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Novel Regulators of Invadopodia in Tumor Metastasis

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

in

Biology

by

Yike Jiang

Committee in charge:

Professor Jing Yang, Chair
Professor Eric Bennett
Professor Gen-Sheng Feng
Professor Amy Kiger
Professor Peter Novick

2018

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Chair

University of California San Diego

2018

DEDICATION

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

EPIGRAPH

Enjoy climbing the mountain.

Tadashi Yanai

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LIST OF ABBREVIATIONS

AChRs: Acetylcholine Receptors

ADAM12: A Disintegrin And Metalloprotease 12

ANXA2: Annexcin A2

ARG: Abl-Related Gene

bHLH: Basic Helix-Loop-Helix

BM: Basement Membrane

BM-MSCs: Bone Marrow-Mesenchymal Stem Cells

CAFs: Cancer Associated Fibroblasts

CAM: Chorioallantroic Membrane

CDC: Center for Disease Control

CTC: Circulating Tumor Cell

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

DCIS: Ductal Carcinoma In Situ

DMEM: Dulbecco’s Modified Eagle Medium

DPP4: Dipeptidyl Peptidase 4

DSP: Dithibis(Succinimidyl Propionate)

DTCs: Disseminated Tumor Cells

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

EGR1: Early Growth Response protein 1

EMSA: Electrophoretic Mobility Shift Assay

EMT: Epithelial-Mesenchymal Transition

ER: Estrogen Receptor

EVH1: Ena/VASP Homolog 1

EVL: Ena/VASP-Like

FAD104: Factor for Adipocyte Differentiation 104

FAK: Focal Adhesion Kinase

FAP: Fibroblast Activation Protein

FGF: Fibroblast Growth Factor

FNDC3B: Fibronectin Type III Domain Containing 3B

FNIII: Fibronectin type III

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green Fluorescent Protein

GIP: Glucose-Dependent Insulinotropic Polypeptide

GLP-1: Glucagon-Like Peptide 1

HER2: Human Epidermal growth factor Receptor 2

HIF1α: Hypoxia-Inducible Factor 1α

HMLE: Human Mammary Epithelial cells immortalized with Large T antigen

IDC: Invasive Ductal Carcinoma

IP/MS: Immunoprecipitation/Mass Spectrometry

IF: Immunofluorescence

IHC: Immunohistochemistry

MARE: MAF Recognition Element

MEFs: Mouse Embryonic Fibroblasts

MET: Mesenchymal-Epithelial Transition

MH-FAP: MycHis-FAP

MMP: Matrix Metalloprotease

MMTV: Mouse Mammary Tumor Virus

MT1-MMP: Membrane-Tethered Matrix Metalloprotease

mTOR: Mammalian Target of Rapamycin

NAs: Nascent Integrin Adhesions

N-WASP: Neuronal Wiskott-Aldrich Syndrome Protein

NCBI: National Center for Biotechnology Information

NFκB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

NPF: Nucleation Promoting Factor

PDGF: Platelet-Derived Growth Factor

PDGFRα: Platelet-Derived Growth Factor Receptor α

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-Kinase

PKC: Protein Kinase C

PR: Progesterone Receptor

PRR: Proline-Rich Region

PRRs: Pathogen Recognition Receptors

PX: Phox Homology

PyMT: Polyoma virus Middle T antigen

RNAi: RNA interference

ROS: Reactive Oxygen Species

RSV: Rous Sarcoma Virus

RTKs: Receptor Tyrosine Kinases

SDS: Sodium Dodecyl Sulfate

SFKs: Src family of non-receptor Tyrosine Kinases

shRNA: small hairpin RNA

siRNA: small interfering RNA

SNAREs: N-ethylmaleimide-Sensitive Factor (NSF)-Attachment Protein Receptors

TGFβ: Transforming Growth Factor β

TGN: Trans-Golgi Network

Tks5: Tyrosine Kinase Substrate 5

TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligands

uPA: Urokinase Plasminogen Activator

VASP: (Ena)/Vasodilator-Stimulated Phosphoprotein

WASP: Wiskott-Aldrich Syndrom Protein

ZEB: Zinc-Finger E-box binding

ZO1: Zonula Occludens

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ABSTRACT OF THE DISSERTATION

Novel Regulators of Invadopodia in Tumor Metastasis

by

Yike Jiang

Doctor of Philosophy in Biology

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Professor Jing Yang, Chair

During metastasis, carcinoma cells acquire invasive abilities to invade locally and to spread to distant organs. The ability to degrade extracellular matrix (ECM) to breakthrough the basement membrane, undergo local invasion, intravasation, and extravasation is crucial for tumor cells to metastasize. Tumor cells acquire ECM

degradation ability through the formation of membrane protrusions termed invadopodia to localize various proteases to cell-matrix contact points. Invadopodia are specialized actin-rich cell protrusions that contain a number of actin regulatory proteins including cortactin, Arp2/3, and N-WASP. The matrix degradation activity of invadopodia has been associated with a number of proteases, including MMPs and membrane type MMPs. Inhibiting essential invadopodial components, including cortactin, Tks5, or MT1-MMP, inhibited breast cancer metastasis *in vivo*, supporting a critical role for invadopodia in tumor metastasis. Our lab previously discovered transcription factor TWIST1 promotes tumor metastasis by inducing invadopodia-mediated matrix degradation and tumor invasion. Additionally, TWIST1 induces the expression of Fibroblast Activation Protein (FAP), a membrane-associated serine protease that have been associated with invadopodia. Furthermore, FAP is essential for focal ECM degradation and is required for TWIST1-mediated breast cancer metastasis *in vivo*, however, the protease activity of FAP is not required for regulating invadopodia function. This dissertation characterized the role of FAP in invadopodia function and metastasis in two new cell lines and found that FAP deficient cells have less percentage of cells forming invadopodia. Further study suggests that FAP is not required for invadopodia initiation but may be required for invadopodia stabilization. To better understand the role of FAP at invadopodia, an immunoprecipitation followed by mass spectrometry was performed to identify FAP interacting proteins. We found FNDC3B, a transient interactor of FAP, that localizes to invadopodia and regulates invadopodia function. Despite FNDC3B deficient cells sharing similar degradation defect as that of FAP, FNDC3B is not required for invadopodia formation. The findings suggest FAP and

FNDC3B have separate functions at invadopodia. We believe that FNDC3B helps promote MT1-MMP vesicle transport to invadopodia while FAP proper MT1-MMP localization once it is at invadopodia. Further studies must be undertaken to better understand the dynamics of FAP and FNDC3B at invadopodia.

Chapter 1

Introduction

1.1 Cancer

Modern day medicine defines cancer as a disease of the body where cells grow uncontrollably and invade into other tissues and if unprevented, will cause death. The earliest record of cancer dates back to 3,000 B.C.; it was found in an ancient Egyptian medical text, called the Edwin Smith Papyrus (Hajdu, 2011). Around 400 B.C., Hippocrates hypothesized that cancer is a disease of natural causes in the body, including excess or deprivation of blood, mucus, bile, and other body secretions, particularly at old age (Hajdu, 2011). Hippocrates first coined the Greek terms *carcinos* and *carcinoma* because cancer growth resembled the spreading of a crab (Hajdu, 2011). 600 years later, Celsus introduced the word cancer, which is Latin for crab.

Cancer is the second leading cause of death in the U.S., following heart disease. The Center for Disease Control (CDC) projects that cancer will surpass heart disease in the near future (Weir et al., 2016). It is estimated that there have been 15 million cancer survivors (Siegel et al., 2018). In 2018, about 1.7 million new cases of cancer are expected to be diagnosed, and about 609,640 Americans will succumb to the disease (Siegel et al., 2018).

Though scholars have tried to characterize the cause of cancer for centuries, it wasn’t until 1775 that an actual cause was identified. A British physician Percivall Pott hypothesized that the high incidence of scrotum cancer in chimney sweepers was due to exposure to a common environmental factor, soot (Hajdu, 2012). Since his discovery, researchers studied cancer tirelessly hoping to understand how this complex disease

came about. In 2000, researchers Douglas Hanahan and Robert Weinberg published a seminal review summarizing the works of countless researchers. They stated that the right combination of genetic mutations is required to activate the multistep process of tumorigenesis. More importantly, they postulated that these mutations collectively should manifest six biological capabilities to promote tumorigenesis. Termed the "Hallmarks of Cancer", these capabilities are: self-sufficiency in growth signals, ability to evade apoptosis, insensitivity to growth-inhibitory signals, sustained angiogenesis, limitless replicative potential, and the ability to metastasize and invade surrounding tissues (Hanahan & Weinberg, 2000). They noted that the activation of each capability indicates the breaching of an anti-cancer defense mechanism innate to the body. In light of new cancer-related discoveries, in 2011, the pair updated the "Hallmarks" to include two enabling characteristics that are crucial for the acquisition of the original six capabilities- "genome-instability and mutation" and "tumor-promoting inflammation". They also added two other hallmarks involved in the pathogenesis of most cancers- "reprogramming cellular metabolism to support neoplastic proliferation" and "the ability to evade destruction by the immune system". Since the latter two hallmarks still require further validation and are not yet fully generalized, they are referred to as "emerging hallmarks" (Hanahan & Weinberg, 2011).

1.2 Breast cancer

Breast Cancer is the most commonly occurring cancer type in women in the US, accounting for 30% of new cancer cases in women each year. It is estimated that 1 in 8 women will develop breast cancer over the course of her life time. In the US in 2018, about 266,120 new cases of invasive breast cancer will be diagnosed in women and

41,400 breast cancer deaths will occur (Siegel et al., 2018). The five-year survival rate of local breast cancer is 95%, while the survival rate for stage IV breast cancer, also known as metastasis, is about 25% (Howlader N, 2011). While there are effective treatment options for earlier stage breast cancer, metastasis remains the main cause mortality in breast cancer patients due to lack of effective treatment options.

Breast cancer can arise from two main types of epithelial cells; ductal, cells lining the milk duct, and lobular, cells from milk-producing glands. Either type can occur as *in situ* or invasive type of cancers. *In situ* cancers are non-invasive, meaning the tumor cells will not spread beyond the cell type that is cancerous. On the other hand, invasive tumors are dysplastic and can invade normal tissue or spread beyond the breast. Ductal carcinoma *in situ*, or DCIS, is a non-invasive carcinoma occurring in the milk ducts. Given time, DCIS may give rise to invasive ductal carcinoma (IDC). IDC is the most common type of breast cancer and it is composed of tumor cells that have lost their epithelial characteristics and have evaded into surrounding tissues.

With the advancements of gene expression profiling, breast cancer further grouped in subtypes based on its estrogen receptor (ER), progesterone receptor (PR), and HER2-expression. Combined with traditional clinicopathological variables including tumor size, tumor grade, and tumor stage, they are used for patient prognosis and disease management (Dai et al., 2015). Tumors with ER and/or PR overexpression have enhanced ligand binding and therefore turn on downstream pathways for cell proliferation and survival. Hormone therapies that block ligand-receptor binding, for example, tamoxifen or reduce the level of ligand, for example, aromatase inhibitors, are effective treatment options. In HER2+ tumors, cells overexpress the epidermal growth

factor receptor, which leads to activation of the PI3K/Akt/mTOR pathway, driving angiogenesis, cell proliferation, survival, and migration to promote tumor growth (Krishnamurti & Silverman, 2014). Breast cancer cells can expression a combination of ER, PR, and HER2, they can be grouped into four subtypes: luminal A (ER+/PR+/HER2-), luminal B (ER+/PR+/HER2+), HER2+, and triple-negative (ER-/PR-/HER2-). Luminal tumors are the most common breast cancer subtype, with luminal A accounting for 54.3% of all breast cancer patients (Dai et al., 2015). Luminal A patients have significantly better prognosis than luminal B patients. Since luminal cancers are hormone receptor positive, they respond better to hormone therapy than tradition chemotherapy. As luminal B cancers are more proliferative, they require combination therapy of hormone and chemotherapy. HER2+ patients have poor prognosis, but can benefit from anthracycline and taxane-based neoadjuvant chemotherapy. Additionally, molecular targeted agents such as anti-HER2 monoclonal antibody, trastuzumab, are available for HER2+ cancers, although trastuzumab is not effect against all HER+ tumors. Triple-negative (TN) tumors have the worst prognosis and are likely to be of tumor grade 3 or higher. Standard treatment for TN patients include chemotherapy, radiation therapy, or non-HER2 targeted therapy. The poor prognosis of HER2+ and triple negative tumors is due to a higher chance of early relapse among patients without complete eradication of tumor cells.

1.3 Breast cancer metastasis

Metastasis is the leading cause of death in breast cancer patients. Metastasis, or the spread of tumor cells to other regions of the body, can be detected at the time of initial diagnosis or during recurrence, which can occur years after initial treatment. It is

estimated that 30% of women with early-stage breast cancer will develop metastasis later on. The 5-year survival rate for women with breast cancer metastasis is 22%. Despite available treatment options for metastasis, therapy specifically targeting metastastic cells is lacking.

The metastatic cascade is a multi-step process and failure to complete any of the steps will result in arrest of the process. The steps include 1) local invasion of surrounding tissue; 2) intravasation into blood or lymphatic systems; 3) travel through the blood or lymphatic systems; 4) extravasation out of blood or lymphatic systems on to secondary organ sites; 5) establishing micrometastasis and ultimately macrometastasis at new site (Scully et al., 2012).

Carcinomas originate from epithelial cells, which is characterized by having cell-cell junction, apical-basal polarity, and cell adhesion to the basement membrane, a type of extracellular matrix (ECM). In order to initiate the metastasis cascade, tumor cells must gain the ability to migrate away from the tumor bulk by altering cell-cell junction through E-cadherin down regulation. Next, to penetrate the tissue boundaries by degrading ECM, tumor cells utilize metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) system (Dano et al., 2005; Egeblad & Werb, 2002). A number of MMPs including -1, -2, -13, and -14 have been implicated in the process of ECM remodeling (Kessenbrock et al., 2010). Integrins are also known to be involved in the regulation of tumor invasion by participating in the activity of MMPs (D. M. Li & Feng, 2011). For example, integrins α5β1 and α3β1 were both reported to upregulate MMP9 activity (Mitchell et al., 2010; Rolli et al., 2003).

Once the tumor cells successfully intravasated into the circulature, they are called circulating tumor cells (CTCs). CTCs must overcome a number of challenges in order to survive the vasculature and eventually extravasate out of it. When cells are not attached to ECM, they normally undergo anoikis, cell-detachment induced apoptosis. CTCs can adapt to the change in environment and gain the ability of anchorage-independent growth through a number of signaling strategies. They include: bypassing integrin signaling via hyperactivation of downstream receptor tyrosine kinases (RTKs), activation of oncoproteins to counter anoikis-inducing pathways, and modification of surface integrin expression (Guadamillas et al., 2011). Additionally, CTCs must also survive the shear force and immunological stress of the circulature (Dasgupta et al., 2017).

