IF	1:50
PDGFR α -FITC	eBioscience 11-1401-80	FACS	1:50
Rap1	EMD Millipore 07-916	WB	1:500
Talin1 (8D4)	Sigma T3287	IF, WB	1:250, 1:2000
Vinculin	Sigma V9131	IF	1:250

Chapter 5: Conclusions and Future Directions

In summary, we have identified a novel mechanotransducing/mechanosensing pathway whereby DDR2 action activates integrin $\beta 1$ downstream of Rap1-GTP driven recruitment of talin1 to adhesions. This promotion of integrin $\beta 1$ activity is correlated to increased cellular contractility and traction force generation in cancer associated fibroblasts (CAFs), which may explain, in part, how collagen is remodeled in tumor stroma to generate aligned fibers and an increasing stiffness gradient which promotes local invasion and metastasis.

Stromal roles of DDR2 in the primary tumor site

Initial insight into the role of DDR2 in the breast tumor stroma came from previous studies in the lab that demonstrated an alteration in the collagen fiber architecture in DDR2 global null mice. Wild type mice had a predominantly TACS-2/3, or aggressive, phenotype while DDR2 global null mice had a predominantly TACS-1, or more benign, phenotype (Corsa et al., 2016). That study went on to show, in a reciprocal transplant, that DDR2 in the host was required for metastasis even when transplanted with wild type tumor. That experiment was not selective for any particular part of the host, but, in combination with the data about stromal architecture alterations, we hypothesized that the most likely cell type that was contributing was cancer associated fibroblasts (CAFs). In this study, we used the stromal specific FSP1-Cre to selectively target CAFs as best we could and demonstrated that stromal DDR2 is required for lung metastasis. We also show that DDR2 action in the stroma promotes matrix remodeling and the generation of a stiffness gradient. However, because FSP1-Cre also targets a population of

hematopoietic cells, most likely macrophages, we cannot conclusively state that the results are due only to DDR2 action in CAFs. Future work will require a co-transplant experiment in which DDR2 +/- CAFs are transplanted with syngeneic wild type or DDR2 null tumor cells into wild type or DDR2 null hosts. Expected results would be that no matter the combination, DDR2 null CAFs would reduce metastasis while DDR2 wildtype CAFs may actually increase metastasis of otherwise previously non-metastatic states, ie- DDR2 null tumor cells in a global null or wild type host. This change in metastasis would be correlated to changes in the tumor associated matrix, including increased collagen fiber alignment and stiffness.

In this study, we propose that DDR2 is a novel mechanosensor or mechanotransducer in CAFs and that this activity leads to matrix remodeling downstream of increased integrin $\beta 1$ activity and intracellular traction force generation. One way CAFs can alter matrix is by pulling on fibers, and it has been shown that even small pulling forces can alter collagen alignment at great distances (Sawhney and Howard, 2002). At this time, we cannot directly link cell contractility and focal adhesion size to *in vivo* collagen fiber alterations, however future studies may be conducted in which contractility is increased in DDR2 null CAFs. For example, we see changes in Rap1-GTP levels that are correlated with talin1 recruitment to adhesions and integrin $\beta 1$ activation. Exogenous expression of constitutively active Rap1 (Katagiri et al., 2000) may bypass the requirement for DDR2 and rescue the phenotype. Co-transplant of these CAFs and controls with wild type tumor cells would allow analysis of stromal changes. Further, it would be interesting to see if we could observe remodeling of existing matrix *in vitro*. However, an experiment like that would require development of a system where one could track which fibers are old fibers which have been remodeled and which fibers are newly produced, ie- by immunofluorescent labeling or similar. It is likely that both processes will happen in the same

location, and teasing them apart will be important. In addition to cell contractility and pulling on fibers, matrix remodeling enzymes also likely play a role (Page-McCaw et al., 2007). DDR2 has been shown in other studies to control expression of MT1-MMP (MMP14) (Majkowska et al., 2017; Zhang et al., 2013). MT1-MMP is a transmembrane protease which activates the pro-enzyme MMP2 (Cathcart et al., 2015). The contribution of MMP expression or activity in stromal remodeling has not been tested for DDR2 in CAFs. It will be important to establish whether DDR2 in CAFs is required for matrix degradation as well.