Following the stress of extravasation, the CTCs must attempt to survive the distant organ environment and establish metastasis. Metastastic colonization proves to be the main bottleneck during metastasis. Cells must survive the lack of survival signals and supportive microenvironment in the host tissue and escape the body's innate immunity (Vanharanta & Massague, 2013). As few as 0.01% of CTCs can effectively give rise to metastasis (Lambert et al., 2017). Many CTCs become dormant and stay as single disseminated tumor cells (DTCs) or only form micrometastasis for months to years. Even CTCs that can give rise to macrometastasis may remain latent for up to years before proliferating. The cause of metastatic latency is still being studied, but some possible explanations include slow attrition over periods of time, persistence in the long-term dormancy stage, and absence of net gain or loss in overall cell number while retaining cell viability (Chambers et al., 2002). The concept of CTCs having to

overcome growth hurdles was first postulated by Stephen Paget more than 120 years ago. In his “seed-and-soil” article, Paget observed that a given type of cancer tends to metastasize to particular organ sites. Based on this observation, he hypothesized that metastasis will only develop if the “soil” (distant site) is suitable for “seeds” (CTCs) survival and proliferation (Valastyan & Weinberg, 2011). Indeed, per his observation, breast metastasis tends to form in the lymph node, bone, brain, liver, and lung.

1.4 Epithelial to Mesenchymal Transition (EMT)

The first step of metastasis requires the tumor cells to break cell-cell junctions, gain migratory and invasive capabilities to leave the tumor bulk, and break through the basement membrane. Tumor cells can do this by turning on a developmental program called epithelial-mesenchymal transition (EMT) (Thiery, 2002).

EMT describes the the transformation of epithelial cells to mesenchymal cells, a process that is required during development throughout the various stage of morphogenesis in many species. In particular, EMT is important for facilitating the formation of a three-layered embryo during gastrulation (Nakaya & Sheng, 2008). Additionally, EMT is also fundamental to many other development processes in the heart, musculoskeletal system, craniofacial structures, and the peripheral nervous system (Thiery, 2002). Not only is EMT integral during development, the program is also reactivated during wound healing, fibrosis, and cancer (Chapman, 2011; Thiery, 2002). The reverse process, mesenchymal-epithelial transition (MET), is an equally important process during, for example, kidney ontogenesis. Since the switch from epithelial to mesenchymal state can be both reversible and irreversible, the term transition is used to describe the phenomenon.

During EMT, epithelial cells lose their cell-cell junctions and apical-basal polarity, reorganize their cytoskeleton, and change cell shape and gene expression in order to enable a more invasive phenotype (Lamouille et al., 2014). During the first step of EMT, cell-cell junctions, which includes tight junctions, adherens junctions, desmosomes, and gap junctions, are broken down and degraded. In tight junctions, decreases in occludin and claudin proteins and protein zonula occludens (ZO1) are observed (Huang et al., 2012). The destabilization of adherens junctions is characterized by the cleavage and degradation of E-cadherin. The transcription of junction proteins like E-cadherin is also repressed, in order to stabilize the loss of epithelial junctions. Since polarity complexes such as PAR and Crumbs are physically and functionally associated with cell junction integrity, loss of cell junctions also leads to the loss of epithelial apical-basal polarity (Huang et al., 2012).

In addition to loss of junction proteins, cells also undergo cortical actin cytoskeleton reorganization in order to facilitate dynamic cell elongation and directional motility (Yilmaz & Christofori, 2009). The reorganized actin cytoskeleton can take shape in the form of membrane projections such as lamellipodia and filopodia to facilitate cell movement or invadopodia to facilitate ECM degradation and cell invasion (McNiven, 2013; Ridley, 2011). An increase in actin stress fiber formation is also observed, which helps facilitate cell contractility and mechanotransduction (Tojkander et al., 2012). The actin rearrangement and change in actin dynamics is regulated by Rho GTPases during EMT (Lamouille et al., 2014). Rac1 and CDC42 promote the formation of lamellipodia and filopodia, while RhoA is responsible for actin stress fiber formation. In addition to changes in actin formation, gene expression impacts changes in intermediate filament

composition, with the repression of cytokeratin and expression of vimentin (Huang et al., 2012).

On a molecular level, the shift from epithelial to mesenchymal phenotype is mediated by transcription modifications that cause the repression of epithelial genes and induction of mesenchymal genes. The transition is mainly driven by several transcription factors: SNAIL, zinc-finger E-box binding (ZEB), and the basic helix-loop-helix (bHLH) transcription factors (Lamouille et al., 2014). These transcription factors may share target similarities, but have specific expression profiles dependent on tissue type. They can also control expression of each other and function cooperatively to promote EMT (Peinado et al., 2007). SNAIL1 and SNAIL2 proteins are known to activate EMT during development, fibrosis, and cancer (Barrallo-Gimeno & Nieto, 2005). They bind to E-box of epithelial genes' promoter via their carboxy-terminal zinc-finger domains to repress transcription (Peinado et al., 2007; Xu et al., 2009). SNAIL proteins can repress E-cadherin expression and cooperate with transcription factor ETS1 to induce MMP expression. ZEB family transcription factors, ZEB1 and ZEB2, bind E-boxes on gene promoters to active or repression genes to promote EMT. Their may interact with additional transcription factors including CTBP, SWI/SNF, PCAF, and LSD1 to carry out their function (Lamouille et al., 2014). Since SNAIL1 can directly bind the ZEB promoter, ZEB expression tends to follow SNAIL1 expression. The bHLH transcription factors that regulate EMT are E12, E47, TWIST1, and TWIST2, together they can form homo- or heterodimers (De Craene & Berx, 2013). Similar to SNAIL1 and ZEB, TWIST1 represses E-caderin expression. Additionally, TWIST1 can also induce N-cadherin and fibronectin expression independent of SNAIL1 (Yang et al., 2010).

EMT can also be initiated by other factors such as components in ECM: collagen, hyaluronic acid, soluble growth factors including TGFβ, fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), to name a few (Lamouille et al., 2014). In particular, TGFβ is involved in many signaling pathways to induce the expression and activity of SNAIL1/2, ZEB1/2, and TWIST1, activate RHO, RAC, and CDC42 GTPases, for example. Hypoxia conditions induces HIF1α, which activates TWIST1 expression and SNAIL1 expression in some cell types (Yang et al., 2008). Contributions from other cells in the tumor microenvironment, by immune cells, endothelial cells, and cancer-associated fibroblasts can all promote EMT.

1.5 Invadopodia

During metastasis, many of the steps including invasion, intravasation, and extravasation involve ECM degradation and remodeling. Tumor cells undergo cytoskeletal rearrangement to form membrane protrusions called invadopodia to facilitate this function. Invadopodia are specialized actin-rich cell protrusions that contain a number of cytoskeletal modulators, adhesion proteins, scaffolding proteins, signaling molecules, and proteases.

In 1985, Guido Tarone and colleagues discovered that Src-transformed chicken embryo fibroblasts caused cytoskeletal rearrangement and formation of ventral membrane protrusions containing actin and tyrosine phosphorylated proteins (Tarone et al., 1985). They termed those structures podosomes or “cellular feet”. That same year, Wen-Tien Chen and colleagues discovered that Src actually localized to the protrusions and the structure is capable of ECM degradation and coined the term “invadopodia” to reflect that ability (Chen et al., 1985). Further studies identified podosome/invadopodia

formation in osteoclasts, macrophages, dendritic cells, endothelial cells, and vascular smooth muscle cells (Gimona et al., 2008). To avoid the confusion regarding the two terms, some researcher in the field are using "podosome" to describe the structure in normal cell types involved in tissue remodeling and immune surveillance, and "invadopodia" for cancer cell invasion and metastasis. Beside a contextual difference, studies suggest podosome and invadopodia are possibly different structures. Although the two have similar overall architecture and function, some morphological and molecular distinctions have been identified. For example, invadopodia can protrude deeper into the ECM and remain stable for longer periods than podosomes. Different lipids seem to be involved in their formation, PI(3,4,5)P3 for podosome and P1(3,4)P2, PI(3)P, and lipid rafts for invadopodia (Buschman et al., 2009; Chellaiah et al., 2001; Oikawa et al., 2008). Additionally, many more proteases are found to localize to invadopodia for ECM degradation, including MMP2, MMP9, seprase, uPAR, ADAMs 12, 15, 19 (Furlan et al., 2007; Gawden-Bone et al., 2010; Linder, 2007). More importantly, other cellular protrusions such as filopodia, lamellipodia, and focal adhesions exhibits minimal ECM degradation and therefore, the co-localization of ventral actin puncta with focal ECM degradation is a valuable way to visually distinguish podosomes and invadopodia from them (Dikovsky et al., 2008).

1.5.1 Structural Components of Invadopodia

Cortactin

Cortactin is an 80/85kDa actin-binding protein that was initially identified in 1991 as a novel Src substrate (Wu et al., 1991). Src interacts with Cortactin via its SH2 domain and phosphorylates Cortactin on the tyrosines located in its proline-rich region

(Sangrar et al., 2007). Upon phosphorylation mediated by Src, Cortactin functions to promote branched actin network formation (Vuori & Ruoslahti, 1995).

Although Cortactin has modest activity for actin nucleation, it seems to carry out the role of nucleation promoting factor (NPF) by interacting with other actin nucleator proteins. The Arp2/3 complex is an essential molecular machinery for nucleating actin filament assembly; Cortactin and N-WASP can both interact with Arp2/3 complex via a similar structural domain (Uruno et al., 2003). N-WASP has higher affinity for free form of Arp2/3 complex, while Cortactin preferentially binds to Arp2/3 and F-actin complex (Uruno et al., 2003). This suggests two roles for Cortactin in actin assembly: 1) initial activation of Arp2/3; 2) stabilization of the branched actin networks (Weaver et al., 2001).

Cortactin proves to be an important protein in all steps of the invadopodia lifecycle. During invadopodia initiation, phosphorylated Cortactin plays an important role by actin as a scaffold to recruit important proteins for invadopodia assembly. Branched F-actin generation is the first step of invadopodia assembly and Cortactin does so by recruiting Arp2/3, Cofilin, and N-WASP to the membrane (Jeannot & Besson, 2017). The interaction amongst Cortactin, Arp2/3, and N-WASP has been characterized above. Cofilin is an actin filament severing protein and generates free actin barbed ends, allowing Arp2/3 to build branched actin (Ichetovkin et al., 2002). Cortactin normally inhibits Cofilin activity and the inhibition is released during invadopodia initiation upon Cortactin phosphorylation on tyrosines 421, 466, and 482 (Magalhaes et al., 2011). Post invadopodia formation, Cortactin dephosphorylation occurs and Cofilin is once again inhibited to allow for invadopodia stabilization and function (Oser et al., 2009).

During invadopodia maturation, as marked by the presence of MMPs at ECM contact points to promote degradation, Cortactin functions by mediating MMP recruitment to the membrane. Specifically, Cortactin can bind the phosphorylated cytoplasmic tail of MT1-MMP (or MT1-MMP) to facilitate its invadopodial trafficking (Lagoutte et al., 2016). Additionally, PKCι-phosphorylated Cortactin can be found on MT1-MMP containing endosomes, and associates with Dynamin-2 to promote MT1-MMP trafficking (Rosse et al., 2014). Role of Cortactin in the trafficking of other proteases such as MMP2 and MMP9 remains to be characterized.

There are two proposed mechanisms of Cortactin's function during invadopodia dissembly. The first suggests that PAK-mediated Cortactin phosphorylation decreases Cortactin binding affinity for F-actin, which may cause invadopodia destabilization (Webb et al., 2006). The second proposes that RhoG phosphorylation of Paxilin promotes invadopodia dissembly by inducing the ERK/Calpain pathway (Badowski et al., 2008; Goicoechea et al., 2017). Since Cortactin is a substrate of Calpin, it may be involved in invadopodia dissembly.

Given Cortactin's role in actin assembly during invadopodia formation, it is no surprise that Cortactin knockdown in MDA-MB-231 cells resulted in inhibition of actin and Cortactin positive puncta formation and reduction in ECM degradation (Artym et al., 2006). In cancer, Cortactin overexpression is well documented and consistently associated with poor prognosis and decreased patient survival in many types of cancer, including breast cancer, head and neck squamous cell carcinoma, hepatocellular carcinoma, and melanoma, to name a few (Jeannot et al., 2017). Cortactin overexpression in NIH3T3 cells resulted in increased cell invasion in a Matrigel Boyden

chamber assay. In vivo studies involved mouse models all demonstrated Cortactin plays an important role in promoting tumor invasion and metastasis (Jeannot et al., 2017).

MENA

Mena is a member of the enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) family, which regulates actin assembly by preventing the capping of filament barbed ends (W. Wang et al., 2007). In vertebrates, there are three Ena/VASP paralogs: Mena, VASP, and Ena/VASP-like (EVL) (Gertler et al., 1996). Ena/VASP proteins contain Ena/VASP homolog (EVH) 1 and 2 domains. The EVH1 domain facilitates Ena/VASP proteins localization and signaling pathway interactions, where as EVH2 domain mediates protein tetradimerization and F- and G-actin binding (Bachmann et al., 1999; Krause et al., 2003).

Mena is found to be upregulated in several cancers, including breast and melanoma, and elevated Mena expression is correlated with increased breast tumor invasiveness (Di Modugno et al., 2004; Di Modugno et al., 2006). In invasive tumors cells, Mena can be differentially spliced, yielding an isoform called $Mena^{INV}$. Both Mena and $Mena^{INV}$ promotes carcinoma cell motility, invasiveness, and lung metastasis, however, $Mena^{INV}$ can further potentiate these abilities in tumor cells (Philippar et al., 2008). During invadopodia formation, $Mena^{INV}$ is recruited to promote Cortactin Tyr421 phosphorylation. $Mena^{INV}$ can prolong invadopodia maturation by displacing phosophatase PTP1B from the invadopodial core and preventing Cortactin dephosphorylation on Tyr421 (Weidmann et al., 2016).

Although Mena and VASP have both been shown to localize to invadopodia, their individual functions at invadopodia have not been elucidated. Rajadurai et al. reported

that SHIP2, a phosphatase important for invadopodia maturation, interacts with both Mena and VASP. SHIP2 is responsible for Mena, but not VASP, localization to invadopodia. Additionally, Mena depletion significantly reduced both ECM degradation and cell invasion whereas VASP depletion had little to no effect (Rajadurai et al., 2016). These findings suggest that Mena is important for invadopodia functions, while VASP role in actin assembly might be elsewhere.

Tks4/5

Tks4 and Tks5 are Tyrosine Kinase Substrate with 4 or 5 SH3 domains, respectively. Tks proteins contain an aminoterminal phox homology domain (PX) for lipid binding, SH3 domains for protein-protein interactions, proline rich regions (PRR), and Src phosphorylation sites (Seals et al., 2005). The PX domain of Tks proteins allows localization to different membrane compartments depending on interactions with specific phosphatidylinositol lipids (Ellson et al., 2002; Sato et al., 2001). In humans, Tks4 and Tks5 share 36% structural similarity (Buschman et al., 2009). They function in recruitment of membrane and cellular components to invadopodia.

In terms of invadopodia function, the Tks proteins play overlapping, but not identical roles. Tks5 plays an essential role as invadopodia scaffold protein. Studies have demonstrated that Tks5 is required for invadopodia formation, ECM degradation, and Matrigel invasion *in vitro* (Iizuka et al., 2016; Seals et al., 2005). Tks4 knockdown has demonstrated similar effects in both NIH3T3-Src cells and melanoma cells (Buschman et al., 2009; Iizuka et al., 2016).

Tks5 is involved during invadopodia initiation in several ways. First, Src phosphorylation of Tks5 promotes its PX domain to bind PI(3,4)P2, this helps tether

invadopodia to the membrane and in turn, stabilize invadopodia formation (Sharma et al., 2013). The PRR of Tks5 interacts with the SH3 domain of Grb2, an adaptor protein important for adhesion-mediated cytoskeletal changes (Oikawa et al., 2008). Additionally, all the SH3 domains of Tks5 can associate with N-WASP to promote actin polymerization. Together with Nck1/2, Tks5 also activates Arp2/3 to promote actin polymerization (Stylli et al., 2009). Tks5 is also involved with recruitment of invadopodia regulators AFAP-110, p190RhoGAP, and Cortactin to invadopodia (Crimaldi et al., 2009).

In addition to its involvement in invadopodia initiation, Tks5 also facilitates the proteolytic activity of invadopodia. The fifth SH3 domain of Tks5 interacts with ADAM 12, 15, and 19 (Abram et al., 2003). Neither Tks4 or Tks5 effects the total secretion of MMP2 or MMP9 (Abram et al., 2003; Seals et al., 2005). It seems the function of Tks proteins in invadopodia depends on the cell type. In NIH3T3-Src cells, Tks5 is required for formation and function of invadopodia while Tks4 regulates ECM degradation through interactions with MT1-MMP (Buschman et al., 2009). On the other hand, in melanoma cells, Tks4 and Tks5 both can control invadopodia formation and surface expression of MT1-MMP (Iizuka et al., 2016).

Tks5's role in tumor metastasis *in vivo* was demonstrated by injecting Tks5 knockdown Ras-transformed human mammary epithelial cells with TWIST1 over expression. Tks5 knock down inhibited both local invasion and lung metastasis formation, agreeing with the *in vitro* observations (Eckert et al., 2011). NIH3T3-Src cells with Tks5 knocked down gave rise to similar number of lung metastasis as control cells, but the metastases were significantly smaller than the controls (Blouw et al., 2008).

Altogether, the *in vivo* data suggests that Tks5 plays a role in early steps of metastasis - during invadopodia formation and could be further required for secondary tumor expansion.

N-WASP

N-WASP is part of the Wiskott-Aldrich syndrome protein (WASP) protein family of key regulators of actin cytoskeleton (Miki & Takenawa, 2003). All WASP family proteins have conserved COOH-terminal domain that can induce actin polymerization through Arp2/3 activation (Millard et al., 2004). Signaling molecules including Nck, Grb2, WISH, Cdc42, and phosphoinositides can release N-WASP from its autoinhibitory conformation. It is known that N-WASP activation by Nck1 is important for invadopodia formation and function (Yamaguchi et al., 2005).

At invadopodia, N-WASP complexes with and activates Arp2/3 for de novo actin polymerization to initiate invadopodia formation. Knockdown of N-WASP can significantly reduce invadopodia formation in invasive and metastatic cancer cells (Yamaguchi et al., 2005). However, N-WASP does not seem to be required for lamellipodia or filopodia formation (Desmarais et al., 2009; A. Li et al., 2010).

1.5.2 Regulatory Components of Invadopodia

Src Kinase

Src family non-receptor tyrosine kinases (SFKs) play important roles in signal transduction during cancer progression. SFKs contain a number of conserved domains, including myristoylated N-terminus, SH3, SH2, and tyrosine kinase domains. SFKs interact with receptor tyrosine kinases (RTKs) to promote cell surface receptor turnover, actin cytoskeleton rearrangements, and cell motility and survival. Some of the RTKs Src

interacts with include EGFR, HER2, PDGFR, IGF-1R, and HGFR. Downstream of Src, signals can be relayed to effectors including phosphoinositide 3-kinases (PI3Ks), Akt, and STATs (Playford & Schaller, 2004; Yeatman, 2004).

Out of the SFKs, Src is the only kinase shown to regulate invadopodia formation. Src was first characterized at invadopodia in 1985, in Rous sarcoma virus (RSV) transformed cells that formed ECM-degrading rosettes. Viral $pp60^{src}$, which is the transforming gene product of RSV, was shown to localize to the rosettes (Chen et al., 1985). Further studies have similarly found that constitutively active c-Src enhances invadopodia formation, as demonstrated by the presence of F-actin and Cortactin puncta (Balzer et al., 2010). In addition, kinase inactive c-Src overexpression in c-Src knockdown cells failed to rescue invadopodia formation and function (Bowden et al., 2006; Kelley et al., 2010).

Given Src's role in stimulating primary actin nucleation, determining the rate of flux at the actin core, and formation of the actin cloud and podosome belt in osteoclasts, it comes as no surprise that Src plays a pivotal role in invadopodia formation. Src phosphorylates many important invadopodia players, including Tks5, Cortactin, N-WASP, dynamin-2, AMAP1, Paxillin, p130Cas, p190RhoGAP, AFAP110, and caveolin (Kelley et al., 2010). Protein kinase C (PKC) can act in concert with Src to promote invadopodia assembly (Saykali & El-Sibai, 2014). Src phosphorylation of Tks5 and Cortactin are important steps during invadopodia initiation.

Src kinase has also been shown to play an important role in tumor cell invasion. C-Src expression in murine embryonic fibroblasts with Src, Yes, and Fyn gene knocked out rescued cell invasion in a Boyden chamber assay, whereas oncogenic Ras did not

(Chan & Chen, 2012). Additionally, Src knockdown in MDA-MB-231 breast cancer cells showed significantly reduced matrix degradation and invasion in matrix-coated chambers (Mader et al., 2011). Lastly, Src inhibitors, including Dasatinib, PP2, SU6656 have successfully blocked MDA-MB-231 cell invasion through Matrigel (Sanchez-Bailon et al., 2012). All these findings points to the importance of Src in tumor cell invasion.

Src kinase activity has also been involved in other steps during metastasis, CTC survival, and metastatic recurrence at the distant organs. The gene signature of the Src pathway activation shows strong correlation with late recurrence of bone metastasis in breast cancer. SFK activation is also crucial for CTCs to maintain survival signal in the presence of CXCL-12 and tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) in the metastatic microenvironment (Zhang et al., 2009). Additionally, outgrowth of dormant metastatic cells in the lung depends on β1-integrin-Src signaling. Src activation promotes ERK-dependent formation of actin stress fibers and activation of survival signals which is critical for metastatic outgrowth (Barkan et al., 2010). Src activation can also confer resistance to ECM detachment associated cell death (anoikis). Research found that lung adenocarcinoma cells with Src hyperactivation are resistant to anoikis (Sakuma et al., 2011). During hypoxic conditions, Src activity as part of the hypoxia-inducible factor 1α (HIF-1α)-STAT3-Src axis activates autophagy to help tumor cells escape cytotoxic T cell-mediated killing (Noman et al., 2011).

The above findings suggest an important role for Src kinase activity during tumor metastasis, Src deletion proves to be effective in inhibiting metastasis. Src deletion in MMTV-PyMT mammary tumor model resulted in reduced CTCs, though no effect on primary tumor formation (S. Wang et al., 2009). *In vivo* studies done using tumor cells

with Src knockdown demonstrated that Src is required for pancreatic cancer cell metastasis to the lung and liver and breast cancer metastasis to the bone (P. C. Chan et al., 2012; Zhang et al., 2009).

Abl family Kinase

The Abl family of non-receptor tyrosine kinases consists of two proteins, Abl and Abl-related gene (Arg). Abl kinases share homologous N-termini which contains the SH2, SH3, and kinase domains, but they differ in their C-terminal domain (Pendergast, 2002). Abl was first known as the oncogene in leukemia that formed as a product of chromosome translocation (Pendergast, 2002). Further studies implicated the kinases in linking diverse stimuli from cell surface growth factor and adhesion receptors to signaling pathways to control cell proliferation, survival, adhesion, migration, and invasion (Meirson et al., 2018).

Abl and Arg kinases are both Src substrates(Srinivasan & Plattner, 2006). Various studies suggest that Arg, not Abl, is important for invadopodia formation. Arg has been shown to localize to invadopodia of NIH3T3-Src cells, as YFP-tagged constitutively active Arg colocalized with Cortactin at invadopodia (Smith-Pearson et al., 2010). On the other hand, kinase-inactive Arg expression disrupted invadopodia formation. Additionally, Arg has also been shown to localize Tks5 to invadopodia of MDA-MB-231 cells (Mader et al., 2011). Mader et al. demonstrated that Arg is not required for invadopodia precursor initiation, but is critical for EGF stimulated invadopodia activation. Src activation of Arg is required for Cortactin phosphorylation and actin polymerization at invadopodia, as loss of Arg function cannot be compensated by Src overexpression (Mader et al., 2011).

Abl and Arg kinase also play important roles during tumor invasion and metastasis. Abl and Arg knockdown significantly reduced ECM degradation and invasion in MDA-MB-231 cells and its metastatic derivatives (Smith-Pearson et al., 2010; Srinivasan et al., 2006). Abl and Arg kinase inhibition by a dual inhibitor significantly reduced MDA-MB-435S breast cancer cell invasion in Matrigel (Srinivasan et al., 2006). Cells expressing Abl and Arg shRNAs or treated with Abl/Arg inhibitors showed fewer circulating tumor cells than control mice in vivo (Gil-Henn et al., 2013).

Integrin-mediated signaling

Integrins are important signaling molecules that reside on the plasma membrane. They interact directly with the ECM to transmit extracellular signals to the cytoplasm, hence “outside-in signaling”; they can also relay status of the cell to the extracellular space, hence “inside-out signaling” (Murphy & Courtneidge, 2011a). Integrins are a large family of heterodimeric transmembrane receptors, typically comprised of an α-subunit and a β-subunit. Extracellularly, integrins bind to different ECM components, and intracellularly, integrins can associate with different cytoplasmic linkers such as α-actinin, vinculin, and talin. Upon ECM binding, integrins can initiate signal cascades carried out by focal adhesion kinase (FAK) or SFKs (Hynes, 2002).

Integrins were first discovered at invadopodia in Rous sarcoma virus-transformed cells (RSVCEF). The cells were cultured on fibronectin-coated crosslinked gelatin beads (FN-beads) and integrin β1 clusters colocalized with invadopodia formation (Mueller & Chen, 1991). Following that discovery, β1 integrin has also been found to localize to invadopodia of LOX melanoma cells and MDA-MB-231 cells (Bowden et al., 1999; Seals et al., 2005). β1 integrin heterodimerize with several α integrins to facilitate

invadopodia formation or function. α3β1 integrin has been shown to complex with a protease called Fibroblast Activation Protein (FAP) in LOX melanoma cells (Mueller et al., 1999). α5β1 integrin is found to be peripherally enriched in invadopodia and is hypothesized to stabilize invadopodia protrusions (Mueller et al., 1999). Activation of α6β1 integrin by laminin peptides or integrin stimulating antibodies induced invadopodia activity and melanoma cell invasion *in vitro* (Nakahara et al., 1996). Additionally, activation of α6β1 integrin was associated with increased phosphorylation and localization of p190RhoGAP (Nakahara et al., 1998). Recently, β1 integrin was shown to interact with and promote Arg kinase phosphorylation of Cortactin to facilitate invadopodia maturation (Beaty et al., 2013). Although the exact mechanism of how integrins regulate invadopodia formation and function is not clear, these findings clearly shows β1 integrin complexes play important roles at invadopodia.

As important signaling molecules mediating cellular responses to changes in ECM, integrins have been implicated in invasive cancers. α2β1, α3β1, α5β1 and α6β1 integrins levels are elevated in GBM tumors compared to normal brains. Antibody-mediated β1 integrin inhibition suppresses primary glioma cell adhesion, migration, and invasion *in vitro*, and β1 integrin knockdown reduced glioma cell invasion *in vivo* (Paulus et al., 1996; Rooprai et al., 1999). αVβ3 integrin is found to localize with MT1-MMP on cell surface of breast carcinoma cells and the complex is capable of activating MMP2 (Deryugina et al., 2001). Interestingly enough, MT1-MMP can also process αV integrin to enhance breast cancer cell motility on vitronectin (Deryugina et al., 2002). Currently, the focus is on integrins' therapeutic potential in anti-angiogensis, however, exploring integrins' role in anti-metastasis treatment may also show promises.

GTPases

The Rho family of GTPases including, Cdc42, Rac1, and RhoA, are important regulators of actin cytoskeleton and plays many essential roles in cell migration and invasion (Stylli et al., 2008). Typically, RhoA stimulates stress fiber formation, Rac stimulates lamellipodia formation, Cdc42 stimulates filopodia formation, and invadopodia formation seems to be regulated by multiple GTPases including, Cdc42, Rac1, RhoA, to name a few (Spuul et al., 2014).

In glioma cells, Rac1 depletion by siRNA significantly inhibited cell invasion through rat brain tissue and Matrigel (Chuang et al., 2004). Further examination revealed that Rac1 depletion in those cells led to a decrease in lamellipodia and invadopodia formation. In EGF-stimulated MTLn3 mammary carcinoma cells, Cdc42 silencing inhibited invadopodia formation (Yamaguchi et al., 2005). Overexpression of dominant active Cdc42 can also induce invadopodia formation in melanoma cells (Nakahara et al., 2003). Mechanistically, it has been shown that Cdc42 regulates invadopodia actin network assembly by stimulating N-WASP and Arp2/3 dependent actin polymerization (Rohatgi et al., 2001). RhoA was shown to localized with F-actin, Cortactin, Tks5 positive invadopodia in NIH3T3-Src cells, and inhibition of RhoA signaling prevented invadopodia formation and ECM degradation (Berdeaux et al., 2004). These findings suggest that Rac1, Cdc42, and RhoA all play important roles at invadopodia. However, it is unlikely that the GTPases function together at once, since RhoA and Rac1 are known to have antagonistic activities (Etienne-Manneville & Hall, 2002). Better understanding of the dynamics of GTPases regulation at invadopodia is much needed.

1.5.3 Proteases Associated with Invadopodia

Several matrix metalloproteinases (MMPs) and serine proteases are associated with ECM degradative ability of invadopodia. MMPs are a family of zinc endopeptidases that are classified based on their substrate specificity. The MMP family not only includes membrane bound or secreted proteins, but also ADAMs. ADAMs, a disintegrin and metalloproteinase, can also be referred to as sheddases, enzymes that cleave the extracellular domain of transmembrane proteins. ADAMs are involved with cleaving growth factors, cytokines, receptors, and adhesion molecules (Edwards et al., 2008). These classes of enzymes have all been implicated in cancer progression and metastasis.