In previous studies of DDR2 global null mice, a decrease in total fibrosis was observed in end stage tumors (Corsa et al., 2016). In this study, despite targeting CAFs, we did not see a difference in fibrosis or total collagen content (Fig. 2.4a, b and Fig. 3.1c). One explanation for this is that CAFs may be induced to increase synthesis of ECM proteins by paracrine factors from tumor or immune cells and that this induction is dependent upon DDR2 expression in those cells. In the case of FSP1-Cre, tumor cells and most immune cells are wild type and, therefore, have normal (if aberrant) DDR2 expression. Analysis of changes in cytokines and paracrine factors from tumor cells and/or immune cells could lend insight into this hypothesis. In this context, it would suggest that DDR2 on CAFs is not necessary for fibrosis in breast cancer.

In addition to cells and matrix proteins, the tumor microenvironment is under the influence of a number of pro-inflammatory, pro-migratory, and pro-survival paracrine factors. Fibroblasts can be activated to CAFs under the influence of TGF- β and PDGF, among others, from tumor and immune cells (Elenbaas and Weinberg, 2001). CAFs secrete factors such as VEGF, IL-6, and EGF to stimulate angiogenesis and inflammation (Kalluri, 2016). Immune cells can secrete pro-fibrotic TGF- β and MMPs which contribute to the desmoplastic reaction (Elkington et al., 2009). In addition, the secretory profile of CAFs is generally

immunosuppressive, which protects tumor cells from immune surveillance and destruction (Kalluri, 2016). The effect of DDR2 in this context, in any cell type, has not been examined, though some evidence exists that paracrine signaling may be affected. In a mixing experiment, DDR2 wild type and null CAFs were co-plated with DDR2 wild type and null tumor organoids. The results indicated that DDR2 was required in both cell types, independently for 3D organoid invasion (Corsa et al., 2016). The experiment did not test whether this effect was due to a paracrine factor, direct contact between cell types, or downstream of matrix remodeling. It would be interesting in future work to gain an understanding of how DDR2 action may impact angiogenesis and immune infiltration in a paracrine manner from tumor cells, CAFs, or immune cells.

An emerging line of thought in metastasis research involves the idea that stromal cells travel with metastasizing tumor and that this co-travel increases the odds that a disseminated tumor cell or group of cells will metastasize (Duda et al., 2010). In the primary site CAFs have been shown to lead tumor cells away from the tumor core (Gaggioli et al., 2007). A role for DDR2 in stromal traveling with tumor cells has not been explored, though there is evidence in our lab and others that DDR2 regulates tumor cell and fibroblast invasion (Corsa et al., 2016; Marquez and Olasso, 2014; Olasso et al., 2002; Olasso et al., 2011b; Zhang et al., 2013).

DDR2 regulation of integrins

We have shown that DDR2 regulates integrin $\beta 1$ activity on collagen through inside-out signaling at timepoints shorter than the time it takes for full DDR2 activation. We see differences in integrin $\beta 1$ activation levels at 15 min, whereas full DDR2 activation takes several hours. This suggests that DDR2 collagen binding is the stimulus for signaling which leads to integrin $\beta 1$

activation, though we cannot say for certain. Further tests using the DDR2 K608E and DDR2 W52A mutants will shed more light and allow us to confirm that DDR2 kinase activity is not required. Previous studies on focal adhesion size, traction force generation, and intracellular tension using pMLC as a readout indicate that kinase activity will not be required (chapter 3). Further, we plan to use the specific peptide for DDR2 as a ligand in similar assays. It was shown by other groups that cells will adhere to DDR2 specific peptide but will not spread due to lack of ligand for integrin engagement (Xu et al., 2012). If we still see integrin $\beta 1$ activation and talin1 recruitment in this context, it would show that DDR2 collagen binding is upstream of both, giving insight into the larger question of whether integrins bind ligand and are then bound by talin1 or vice versa.