MT1-MMP (MMP14)

MT1-MMP, also known as MMP14, is a membrane-anchored metalloproteinase. Out of the 28 MMPs total, MT1-MMP is considered to be the central player in invadopodia-mediated ECM degradation. MT1-MMP can cleave several substrates *in vitro*, including fibronectin, collagen type I, II, and II, laminins, vitronectin, and aggrecans (d'Ortho et al., 1997; Fosang et al., 1998; Koshikawa et al., 2000; Ohuchi et al., 1997). MT1-MMP can activate MMP2 and MMP13 and together with MMP2, MMP3, MT1-MMP can activate MMP9 (Deryugina et al., 2001; Toth et al., 2003).

MT1-MMP is expressed by many cell types and MT1-MMP-null mice exhibit defects in skeletal development, soft tissue fibrosis, angiogenesis, submandibular gland development and lung development (Itoh, 2015). The craniofacial abnormality is due to loss of MT1-MMP inactivation of ADAM9, and active ADAM9 can cleave FGFR2 to inactivate it (K. M. Chan et al., 2012). Additionally, MT1-MMP is a cellular migration and

invasion promoter and has been implicated in many aspects of disease, including cancer cell invasion, metastasis, angiogenesis, wound healing, inflammation, rheumatoid arthritis and many more (Itoh, 2015). In the context of cancer metastasis, MT1-MMP has been shown to be required for invadopodia function. MT1-MMP localizes with Cortactin at invadopodia and its knockdown only moderately effected invadopodia formation but severely reduced ECM degradation (Artym et al., 2006).

Given MT1-MMP's main role in facilitating matrix degradation at invadopodia, it's proper recruitment to the structure is essential. MT1-MMP delivery requires membrane fusion machinary including vSNARE, VAMP7, Rab 7, and Rab8 (Yu et al., 2012). It was reported that the cytoplasmic domain of MT1-MMP is necessary for N-WASP facilitated delivery from late endosomes to invadopodia (Yu et al., 2012). Another study saw that vesicle transport of MT1-MMP is triggered by β1 integrin-matrix interactions and required the activity of Rab8 GTPase (Bravo-Cordero et al., 2007). Following its activity, MT1-MMP needs to be internalized in order to recycle the protein for further function. MT1-MMP can also be internalized by clarthrin- and caveolae-mediated endocytosis in order to recycle MT1-MMP back to invadopodia as needed (Murphy et al., 2011a). The clathrin-dependent endocytosis requires LLY573 peptide region on MT1-MMP to interact with adaptor protein 2, a component of clathrin-coated pits (Uekita et al., 2001). Additionally, MT1-MMP can be palmitoylated at Cys574, and this lipid modification is critical for MT1-MMP clathrin-dependent endocytosis and cell migration (Anilkumar et al., 2005).

Src kinase has been shown to regulate MT1-MMP delivery to invadopodia. Src-mediated phosphorylation of the AP2 clathrin adaptor binding domain of MT1-MMP

reduces the speed of its endocytosis and increase ECM degradation (Poincloux et al., 2009). Additionally, Src phosphorylation of MT1-MMP at Tyrosine 573 is required for tumor cell proliferation, invasion in 3D, and tumor growth in vivo (Nyalendo et al., 2007). Further studies have also implicated this tyrosine phosphorylation site with mono-ubiquitination of Lys581, which facilitates MT1-MMP cell surface trafficking and invasion in collagen matrix (Eisenach et al., 2012).

14 out of 28 MMPs have been implicated in cancer development and progression. MT1-MMP expression is positively correlated with tumor progression, metastasis, and poor prognosis in breast, lung, melanoma, colorectal cancers, and squamous cell carcinomas (Egeblad et al., 2002). Using orthotopic transplant model or mammary fat pad injection model of MMP14 deficient tumor cells, researchers showed that loss of MMP14 significantly reduced metastasis but no effect on primary tumor growth (Perentes et al., 2011; Szabova et al., 2008).

ADAMs

The ADAM family of metalloproteinases contain metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains (Seals & Courtneidge, 2003). ADAMs can bind and modulate integrin functions, release and activate transmembrane growth factors (Stylli et al., 2008). The enzymes are involved with numerous biological events including cell adhesion, cell fusion, cell migration, myoblast fusion, membrane protein shedding and proteolysis (Seals et al., 2003).

In particular, ADAM12 has been associated with disease stage in breast and bladder cancer (Frohlich et al., 2006; Narita et al., 2012). ADAM12 is a known candidate cancer gene and found to be overexpressed in malignant breast tissue and metastatic

lymph nodes, human glioblastoma, lung adenocarcinoma, and head and neck cancer (Diaz et al., 2013). Crossing mice expressing ADAM12 under the control of a mammary gland specific promoter with MMTV-PyMT background led to significant increase in tumor growth and lung metastasis (Kveiborg et al., 2008). Additionally, a prostate cancer mouse model demonstrated that ADAM12 is essential for prostate tumor growth (Peduto et al., 2006).

ADAM12 has been shown to localized to invadopodia with Tks5 and mediate outside-in signaling to drive the formation of invadopodia clusters (Abram et al., 2003; Albrechtsen et al., 2011). Additionally, hypoxia can active Notch signaling which increases ADAM12 levels. ADAM12 uses its sheddase activity to induce the ectodomain shedding of pro-HB-EGF, which becomes HB-EGF to bind EGFR and thereby stimulating invadopodia formation (Diaz et al., 2013). Eckert et al. directly linked ADAM12 to master regulator of EMT and metastasis TWIST1 by demonstrating that ADM12 is a downstream effector of TWIST1 and is required for mediating TWIST1-induced metastasis in vivo (Eckert et al., 2017).

Serine Proteases

Serine proteases are type II integral membrane proteins with 6 amino acid (aa.) cytoplasmic tails followed by 20 or 22 aa. transmembrane domain, N-glycosylation- and cysteine-rich substrate binding domain, and about 200 aa. of catalytic domain in the C-terminus (Chen et al., 2003). Dipeptidyl Peptidase (DPP) IV and Fibroblast Activation Protein (FAP) are two members of the serine protease family that have been associated with invadopodia. The two proteins share a 68% homology at their catalytic domains (Chen et al., 2003). The catalytic activity between the two proteases have slight

differences, DPP4 has only exopeptidase activity, while FAP exhibits both exo- and endopeptidase activity, due to a key alanine reside that shapes that active site (O'Brien & O'Connor, 2008). FAP has been shown to heterodimerize with DPP4 at invadopodia, although it is not clear what is the role of the complex there. Additionally, α3β1 integrin can associate with and provide a docking site for FAP to anchor to invadopodia (Mueller et al., 1999). Is it not known if the catalytic activity of FAP and DPP4 are required for invadopodia-mediated matrix degradation. But in vitro assays have demonstrated following initial collagen I cleave, FAP can further digest the substrate into smaller fragments, suggesting that FAP may work sequentially with other proteases to help breakdown partially degraded ECM components (Christiansen et al., 2007).

DPP4 is ubiquitously expressed throughout all tissues, and is known for its role in blood glucose regulation, leukocyte migration, angiogenesis, and T-cell activation (Chen et al., 2003). For example, two of DPP4’s substrate, Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are important hormones of the glucose regulation pathway (Chen et al., 2003). DPP4 inhibitors led to increased levels of GLP-1 and enhanced insulin secretion and improved glucose tolerance in normal and diabetic animals (Chen et al., 2003). FAP, on the other hand, is not expressed in healthy adults. FAP expression is detected in fetal mesenchymal tissues, during wound healing, fibrosis, and tumor cells and tumor stroma. Several groups have shown that FAP is expressed in reactive stromal fibroblasts of human breast cancer. FAP is also expressed in a number of aggressive cancers, including breast, colon, ovarian cancers, and malignant melanoma. However, FAP expression in cancer cells have been associated with both longer and shorter patient survival (O'Brien et al., 2008). *In vivo*

experiments have demonstrated FAP's role in tumor invasion: FAP deletion inhibited tumor growth in a Kras-driven model of endogenous lung cancer and color cancer (Santos et al., 2009). Pharmacological inhibition of FAP activity also similarly decreased tumor growth in these models, suggesting that FAP is a good target for therapeutic intervention of primary tumor growth (Santos et al., 2009). Given the potential of FAP's role at invadopodia, its function in tumor cell invasion and metastasis *in vivo* remains to be answered.

1.5.4 Physiological Role of Invadopodia

As mentioned in an earlier chapter, podosomes and invadopodia are similar structures that carry out similar functions in different cell backgrounds. Having extensively explored the role of invadopodia in tumor invasion and metastasis, this chapter aims to examine the physiolocal functions of podosomes, the invadopodia equivalence in non-diseased cells.

Podosomes play important roles in hematopoietic lineage, embryonic and postnatal development, angiogenesis and vasculature remodeling, post synaptic membrane maturation, antigen sampling and recognition, and cell-cell fusion mechanisms. Osteoclasts, which are derived from monocytes, were the first normal cell type described to form podosomes (Vaananen et al., 2000). Osteoclasts functions to resorb the bone matrix through formation of podosomes to form a tight attachment to the bone matrix to degrade and resorb the substrate. Loss of podosomes in the hematopoietic lineage is well documents in human disease. Mutations in the WASP gene causes Wiskott-Aldritch syndrome, which manifests as severe immunodeficiency, eczema, and thrombocytopenia (Ochs & Thrasher, 2006).

During embryogenesis and postnatal development, podosomes have also been implicated with cell migration required to facilitate proper cellular differentiation (Kurosaka & Kashina, 2008). The first study of podosomes in embryogenesis were performed in zebrafish embryos. Tks5 knockdown resulted in decreased head and eye size, cardiac malformation, and lateral line and pigmentation irregularities (Murphy et al., 2011b). Specifically, the defect originated from neural crest cells and the loss of their ability to migrate correctly in 3D, to pattern the embryo and to generate tissue in the appropriate position (Murphy et al., 2011b). Other disease that may be due to improper podosome function during embryogenesis includes Frank-ter Haar syndrome, hearing impairment, congenital eye diseases, and reduced bone mineral density (Paterson & Courtneidge, 2018).

Recent discoveries have implicated podosomes in angiogenesis where they facilitate basement membrane degradation for the formation of new blood vessels in the sprouting angiogenesis mechanisms. Arp2/3, WIP, and N-WASP positive podosome structures were observed on the ventral side of endothelial cells and are shown to be capable of degradation (Moreau et al., 2003). In vivo immunoelectron microscopy analysis in mice showed the formation of Cortactin and Tks5 positive podosomes in the aorta (Quintavalle et al., 2010). When stimulated with TGF-β, aortic endothelial cells form podosomes containing MT1-MMP and MMP9 to facilitate local degradation and invasion through the matrix (Varon et al., 2006). These findings suggest an important role for podosomes during vascular remodeling and healing. Additionally, podosomes may facilitate sprouting angiogenesis by serving as a recognition site for new blood

vessel synthesis or creating an open space for angiogenesis, although the exact mechanism is not known (Paterson et al., 2018).

During synaptogenesis or new synapse creation, podosomes are found on myotubes at sites of Acetylcholine receptors (AChRs) clusters. Podosomes are believed to help remodel the AChRs clusters, a critical step during synapse maturation (Proszynski et al., 2009). Synapse podosomes were found to contain F-actin, Arp2/3, Cortactin, Tks5, vinculin, and Src (Proszynski et al., 2009). During neuron development, neuronal growth cones have been observed to form podosome-like protrusions that contain a number of invadopodia components and are capable of ECM degradation (Santiago-Medina et al., 2015). The formation of the podosomes allow proper extension of the motor neuron axons into the peripheral myotome tissue during development in vivo (Santiago-Medina et al., 2015).

Podosomes are also formed on dendritic cells and are believed to play a role in adaptive immunity. Dendritic cells express pathogen recognition receptors (PRRs) in order to bind pathogen antigens and present them to T cells for elimination. Studies suggest that podosomes are involved in pathogen antigen endocytosis by the PRRs (Paterson et al., 2018). Cell fusion is a process important for the formation of multinucleated cells such as during fertilization, osteoclastogenesis, and myotube formation (Paterson et al., 2018). Tks5, was found to be important for podosome formation, which is required for osteoclast fusion (Oikawa et al., 2012).

1.5.5 Therapeutic implications

Given the role of invadopodia in promoting tumor invasion and multiple steps during metastasis, it is an attractive therapeutic target for cancer treatment. As the main

player in carrying out ECM degradation, MMPs were first identified as promising targets for tumor progression. There have been some orally bioavailable “second generation” MMP inhibitors (MMPIs) tested in phase III clinical trials for patients with late stage cancers (Overall & Kleifeld, 2006). Despite showing a strong correlation between metastatic potential and tumor cells’ ability to degrade ECM, none of the MMPIs were clinically effective in slowing disease progression (Liotta et al., 1980). Since MMPs also play important physiological roles in patient protection or homeostasis, more selective MMPIs that do not interfere with host defense needs to be developed. Due to its specific expression in diseased adults, FAP have also been targeted for therapeutic interventions of tumor growth and metastasis. In 2003, a humanized monoclonal antibody for FAP, Sibrotuzumab, was tested in Phase I/II trails and failed to demonstrate any therapeutic effects in metastatic colorectal cancer patients (Hofheinz et al., 2003). Interestingly enough, Sibrotuzumab does not seem to block any cellular or protease function of FAP (Hofheinz et al., 2003). In 2007, Talabostat, a protease inhibitor of FAP was tested in clinical trials and saw lack of significant effect in patients with metastatic colorectal cancer receiving Talabostat alone or in melanoma patients treated with Talabostat and cisplatin combination (Eager et al., 2009; Narra et al., 2007).

Src family kinases, especially Src, has been to shown to be active in a number of human tumors and are considered great anti-cancer targets. Some of the SFKs inhibitors compete for its ATP binding site or inhibit kinase activity to prevent SFKs from phosphorylating target proteins (Stylli et al., 2008). Given SFKs involvement in diverse pathways and functions in many processes involved in cancer, it is a challenge to

pinpoint whether targeting Src inhibits cell invasion by preventing invadopodia formation or through other mechanisms.

Since many of invadopodia proteins also play important functions in various cellular processes such as proliferation, apoptosis, and migration, it would be difficult to attribute the effect of targeting them to their role at invadopodia. The timing of anti-invadopodia treatment is also critical during therapy. The purpose of invadopodia inhibition is to prevent primary tumor cells from successfully metastasizing, so combination with treatment that targets the primary tumor might be more effective. This may also explain why using invadopodia inhibitors to target metastatic disease yields no significant efficacy. Gene expression profiling or biomarkers for high risk metastasis groups would identify patient groups that can benefit from combination therapy of invadopodia inhibitors and chemotherapy.

1.6 References

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CHAPTER 2

Fibroblast Activation Protein (FAP) Regulates Invadopodia Function and Promotes Tumor Metastasis

2.1 Introduction

Metastasis is the main cause of death in cancer patients. Metastasis is a complex process involving the dissemination of tumor cells from the primary tumor mass to distant organs such as the lungs, liver, and bone (Murphy & Courtneidge, 2011). Briefly described here, cancer cells must gain invasive and migratory abilities to intravasate into the lymph and blood systems, travel through the vasculature, and extravasate from the vessel into the distant organ, where they eventually form macrometastasis. During the intravasation and the extravasation process, the ability to degrade the extracellular matrix (ECM) is crucial for tumor cells metastasis.

Tumor cells acquire the invasive ability through the formation of membrane protrusions to localize various proteases to cell-matrix contact points for ECM degradation. These structures, termed invadopodia, are actin-rich cell protrusions that contain a number of actin regulatory proteins such as Cortactin, Arp2/3, and N-WASP (Gligorijevic et al., 2012). In addition, Tks4 andTks5 are important adaptor proteins that regulate invadopodia structure and function (Gligorijevic et al., 2012). Invadopodia also contain numerous proteases, including: metalloproteinases, MMP2 and MMP9, membrane type MMPs, MT1-MMP, transmembrane serine proteases, FAP and DPP4, and disintegrin and metalloproteinases (ADAMs) to carry out ECM degradation. Being distinct from other actin-based protrusions such as lamellipodia and filopodia, invadopodia are mostly found in invasive cell types: macrophages, osteoclasts, and

invasive tumor cells (Gligorijevic et al., 2012). Using intravital imaging techniques on metastatic tumor xenograft in mice, a number of studies have demonstrated invadopodia formation during both the intravasation and extravasation steps of tumor metastasis (Ito et al., 2001; Murphy et al., 2011; Stoletov et al., 2010). In addition, suppressing invadopodia function by inhibiting essential invadopodia components, Cortactin, Tks5, or MT1-MMP, prevents breast cancer metastasis in vivo (Eckert et al., 2011; Li et al., 2001; Perentes et al., 2011). Altogether, the data suggest that invadopodia-mediated ECM degradation is essential for tumor invasion and metastasis.

Previously, our lab has discovered that transcription factor TWIST1 promotes breast cancer metastasis (Yang et al., 2004). TWIST1 promotes epithelial to mesenchymal transition in the tumor cells, allowing the cells to be more invasive and migratory. TWIST1 expression is associated with metastasis and poor survival rates in melanoma, neuroblastoma, prostate cancers, breast cancers, and gastric cancers (Peinado et al., 2007). Our further studies demonstrate that TWIST1 promote tumor metastasis by regulating invadopodia formation and function in breast cancer cells (Eckert et al., 2011).

Since TWIST1 is a transcription factor that regulates the expression of a large number of genes, we aimed to identify downstream targets of TWIST1 that can directly regulate invadopodia formation or function. We performed a DNA microarray using an inducible TWIST1-ER system to identify gene expression changes associated with invadopodia function (Mani et al., 2008). One particular candidate, Fibroblast Activation Protein (FAP), mRNA expression was increased 4-fold at 3 days post-TWIST1 induction, and increased 10-fold at 15 days. FAP protein expression also increased 3

days post-TWIST1 induction.

FAP is a type II integral serine protease whose expression is restricted to the embryonic mesenchyme and is not observed in normal adult tissues. However, FAP is highly expressed in activated tumor-associated fibroblasts (O'Brien & O'Connor, 2008), but has also been shown to be expressed in tumors of epithelial origin (Chen et al., 2006). FAP has been previously shown to associate with $\alpha_3\beta_1$ integrin, DPPIV, MMP-2, membrane-type 1 MMP (MT1-MMP) and uPA at the invadopodia of human malignant cells (O'Brien et al., 2008). However, whether FAP is involved in invadopodia formation and/or function is unknown. Our initial studies characterized FAP localization in breast cancer cell lines. Further characterization demonstrated that FAP is essential for focal ECM degradation and is required for TWIST1-mediated breast cancer metastasis *in vivo*. We performed gelatin degradation assay using FAP protease inhibitor, M83, and observed that inhibition of FAP protease activity does not affect ECM degradation (Figure 2-1).

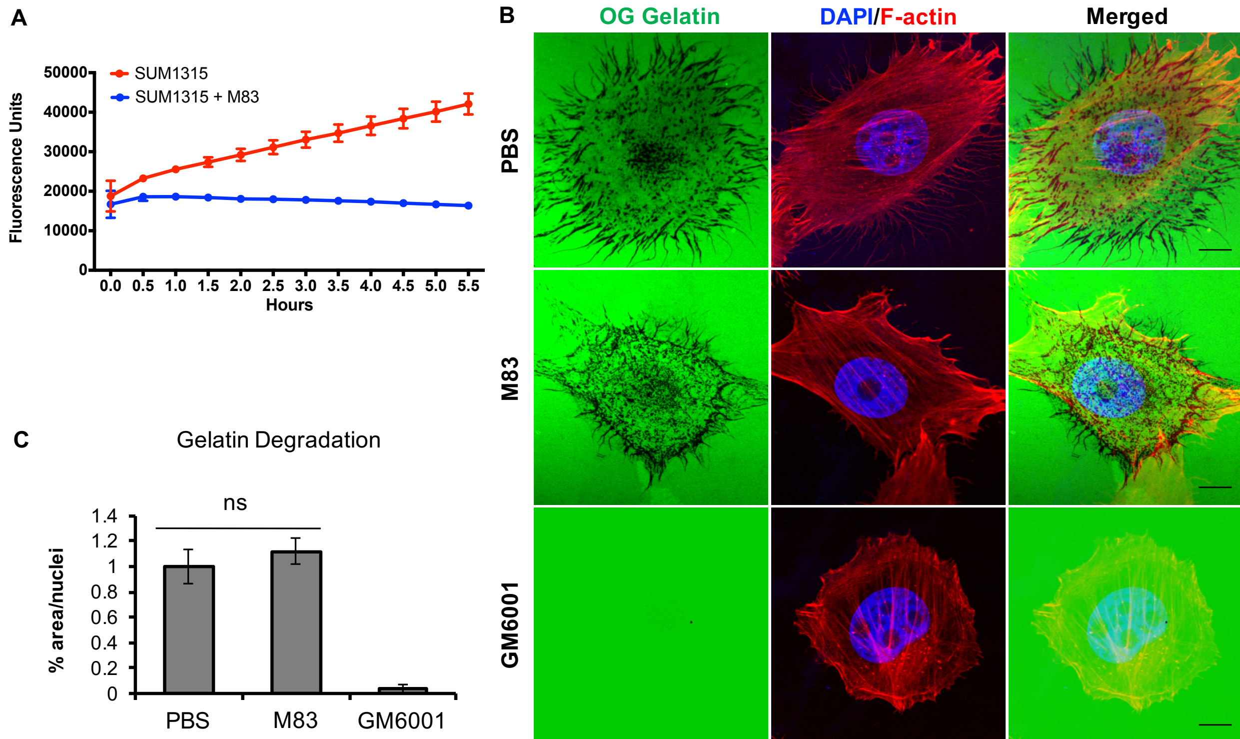

Figure 2-1: Inhibition of FAP's proteolytic activity does not reduce ECM degradation. A) Enzymatic assay to measure effect of M83, a FAP protease inhibitor, on FAP's proteolytic activity. B) Sample images of gelatin degradation assay using SUM1315 cells treated with M83 or GM6001: pan-MMP inhibitor (scale bar: 10μm). C) Quantification of gelatin degradation images (20 images per group).

We generated FAP point mutants that inhibited its proteolytic activity: The S624A mutant renders the proteolytic activity completely dead, and the A657S mutant has partial proteolytic activity (Figure 2-1-A). Wild-type and mutant FAP constructs were then transfected into FAP knockdown cells and the ability of the cells to degrade gelatin was assessed. FAP mutants could restore ECM degradation in FAP knockdown cells to a similar level as the wild type FAP constructs (Figure 2-2-B, C). Along with the FAP inhibitor treatment, these findings suggest that the proteolytic activity of FAP is not required for ECM degradation.

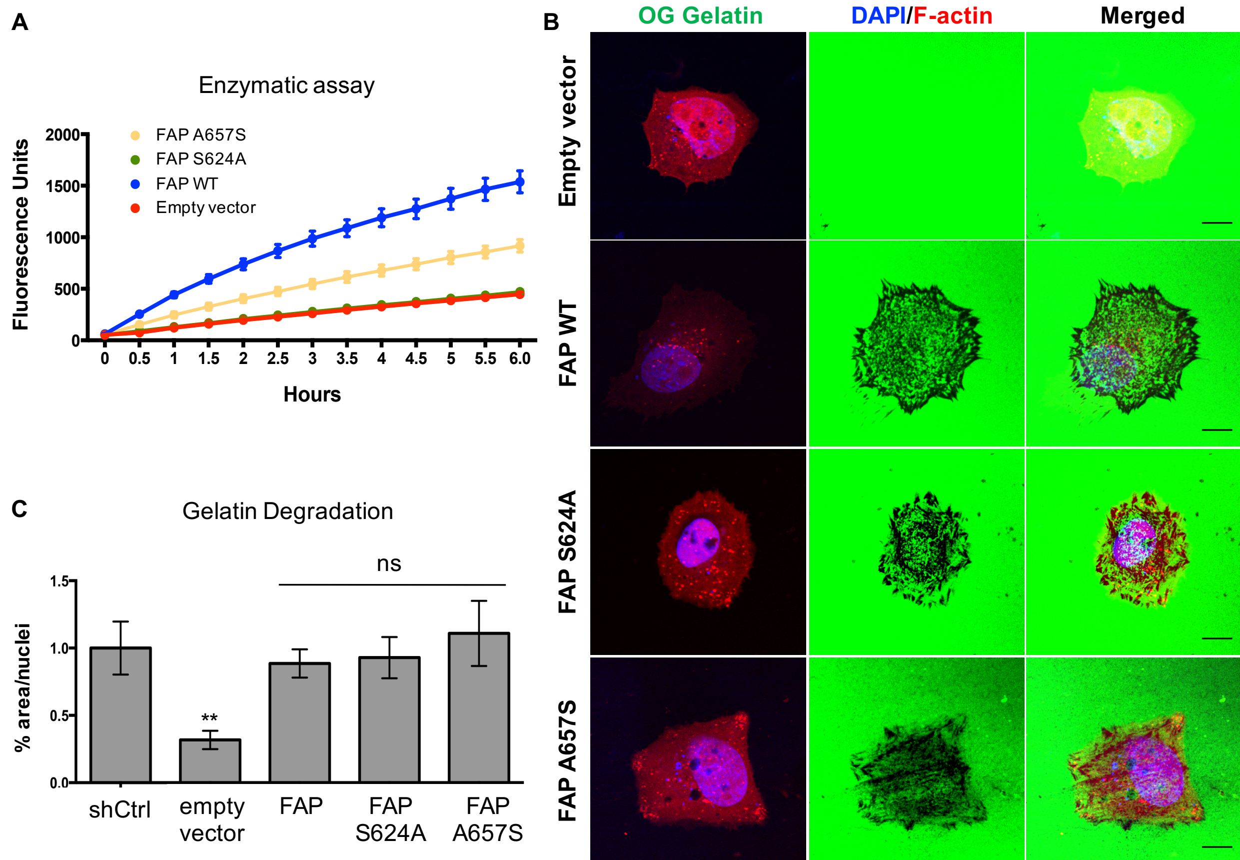

Figure 2-2: Proteolytic activity of FAP is not required for ECM degradation. A) Enzymatic assay to measure effect of proteolytic activity of FAP point mutants. B) Sample images of gelatin degradation assay performed on FAP knockdown cells transfected with wild-type or FAP point mutants (scale bar: 10µm). C) Quantification of gelatin degradation images (20 images per group). **, $P \leq 0.01$

A closer examination at FAP's structure reveals several interesting discoveries. FAP is a large integral membrane protein with the majority of the protein in the extracellular region (O'Brien et al., 2008). The middle of the protein is made up of 8 beta-propeller domains, which is capable of mediating protein-protein interactions. These observations suggest FAP may be involved in recruitment of other proteins such as proteases to invadopodia. Proper invadopodia function is orchestrated by numerous proteins and understanding the process in its entirety is a daunting task. In order to study the mechanism by which FAP regulates invadopodia function, we took two

approaches to characterize the interactions between FAP and other invadopodia proteins. The first approach, described in this chapter, is to determine the role of FAP in invadopodia dynamics. The second approach aims to identify novel FAP interacting proteins that functions at invadopodia, will be discussed in chapter 3.

2.2 FAP Localization in Cancer Cell Lines

Previous work in the lab characterized FAP localization and function in invadopodia using human epithelial cells with TWIST1 over expression (HMLE-TWIST1) or breast cancer cell line, SUM1315. Both cell lines posed limitations for further characterizing FAP's role at invadopodia. Therefore, we searched additional cancer cell lines to find better models for studying invadopodia dynamics *in vitro*. We selected SCC61, a head and neck squamous carcinoma cell line, and MDA-MB-231, an invasive triple negative breast cancer cell line, both of which form robust invadopodia for *in vitro* imaging purposes (Branch et al., 2012; Iorns et al., 2012). Given the role of Src kinase in initiating invadopodia formation, we decided to overexpress constitutively active hSrc (Src Y530F) in MDA-MB-231 cells (MDA-MB-231-Src) to better accompany our studies (Destaing et al., 2008; Sanchez-Bailon et al., 2012).

First, FAP localization and expression in SCC61 and MDA-MB-231 cells were characterized. Co-localization of the actin-binding protein Cortactin with F-actin puncta is used to properly identify invadopodia (Bowden et al., 2006). We found FAP that colocalizes with F-actin and Cortactin positive puncta in both cell lines, demonstrating that FAP is found in invadopodia of these cells (Figure 2-3).