Integrins are activated bidirectionally; outside – in through direct ligand engagement or inside – out downstream of signaling pathways which recruit and activate talin1. Regardless of route, integrins are not fully activated until they are bound by talin1 (Kim et al., 2011). We have shown here that DDR2 regulates integrin $\beta 1$ activity without influencing the amount of integrin $\beta 1$ in protrusions. We have further shown that DDR2 action stimulates Rap1-GTP activity, which is correlated to talin1 recruitment to adhesions. Talin1 can be activated by a few pathways, including downstream of PIP₂ (Martel et al., 2001), RIAM (Yang et al., 2014), G α 13 (Schiemer et al., 2016), or Kank2 (Klapholz and Brown, 2017; Sun et al., 2016). These pathways may all depend upon one another or may only exist in one cell type. For example, in platelets, PIP₂ and G α 13 are required for talin1 activation but RIAM is not (Schiemer et al., 2016; Stritt et al., 2015). We do not yet know in CAFs which of or if these pathways are required. Experiments are under way now to test these questions.

An interesting fact about integrins is that *in vivo*, ligand is constantly available for fibroblasts; collagen is one of the most highly expressed protein in the body. Some argue that in this setting, outside – in signaling trumps inside – out signaling simply due to stochastic availability of high affinity integrins (Klapholz and Brown, 2017). Our work here may suggest that inside – out signaling does play a large role *in vivo* as we see dramatic differences in ECM collagen alignment and stiffness in our system, though we still cannot say for certain that a DDR2 – integrin β 1 activation pathway is the cause for the change. Future work will have to be done to conclusively link CAF integrin β 1 activation and cellular contractility to changes in collagen architecture *in vivo*. Another way that integrins transduce force and strengthen adhesions is through increased avidity or clustering, a phenomenon which requires Kindlin2 (Kahner et al., 2012; Montanez et al., 2008). It is not yet known whether DDR2 action plays a role in this process or if DDR2 affects Kindlin2 expression or localization. Experiments are under way now to test this possibility.

Downstream signaling and regulation of DDR2

It is clear that DDR2 plays many roles in cancer and other diseases so it will continue to be important to discover the signaling pathways downstream of DDR2, both kinase dependent and independent. The Longmore lab has previously shown that DDR2 stabilizes the EMT factor Snail1. DDR2 activation by collagen led to the nuclear accumulation of Snail1 and protection from degradation in a src/ERK2 dependent manner. This stabilization was found to promote EMT and facilitate metastasis in a 4T1 transplant model of breast cancer (Zhang et al., 2013). The lab has gone on to show that Snail1 in CAFs is activated by mechanical signals and that this activation promotes fibrosis (Zhang et al., 2016). It is not yet known whether DDR2 signaling to

Snail1 is required for the pro-fibrotic phenotype. Further, it is not known whether Snail1 is required in CAFs for integrin β 1 activation. The possibility, however, is unlikely as Snail1 is stabilized downstream of DDR2 activation, and we see effects on integrin β 1 within 15 min. Other possible kinase dependent or independent pathways that have yet to be explored include crosstalk with other RTKs. Crosstalk between RTKs is common and can lead to differential effects of and resistance to treatment (Stommel et al., 2007), but no such phenotype has yet to be described for DDR2.

The activation kinetics of DDRs are extremely long, on the order of hours, and it is not entirely clear how it occurs. For both DDR1 and DDR2, it appears that Src must phosphorylate tyrosine residues in the activation loop for activation (Lu et al., 2011; Yang et al., 2005). For DDR1, there is evidence that the receptor is quickly internalized into endosomes after collagen stimulation but before it is fully activated. It is not clear if the ligand is retained or if there is some ‘memory’ of ligand binding (Mihai et al., 2009). It seems unlikely but not impossible that the receptor would be internalized with collagen peptides bound to it, a process which would require coordinated proteolysis of collagen fibers. It is not known whether DDR2 also undergoes receptor internalization after collagen stimulation, though the question is interesting.