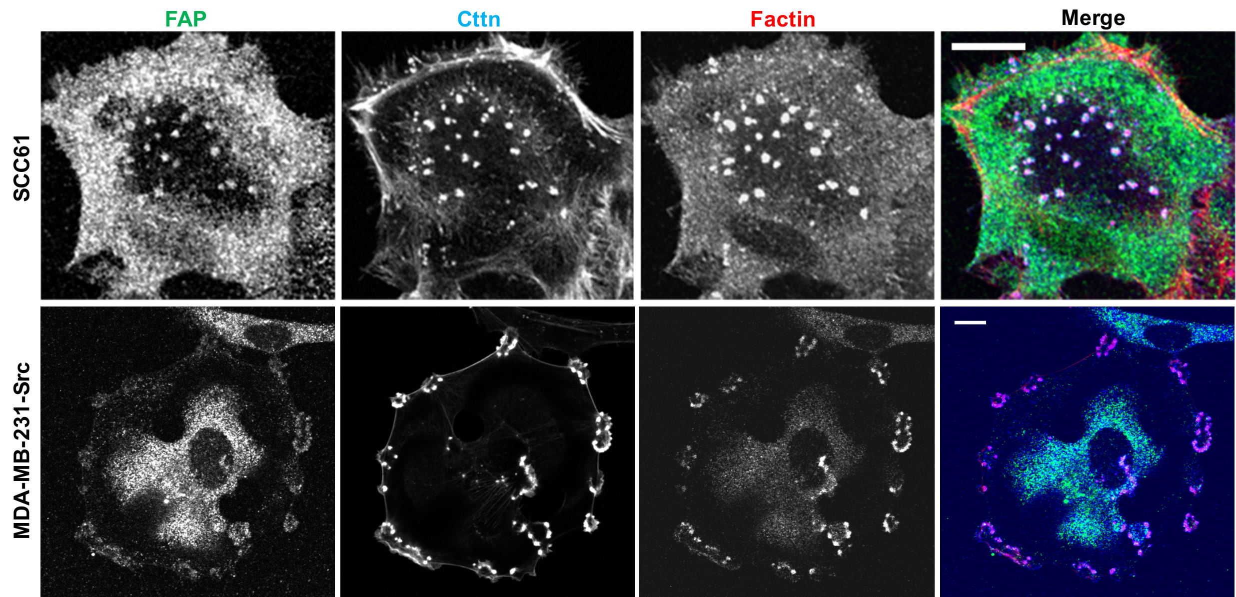

Figure 2-3: FAP localizes to invadopodia in SCC61 and MDA-MB-231-Src cells. Cells were stained for FAP, Cortactin, and F-actin. (scale bar: 10μm)

2.3 FAP is Required for ECM Degradation *In Vitro*

To assay invadopodia-mediated ECM degradation *in vitro*, gelatin-degradation assay is used. In short, cells were seeded on top of glass coverslips coated with fluorescently-labeled gelatin. After the optimal degradation period, cells were fixed and areas of degradation were imaged and quantified. Our lab previously demonstrated that FAP is required for ECM degradation in HMLE-TWIST1 and SUM1315 cell lines via the gelatin degradation assay. We set out to test whether FAP also regulated ECM degradation in the SCC61 and MDA-MB-231-Src cell lines. ShRNA-mediated FAP knockdowns were generated in both cell lines (Figure 2-4).

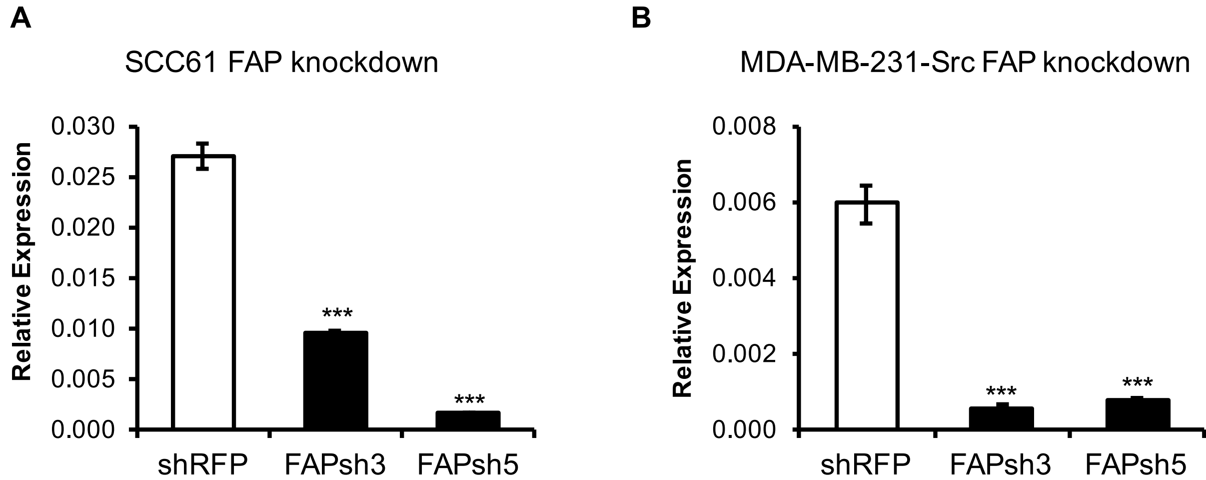

Figure 2-4: FAP knockdown cells were generated using shRNAs. A) qPCR was performed to measure FAP mRNA expression SCC61 cells. B) qPCR was performed to measure FAP mRNA expression MDA-MB-231-Src cells. ***, P ≤ 0.001

Gelatin degradation assays performed using FAP knockdown cells showed significantly reduced ECM degradation in SCC61 cells, suggesting that similar to previous findings, FAP also plays an important role in regulating invadopodia function in these cells (Figure 2-5).

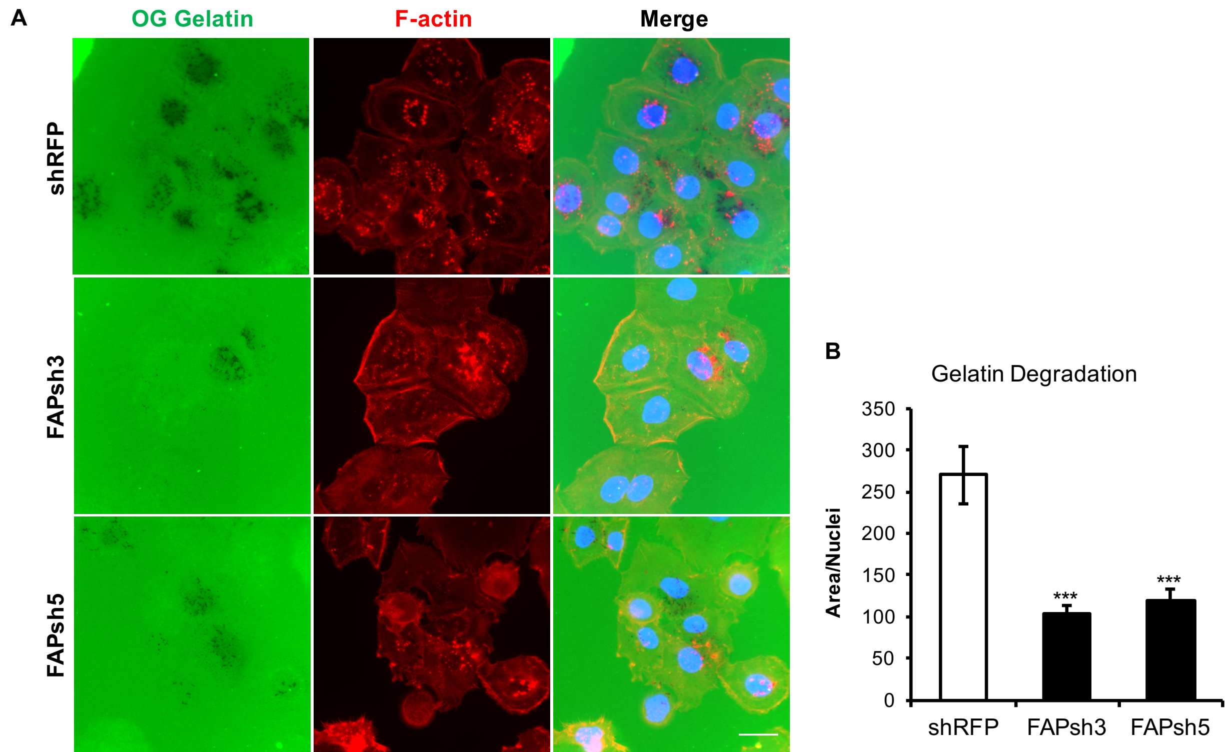

Figure 2-5: FAP is required for invadopodia-mediated ECM degradation in SCC61 cells. A) Sample images of gelatin degradation assay using SCC61 knockdown control and FAP knockdown cells (scale bar: 50µm). B) Quantification of gelatin degradation images (20 images per group). ***, P ≤ 0.001

FAP knockdown in MDA-MB-231-Src cells also exhibited similar defect in ECM degradation, suggesting FAP also plays an important role in regulating invadopodia function in these cells (Figure 2-6). Overall, the results demonstrate FAP knockdown exhibits interesting phenotype in these cells, and the two cell lines are good models for further *in vitro* characterization of FAP's function at invadopodia.

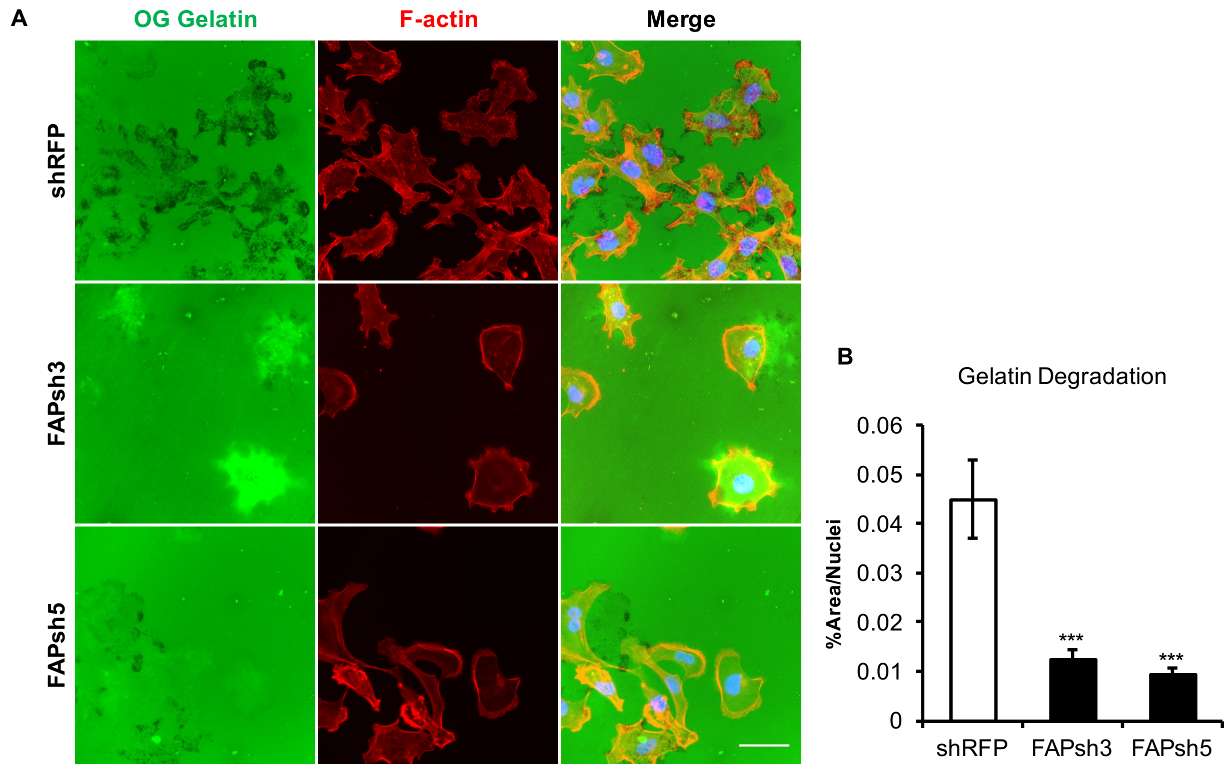

Figure 2-6: FAP is required for invadopodia-mediated ECM degradation in MDA-MB-231-Src cells. A) Sample images of gelatin degradation assay using MDA-MB-231-Src control and FAP knockdown cells (scale bar: 50μm). B) Quantification of gelatin degradation images (20 images per group). ***, $P \leq 0.001$

2.4 FAP is not Required for Invadopodia Initiation but Stabilization

Given our previous finding that FAP deficient cells exhibit defect in ECM degradation, we next wanted to determine whether loss of FAP caused a defect in invadopodia formation or recruitment of MMPs. Invadopodia formation was measured in both control and FAP depleted SCC61 cells using Alexa 546-Phalloidin to stain for F-actin. In SCC61 cells, loss of FAP expression significantly impacted invadopodia formation, which was quantified as percentage of cells that could form 5 or more invadopodia (Figure 2-7). This finding suggests that FAP is either an important player during invadopodia initiation or involved with stabilizing invadopodia formation.

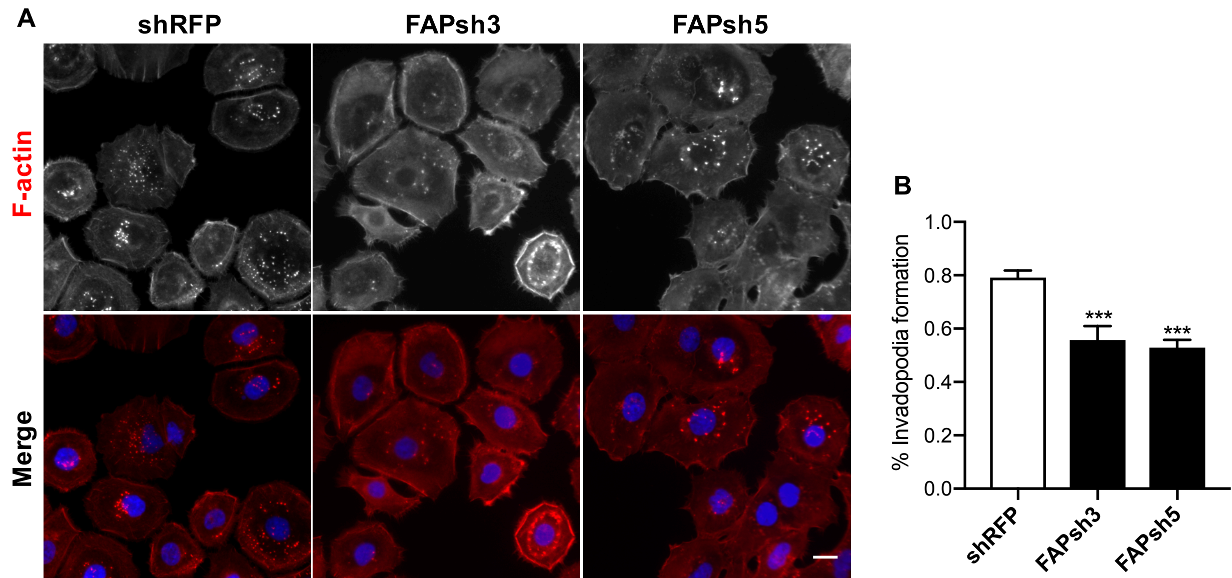

Figure 2-7: FAP knockdown effects invadopodia formation. A) Sample images of F-actin staining by Phalloidin in SCC61 control and FAP knockdown cells (scale bar: 20μm). B) Quantification of gelatin degradation images (20 images per group). ***, $P \leq 0.001$

To further test whether FAP regulates invadopodia initiation or invadopodia stabilization, we compared invadopodia initiation and stabilization in SCC61 control and FAP deficient cells. We utilized PP2, a Src kinase inhibitor that prevents invadopodia formation, to synchronize invadopodia formation by removing PP2 after cell attachment to matrix. Cells were fixed 5 minutes, 10 minutes, 25 minutes, 60 minutes, and 80 minutes post PP2 removal and the amount of Tks5 localization to invadopodia was quantified by ImageJ. Within the first 80 minutes following PP2 removal, there was no significant difference in Tks5 localization to F-actin between the control and FAP knockdown cells (Figure 2-8). However, in cells without PP2 treatment, there is significantly less Tks5 localization to invadopodia of FAP deficient cells. This is also correlated with a decrease in the ability of these cells to form invadopodia as compared to control cells. These findings suggest that FAP does not regulate invadopodia

initiation, but may be involved in the stabilization of already formed invadopodia. However, this might not completely explain why fewer FAP deficient cells form invadopodia over all. Additional studies to characterize FAP's role at invadopodia is needed.