Because the kinetics of DDR2 autophosphorylation and activation are so uncommon, research into other methods by which DDRs acts are emerging. Of these possibilities, clustering of the receptors into multimers seems to be the most likely. Clustering of receptors can have several effects, including the ability to form more contacts with ligand and to locally pool signaling partners near the membrane. DDR2 is known to interact with src as well as other proteins containing SH2 or PTB binding domains, however these interactions depend on tyrosine phosphorylation (Ikeda et al., 2002). It is not known whether DDR2 is capable of acting as a co-

receptor for other transmembrane proteins or RTKs, nor it is known whether clustering itself recruits other proteins. DDR1 has been shown to cluster within minutes of collagen binding (Mihai et al., 2009), suggesting that clustering is a kinase independent function. Further, DDR1 was recently shown to influence collagen contraction, and clustering as well as direct interaction with non-muscle myosin IIa (NMAII) was required, however they determined that the phenotype was kinase dependent (Coelho et al., 2017). It is possible that collagen binding creates clusters of DDR2 as well and that is how signaling downstream of DDR2 recruits talin1 and activates integrin β 1 in CAFs. Future studies could use the DDR2 W52A mutant to determine if collagen binding is required for clustering, and, further, use the new DDR2 T96/98A mutant (binds collagen but does not cluster) to determine if collagen binding is sufficient to activate integrin β 1 or if clustering is necessary. Collagen binding only could support a mechanism by which potential DDR2 conformational changes are required for kinase independent signaling. More research is needed to fully understand the kinase dependent and independent functions of DDRs.

DDR2 in other cell types

The Longmore lab has now demonstrated roles for DDR2 action in multiple cell types in breast cancer metastasis. In initial studies, a DDR2 null mouse was used to assess whether there was a global defect in breast cancer metastasis related to DDR2 expression. In both a transplant and genetic MMTV-PyMT model of breast cancer, DDR2, in general, was found to be essential for lung metastasis (Corsa et al., 2016). DDR2 is not expressed on normal breast epithelial cells, but it is aberrantly expressed in 71% of invasive breast cancer tumor cells (Zhang et al., 2013), so studies first focused on DDR2 action in tumor cells. Using MMTV-Cre and K14-Cre, the luminal and basal epithelial cells were targeted in the MMTV-PyMT model. Analysis of end

stage animals showed that while luminal DDR2 expression was dispensable in lung metastasis, basal DDR2 expression was critical. Since K14⁺ basal cells are invasive and contractile, they went on to show that DDR2 expression in these cells was necessary for local invasion (Corsa et al., 2016). One potential complication with this study is that K14⁺ progenitors give rise to both the luminal and basal stem cell populations in normal breast development, so it possible that K14-Cre targets both the luminal and basal epithelial compartments (Visvader and Stingl, 2014). While it is clear through studies using MMTV-Cre that DDR2 in the luminal compartment alone is not sufficient to drive metastasis, it is not yet clear that DDR2 action in basal cells is sufficient. Further studies using an inducible K14-Cre could bypass developmental K14 expression in progenitors and restrict DDR2 deletion to the basal cells and basal progenitors.

This study utilizes FSP1-Cre to delete DDR2 from fibroblasts as efficiently as possible. Unfortunately, it is likely that FSP1-Cre also targets a subset of CD45⁺/F4/80⁺ macrophages. While we can correlate *in vitro* phenotypes to *in vivo* collagen derangements, we cannot say with absolute certainty that the reduction in lung metastasis is directly due to DDR2 expression in CAFs. Since CAFs are an extremely heterogeneous population with cells from multiple sources, it is unlikely that a Cre will ever be derived which selectively target CAFs. One experiment that remains to be done is a co-transplant mixing experiment where DDR2 +/- CAFs are co-injected with wild type and DDR2 null tumor cells into wild type and DDR2 null mice. This experiment, while imperfect, would allow interrogation of DDR2 in CAFs more specifically.

Systems in which DDR2 contribution to breast cancer metastasis have not yet been explored include the immune system and endothelial cells. DDR2 is expressed in some cells of the myeloid lineage including macrophages (Ferri et al., 2004), dendritic cells (Lee et al., 2007), and neutrophils (Afonso et al., 2013). In neutrophils, DDR2 expression was required for 3D

migration (Afonso et al., 2013). Immune cell contribution to cancer is profound, especially in the innate inflammatory response that is mediated by macrophages and neutrophils. Macrophages can be pro – or anti – tumorigenic depending on class (DeNardo et al., 2009; Zhu et al., 2014). Dendritic cells present antigen to the adaptive immune system, which modulates the T cell response (Quintana, 2017). The role for DDR2 in these cell types has not been explored, though Snail1 has been implicated as important for some immune cell functions. Since DDR2 is an upstream regulator of Snail1 (Zhang et al., 2013) and required in neutrophils for invasion, it may be involved in the innate immune response in cancer. Future studies could include using LysM-Cre to delete DDR2 in macrophages and neutrophils in a genetic or transplant model of breast cancer, however the cell type specificity would still be low (Clausen et al., 1999). Reciprocal bone marrow transplants or specific implantation of modified immune cells could abrogate that problem.

Another important process necessary for metastasis is angiogenesis. In order for cells to escape the primary site, they must be able to enter the bloodstream or lymphatics. Blood vessels which develop in solid tumors under stimulation by growth factors such as VEGF are often disorganized and leaky, allowing easier intravasation of invading tumor cells (Kerbel, 2008). There is evidence that DDR2 expression is upregulated in tumor endothelial cells and that it plays a role in angiogenesis (Zhang et al., 2014). The authors in that study utilize a DDR2 global null mouse for their studies, so using a more Cre to more specifically target endothelial cell in a breast cancer model would be interesting. Further, there is increasing evidence that DDR2 regulates VEGFR levels in endothelial cells, suggesting a possible mechanism by which DDR2 action may regulate tumor angiogenesis (Zhang et al., 2014; Zhao et al., 2016).

DDR2 in downstream metastatic processes

The bulk of our work in describing the effect of DDR2 on breast cancer metastasis has focused on the primary site. In the primary site, tumors must invade away from the tumor core, through the tumor associated stroma, and intravasate into the blood stream or lymphatics. At this time, we cannot rule out a role for DDR2 in other steps of the metastatic cascade. We have shown that DDR2 depleted tumor cells do not grow in the lung as well as wild type cells in a tail vein injection model (unpublished, Corsa), however this experiment does not necessarily parse out whether the difference was due to problems with extravasation, seeding, or outgrowing in the lung. Lung colonization in this experiment was detected by bioluminescence, and no differences in initial lung photon flux were observed. This suggests that DDR2 depleted tumor cells could extravasate as efficiently as wild type. In other unpublished work from our lab, we have tail vein injected both wild type and DDR2 global null mice with wild type tumor and see no differences in lung tumor burden. This suggests that no intrinsic defect lies in the lungs of DDR2 global null mice that may explain differences in lung metastasis.

While we have mostly focused on lung metastasis in the Longmore lab, other sites of breast cancer metastasis are common in women, particularly the bone (American Cancer Society, Facts & Figures, 2017). DDR2 has profound effects on bone development and skeletal growth (Bargal et al., 2009; Ge et al., 2016), however no work has been done to determine if DDR2 effects bone metastasis in breast cancer. Future studies could utilize existing models in the lab to determine if any differences in bone metastasis exist, and, if so, what mechanisms may underlie those differences.

DDR2 in other cancers and diseases

DDR2 expression or mutation has been implicated in several cancers and diseases. Several mutations have been described for DDR2 in non-small cell lung cancer and squamous cell carcinoma of the lung (Hammerman et al., 2011; Rikova et al., 2007) as well as aberrant expression in ovarian cancer (Divine, et al, 2015), nasopharyngeal cancer (Chua et al., 2008), and aggressive thyroid cancer (Rodrigues et al., 2007). DDR2 has now been shown to have effects on collagen deposition and collagen remodeling, so it is likely that any cancer or pathology involving fibrosis may involve DDR2. Interestingly, in liver fibrosis, loss of DDR2 was found to promote increased fibrosis in a model of carbon tetrachloride injury. The authors proposed that this effect was due to dysregulation of paracrine signaling between hepatic stellate cells and macrophages, suggesting that, in some cases, DDR2 action modulates or limits deposition of collagen (Olaso et al., 2011a). In other systems, it has been suggested that DDR2 expression increases fibrosis, such as in a bleomycin induced model of lung fibrosis or alcoholic liver disease (Luo et al., 2013; Yang et al., 2013). It is clear that further work is required to tease out the relative contribution of DDR2 to fibrosis, and research in fibrotic cancers such as pancreatic cancer is warranted.