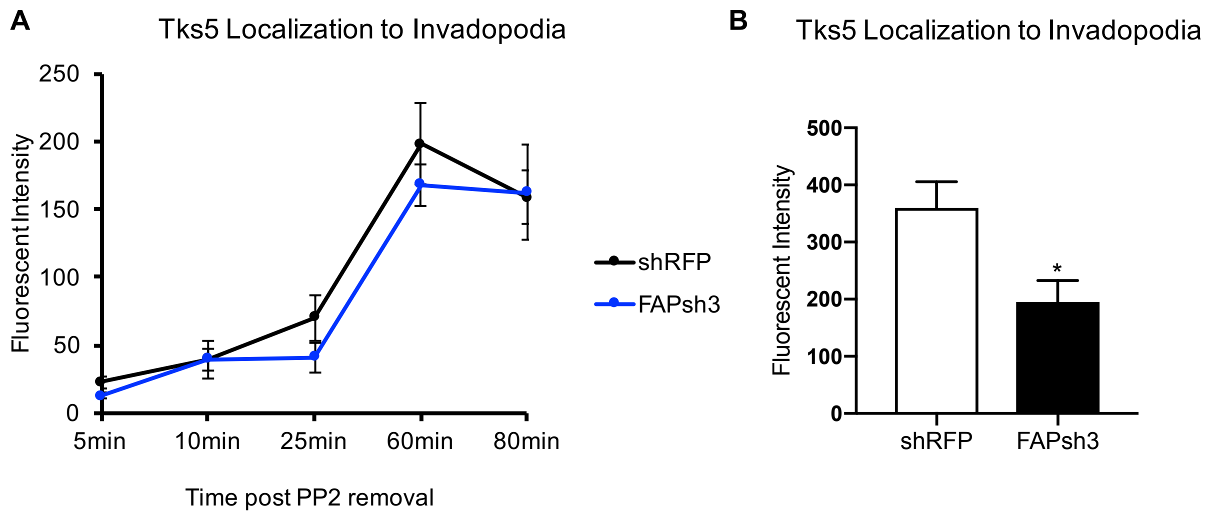

Figure 2-8: FAP is not required for invadopodia initiation but stabilization. A) Tks5 localization to invadopodia of SCC61 cells post PP2 removal as quantified by ImageJ. B) Tks5 localization to invadopodia in SCC61 cells without PP2 treatment. *, $P \leq 0.05$

2.5 Conclusion

In this chapter, we reported a novel role for Fibroblast Activation Protein (FAP) in invadopodia-mediated ECM degradation. FAP is a downstream target of transcription factor TWIST1, a master regulator of EMT and metastasis. We demonstrated that FAP is required for invadopodia-mediated matrix degradation and breast cancer metastasis *in vivo*. Moreover, the proteolytic activity of FAP is not required for invadopodia function. Though these observations have previously been characterized in the lab, more optimal cell lines were used in this study in order to allow for better characterization of FAP at invadopodia.

Transcription factor TWIST1 is a well-known regulator of EMT and metastasis. Our lab previously demonstrated that TWIST1 regulates metastasis by promoting invadopodia formation and function (Eckert et al., 2011). We built on this previous work by identifying FAP as a downstream target of TWIST1. Given that TWIST1 does not induce the expression of other important invadopodia proteins such as Cortactin, Tks4/5, MT1-MMP, the TWIST1/FAP pathway warranted further investigation. A closer examination of FAP induction by TWIST1 showed that FAP mRNA expression was increased 4-fold at 3 days post-TWIST1 induction, showing a delay FAP induction following TWIST1 expression and suggesting that FAP is an indirect target of TWIST1. In an effort to identify intermediates of the TWIST/FAP pathway, we used a multiple sequence alignment tool from BCBI DCODE and identified two basic leucine zipper transcription factors, BACH2, and MAFB, that have two consensus binding sites on the human FAP promoter (Pennacchio et al., 2007). Maf proteins have DNA-binding abilities, but lack any transactivation activity. They heterodimerize with Bach family of proteins to regulate transcription, reported roles for this complex includes B-cell specific transcriptional repression (Muto et al., 2004; Muto et al., 2002; Oyake et al., 1996). Similar to TWIST1, MAFB is also expressed in migrating neural crest cells in development and mutations in MAFB is associated with craniofacial defects (Beaty et al., 2010; McGonnell et al., 2001). These findings suggest MAFB/BACH2 likely act downstream of TWIST1 to directly regulate FAP expression. To test this, time points FAP mRNA and protein expression following BACH2/MAFB induction will be collected and quantified. Additionally, luciferase reporter assays using the FAP promoter or mutations in the putative BACH2/MAFB binding sites will help establish whether

BACH2/MAFB directly binds FAP promoter and whether the binding occurs at the putative sites. Identifying regulators of FAP expression will help us better understand how a protein with such tissue specific expression is induced during tumorigenesis.

Our studies demonstrate a protease-independent role for FAP during invadopodia-mediated ECM degradation. However, the exact role of FAP at invadopodia is unclear. In FAP knockdown cells, few percentage of cells can successfully form invadopodia, suggesting that FAP impacts invadopodia formation in a subset of the cells. Additionally, FAP is not required for the recovery of invadopodia initiation following PP2 removal, but may be required for stable invadopodia formation, as evidenced by the reduction in Tks5 localization to invadopodia in FAP knockdown cells. These findings suggest FAP may regulate invadopodia stabilization. To characterize the role of FAP in invadopodia dynamics, time lapse live imaging will be performed using Lifeact-mCherry. Lifeact is a 17-amino acid peptide that binds F-actin and does not interfere with actin dynamics (Riedl et al., 2008). mCherry allows live cell visualization of F-actin dynamics without additional sample preparations. Using this system, the average lifetime of and distance traveled by invadopodia in control and FAP knockdown cells will be measured. Since Tks5 and Cortactin are important proteins throughout invadopodia formation, characterizing the dynamic relationships between FAP and Tks5 and Cortactin will help us better understand FAP. Time lapse live imaging will be performed on SCC61 control and FAP knockdown cells overexpressing fluorescently labeled Tks5, Cortactin, and Lifeact-mCherry to determine the dynamics of these proteins at invadopodia.

In addition to acting as a scaffold protein during invadopodia initiation, FAP may also regulate invadopodia formation through interactions with integrin β1. As important signaling molecules that facilitate ECM and cellular communications, integrins are in great position to regulate invadopodia formation. Integrin β1 has been found to localize to invadopodia of a number of cancer cell lines like LOX melanoma cells and MDA-MD-231 cells (Bowden et al., 1999; Seals et al., 2005). Furthermore, α3β1 integrin has been shown to complex with FAP in LOX melanoma cells (Mueller et al., 1999). Our preliminary studies also showed a defect in β1 integrin recruitment to invadopodia in FAP knockdown cells (not shown). These findings suggest that the β1 integrin-FAP interaction at invadopodia is important and may be required for stabilization of the protrusion for proper invadopodia formation. Additional studies are required to further understand the relationship between β1 integrin and FAP and whether their interaction is important for invadopodia stabilization. First, immunoprecipitation and immunofluorescent staining studies will be performed to test whether β1 integrin and FAP interact and localize together at invadopodia. Next, live imaging of SCC61 cells treated with antibodies preventing β1 integrin-FAP interaction or expressing FAP mutants that cannot interact with β1 integrin will be used to measure the effect of blocking β1 integrin-FAP interaction on invadopodia formation.

The above hypotheses suggest that FAP may serve multiple functions at invadopodia. To test whether that's true, the timing of FAP recruitment will be characterized. SCC61-Lifeact-mCherry cells expression fluorescently-tagged FAP, Tks5 or MT1-MMP will characterized via time lapse live imaging. We can use PP2, a Src kinase and therefore invadopodia formation inhibitor, to synchronize invadopodia

formation by removing PP2 after cell attachment to matrix. FAP recruitment to invadopodia will be compared to the onset of F-actin polymerization and Tks5 and MT1-MMP recruitment to F-actin. Knowing the timing of FAP recruitment to invadopodia will help us better understand during which step of invadopodia formation it is involved in.

Previous in vivo studies indicated the importance of FAP in TWIST1-mediated metastasis. Given that FAP is required for invadopodia-mediated matrix degradation, breaching basement membrane (BM), intravasation, and extravasation are all possible steps required FAP regulation. Further experiments are need to better understand which step of metastasis FAP is required. Following primary tumor formation by control and FAP knockdown cells, counting the number of circulating tumor cells would help determine whether FAP regulates BM breaching and intravasation. Tail vein injection of control and FAP knockdown cells would allow us to identify whether FAP regulates intravasation or extravasation step of metastasis. Given its unique expression pattern and function in breast cancer metastasis, there is no doubt FAP is a promising therapeutic target. A better understanding of the protease-independent role of FAP at invadopodia will bring us closer towards an effective anti-metastasis treatment.

2.6 Materials and Methods

2.6.1 Cell Lines and Cell Culture

Human mammary epithelial cells (HMLE), HMLE-TwistER, and the SUM1315 cell line were cultured as previously described (Yang et al., 2004). The SCC61 cell line was cultured in DMEM with 10% FBS and 0.5ug/ml hydrocortisone. The MDA-MB-231 cell line was cultured in DMEM with 10% FBS. All cell lines also had 1% penicillin and

streptomycin in the culture media. Viral production and infection to produce stable knockdown cell lines was carried out as previously described (Eckert et al., 2011).

2.6.2 Plasmids

Control shRNAs in the pLKO.1 vector were obtained from the Sabatini lab (Addgene) and purchased from Sigma. ShRNAs targeting FAP were purchased from Sigma and have the following targeting sequences: FAP sh3: GCATTGTCTTACGCCCTTCAA; FAP sh5: TGATAATCTTGAGCACTATAA.

2.6.3 Western Blotting

Cells at 80-90% confluency were washed with cold PBS and lysed in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10 mM NaF, 1% Triton X-100), with 100 μM Na_3VO_4, PMSF, and the Protease Inhibitor Cocktail Set III (Calbiochem) diluted 1:200. Protein lysates were boiled in 50mM DTT LDS sample buffer at 70°C for 10 minutes and then ran on 4-12% pre-cast gels (PAGEgel). Antibodies used for western blotting were diluted in 1% BSA in PBST (1X PBS+1% Tween 20) + Sodium Azide.

The following antibodies were used for western blotting:

FAP (1:500, D8, Vitatex), GAPDH (1:2500, GeneTex).

2.8.4 Quantitative Real-Time PCR

Total RNA from cells at 80-90% confluency using the NucleoSpin RNA II kit (Macherey-Nagel), and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNAs were analyzed in triplicate using the SYBR-Green PCR Mix. Relative mRNA levels were determined by $2^{-(Ct-Cc)}$, where Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH values. Primers used for qRT-PCR are as follows:

HPRT:
Forward: ACGTCTTGCTCGAGATGTGA
Reverse: TCCCCTGTTGACTGGTCATT

FAP:
Forward: AATGAGAGCACTCACACTGAAG
Reverse: CCGATCAGGTGATAAGCCGTAAT

2.8.5 ECM Degradation Assays

ECM degradation assays were carried out as previously described (Artym et al., 2009; Eckert et al., 2011).

2.8.6 Immunofluorescense

Prior to cell seeding, coverslips were coated with 100ug/ml rat tail collagen (Corning) diluted in DPBS for 30mins at 37°C, then washed once in 1X DPBS. Cells were seeded on the coated coverslips for at least 48 hours and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at 37°C. Cells were washed 3 times with 1X PBS, permeabilized with 0.25% Triton X-100 in PBS for 5 minutes, and then blocked with 1% BSA in PBS for 1 hour. Samples were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 hour at room temperature. Samples were then washed 3 times with 1X PBS, then mounted with VECTASHIELD Anti-fade Mounting Medium with DAPI (Vector Laboratories) prior to imaging. Confocal images were acquired using an Olympus FV1000 with 405, 488, 555, and 647 laser lines. Images were linearly analyzed and pseudo-colored using ImageJ analysis software. Primary antibodies used for ICC include Cortactin (1:200, 4F11, Millipore), FAP (1:500, D28, Vitatex). Secondary antibodies used include Alexa Fluor 488 and 546 (Invitrogen). Alexa 546-Phalloidin was used to label F-actin (Invitrogen).

2.8.7 FAP Enzymatic Activity Assay

SUM1315 cells were plated in 96-well plates 48 hours prior to assay, and washed 3 times with 1X PBS. The wash solution was replaced with fresh PBS, and either additional PBS or 10 μM M83 inhibitor was added. 125 μM C95 substrate was added to all wells, and then fluorescence emission was measured at 360/460 nm excitation/emission wavelengths. The M83 inhibitor and the C95 substrate were generously provided by Dr. Patrick McKee.

2.8.8 Statistical Analysis

All statistical analysis was carried out using Prism8 software. Student t-test was performed to determine statistical significant between two test groups. One-way ANOVA test was carried out to determine statistical significance of three or more test groups.

2.9 Acknowledgements

Chapter 2, in full, is currently being prepared for submission for publication of the material. Pathak N., Santiago-Medina M., Jiang Y., Li D.M., Jackson K.W., Christiansen V.J., McKee P.A., Yang J. Fibroblast Activation Protein (FAP) regulates invadopodia activity and promotes tumor metastasis. *In preparation.* The dissertation author was one of the primary investigator and co- first author of this material.

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CHAPTER 3

Identification of Novel Regulator of Invadopodia Function and Tumor Invasion

3.1 Introduction

As described in chapter 2, in an effort to identify downstream targets of transcription factor TWIST1 that regulates invadopodia function, we discovered Fibroblast Activation Protein (FAP). We demonstrated that FAP is required for invadopodia function; cells with FAP knocked down exhibited significant reduction in ECM degradation as measured by the gelatin degradation assay. Furthermore, gelatin degradation assays performed using FAP protease inhibitor or FAP point mutants that block its proteolytic activity showed no significant difference in ECM degradation. These results indicate that the proteolytic activity of FAP is not required for invadopodia function.

To understand how FAP regulates invadopodia function independent of its protease activity, we decided to further examine its protein structure. FAP is a large transmembrane protein made up of 760 amino acids (aa.). FAP homodimerization is required for its proteolytic activity (Wonganu & Berger, 2016). Of the 760 aa., only 6 aa. makes up the cytoplasmic tail, followed by a 21 aa. single-pass transmembrane domain. The rest of the protein is in the extracellular region, made up of two domains: the beta-propeller domain and the catalytic domain (O'Brien & O'Connor, 2008). According to the crystal structure of FAP, there are 8 beta-propeller blades found in each FAP monomer (Figure 3-1). The 8-bladed beta-propeller domain is proposed to act as structural protein that can mediate protein-protein interactions.