DDR2 mutation is a cause of dwarfism called SMED-SL, however no other collagen disorders have been associated with DDR2 expression or mutation (Bargal et al., 2009). However, DDR2 has been implicated in osteoarthritis (OA) where upregulation of the protein was found in chondrocytes in joints affected by OA. This upregulation caused increased expression and activity of MMP-13, enhancing the degradation of articular cartilages (Xu et al., 2007; Xu et al., 2005). OA can develop due to genetic causes or from injury and surgery. In both cases, DDR2 expression and concomitant MMP-13 upregulation were observed in mice and

human patients (Sunk et al., 2007). While it is unknown how DDR2 upregulation occurs in articular cartilages, the seemingly consistent finding that MMP-13 upregulation associated with DDR2 expression makes the pathway a potential therapeutic target for OA.

Therapeutic potential of targeting DDR2

Despite improvements in detection, diagnosis, and treatment, breast cancer metastasis remains the second leading cause of cancer related death in women in the United States (American Cancer Society). Developing treatments that specifically target the metastatic process, in addition to treating tumor growth itself, will be essential to reducing metastasis related death. Breast cancers are typically detected early, but 1 in 10 women will still develop metastasis within five years and 1 in 5 with ten years. We can now show that DDR2 regulates breast cancer metastasis in at least two different cell types, the basal epithelial cells (Corsa et al., 2016) and stromal cells expressing FSP1. These studies are one of the first to show potential efficacy for targeting a single protein in multiple compartments. If treatments targeting DDR2 make it through clinical trials, the ability to hit at least two pathways by which tumor cells metastasize can only increase the odds of treatment success.

While we have shown that DDR2 action in breast tumor and stromal cells is essential for metastasis, we do not show any effect of DDR2 on tumor growth or tumor latency. This would suggest that treatment of breast cancer with a DDR2 inhibitor alone would never be feasible. In practical terms, the tumor would likely be surgically removed and/or treated with other drugs such as immunotherapies or chemotherapy in addition to DDR2 inhibition. Hopefully by doing so, not only could we treat the tumor itself but also allay any downstream metastatic processes. It is currently unknown what effects DDR2 action may have on metastatic but dormant cells. More

work will need to be done in the future to determine if DDR2 inhibition would help treat metastatic recurrence.

Most cancers have mutations in receptor tyrosine kinases which promote cell growth and survival. For this reason, many tyrosine kinase inhibitors have been developed which target and inhibit the kinase domain of this class of receptors. However, the pitfalls of this strategy have been problems with specificity, toxicity, and development of resistance (Lin and Shaw, 2016; Zhu et al., 2011). DDR2 is unique in that it binds an insoluble substrate, fibrillar collagen (Shrivastava et al., 1997; Vogel et al., 1997), and the possibility exists that it may be possible to interfere with collagen binding rather than kinase activity. It would also allow for the development of compounds which do not need to enter the cell, which would improve selectivity and reduce toxicity. Importantly, there is new evidence that DDRs may have kinase independent functions that a kinase inhibitor would not affect (Hammerman et al., 2011). In this study, we show that kinase activity is not required for matrix remodeling, only collagen binding. Therefore, development of inhibitors which can also target the kinase independent effects of DDR2 will be beneficial.

In currently unpublished work from the Longmore lab, Grither, et al, have developed a novel compound which inhibits DDR2 by promoting receptor dissociation from collagen ligand. In early studies, the compound has been shown to have efficacy *in vitro* and *in vivo* and further development is planned for future studies.

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