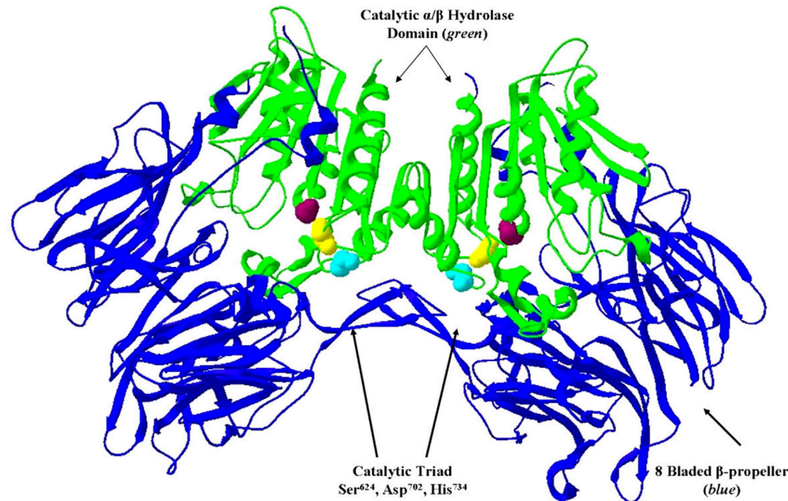

Figure 3-1: Crystal Structure of FAP Dimer. Eight blades of beta-propeller shown in blue. The catalytic domain is labeled in green. Figure is from O'Brien & O'Connor, 2008.

Combining our preliminary data and our understanding of FAP's protein structure, we hypothesized that FAP may function as a structural scaffold to help recruit other proteins such as proteases to invadopodia. In order to test this hypothesis, we took two approaches to characterize the interactions between FAP and other invadopodia proteins. The first approach, described in chapter 2, was to determine the dynamics of FAP with known invadopodial proteins, including Cortactin, Tks5, and MT1-MMP. The second approach, discussed in this chapter, aimed to identify novel FAP interacting proteins that functions at invadopodia.

3.2 Generating MycHis-FAP

To identify novel FAP interacting proteins, we took a proteomics-based approach compromised of immunoprecipitation followed by mass spectrometry (IP/MS). We generated C-terminal Myc-His tagged FAP (MH-FAP) in the pCDNA4B plasmid, which can be used for transient expression in cells. The MH-FAP protein was characterized to assess whether C-terminal tagging of FAP would effect protein folding or prevent

protein-protein interactions at invadopodia. Transient transfection of MH-FAP in HEK293T cells showed comparable level of expression as wild type FAP (Figure 3-2).

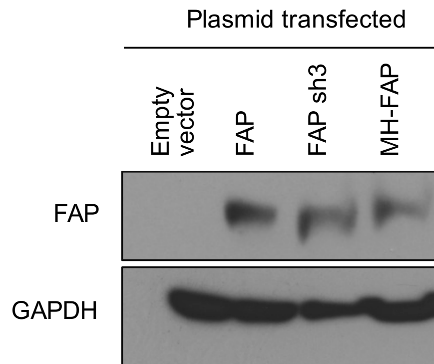

Figure 3-2: MH-FAP is expressed. Western blot of HEK293T cells lysates containing transiently transfected with wild type FAP, shRNA#3 knockdown resistant FAP, and MH-FAP.

Since the purpose of the IP/MS is to identify proteins interacting with FAP at invadopodia, we first tested whether MH-FAP localized to the structure. MH-FAP was expressed in Hs578T cells, a breast cancer cell line that forms robust invadopodia. We saw MH-FAP protein co-localizing with Cortactin and F-actin at invadopodia, indicating the tagged protein exhibits proper cellular localization (Figure 3-3).

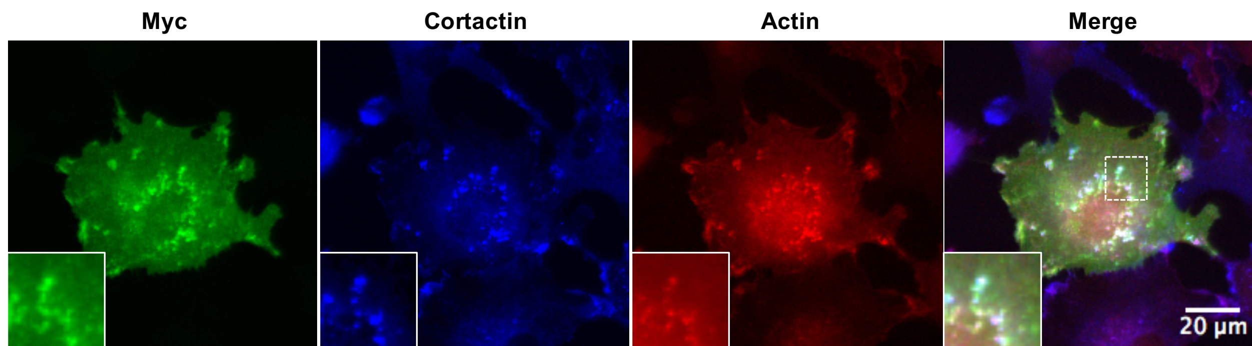

Figure 3-3: MH-FAP localizes to invadopodia in breast cancer cells. MH-FAP construct was transiently transfected in Hs578T cells and stained for Myc (MH-FAP), Cortactin, and F-actin. Sample areas of colocalization shown in inset.

Next, to determine whether MH-FAP exhibits proper protein folding, we performed an enzymatic assay to measure the proteolytic activity of FAP. It is known

that homodimerization is required for FAP's catalytic function, and proper protein folding is a prerequisite for homodimerization and therefore catalytic function (Wonganu et al., 2016). To that end, measuring the catalytic activity of MH-FAP protein would indicate whether the protein is folded correctly. Cells expressing MH-FAP displayed similar level of enzymatic activity as cells expressing wild-type FAP (Figure 3-4). This finding indicates that the Myc-His tag does not impact FAP folding.

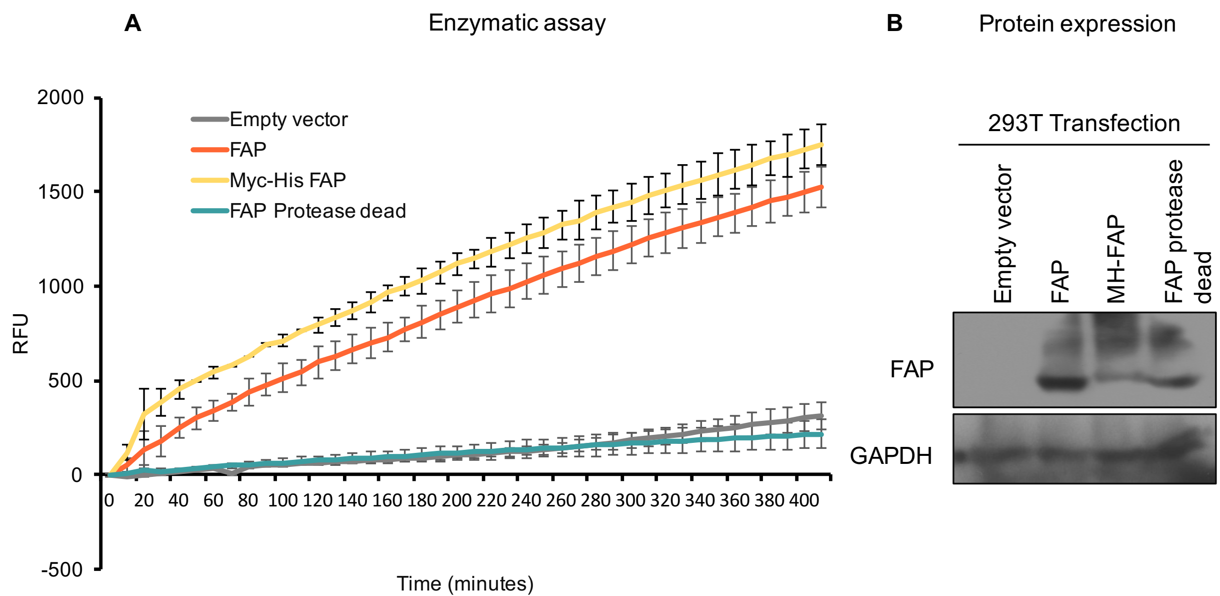

Figure 3-4: MH-FAP exhibits proper protein folding. A) Enzymatic activity to measure the functionality and protein folding of MH-FAP. B) Western blot of protein lysates collected from cells used in the enzymatic assay.

Lastly, to determine whether MH-FAP was functional at invadopodia, we measured the ability of MH-FAP to rescue ECM degradation in SUM1315 cells with FAP knocked down. MH-FAP rescued ECM degradation similar to the level achieved by wild type FAP, suggesting it was functional (Figure 3-5). All together, the findings demonstrate that MH-FAP behaves similarly as wild type FAP and can be used as the bait in the IP/MS.

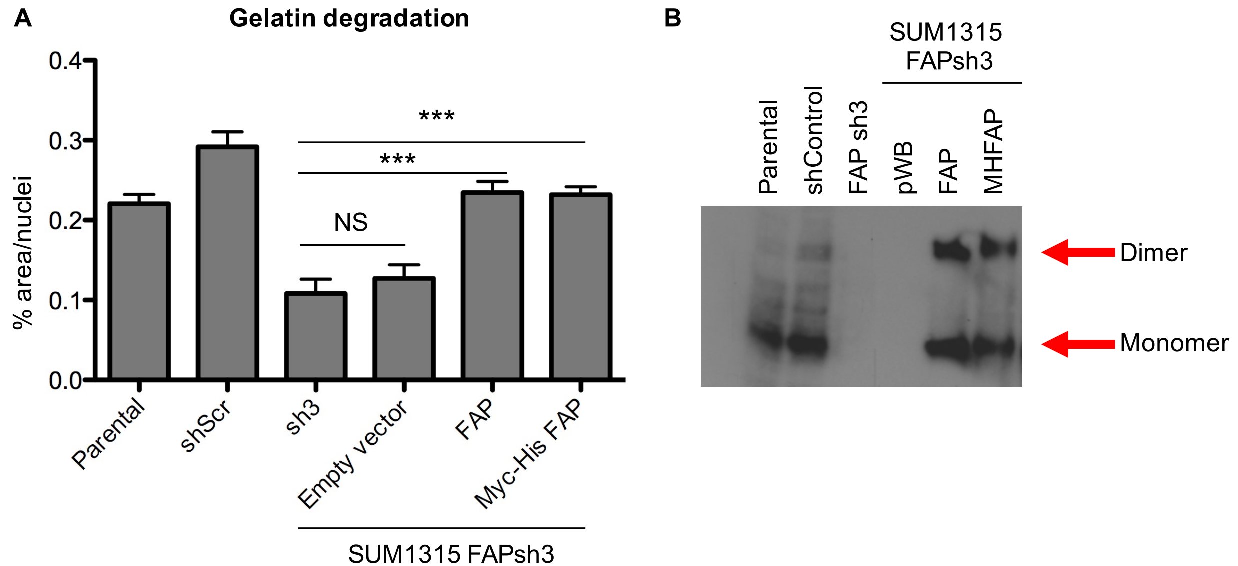

Figure 3-5 MH-FAP is a Functional Construct. A) MH-FAP can rescue ECM degradation defect in FAP knockdown cells. ***, P ≤ 0.001. B) Western blot of wild-type and MH-FAP expression in SUM1315 FAP knockdown cells.

3.3 Identification of FAP Interacting Proteins

Our initial IP/MS yielded no known invadopodia proteins, we suspected that the protein interactions may be transient and low affinity. In order to enrich for those interactions and remove background, we utilized dithibis(succinimidyl propionate) DSP to crosslink proteins to MH-FAP prior to stringent imidazole and NaCl wash conditions (Figure 3-6). DSP is a membrane-permeable reversible crosslinker and has been used to enrich for low-affinity, transient protein interactions and minimize non-specific backgrounds in IP/MS (Smith et al., 2011).

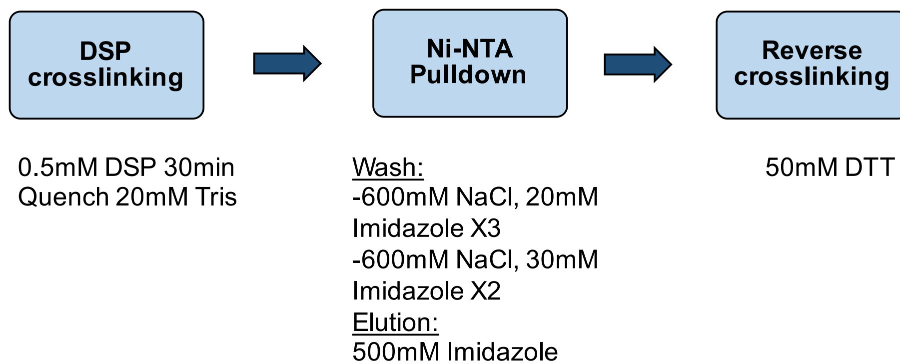

Figure 3-6: Workflow of crosslinking IP. The IP workflow includes DSP crosslinking, reaction quenching, stringent NaCl and Imidazole wash conditions, and reverse crosslinking.

Using DSP to enrich weak and transient binding partners of invadopodia, we successfully performed the IP/MS. From the mass spectrometry data, we subtracted from the MH-FAP IP results list proteins that were also identified in the parental negative control group. Upon examining the proteins that are unique to the MH-FAP sample, the bait itself had the highest number of peptide counts (26 counts), validating the experimental set up. We then removed nuclear proteins and common protein contaminants based on CRAPome database from the list. Lastly, we selected for proteins that may function in cell motility and have been implicated in tumor invasion. Fibronectin type III domain containing 3B (FNDC3B) stood out as an attractive candidate.

FNDC3B, also known as Factor for Adipocyte Differentiation 104 (FAD104), has been reported to be an important regulator of adipocyte differentiation (Tominaga et al., 2004). Knockdown of FNDC3B dramatically repressed adipogenesis in cell lines, and FNDC3B-deficient mice all died at day 1 after birth (Nishizuka et al., 2009). Additionally, cell adhesion and wound healing assays performed with FNDC3B-deficient MEFs

exhibited reduction in stress fiber formation, delayed cell adhesion, spreading, and migration (Nishizuka et al., 2009). According to 5547 tumor samples from TCGA, the FNDC3B gene resides in chromosome location 3q26, which contains 20 genes that have been reported to be amplified many types of cancer including lung squamous cell, serous ovarian, cervical squamous cell, head and neck, pancreatic, to name a few (Hagerstrand et al., 2013). Overexpression of FNDC3B can transform epithelial cells *in vitro and* induce EMT. FNDC3B also promotes cell migration and invasion in esophageal squamous cell carcinoma and hepatocellular carcinoma cells (Lin et al., 2016; Yang et al., 2017). Overall, these findings suggest FNDC3B is implicated in tumor invasion and may do so through regulating invadopodia function.

3.4 FNDC3B Interacts Transiently with FAP

The mass spectrometry result suggests FAP and FNDC3B interactions with each other. To validate this interaction, we immunoprecipitated endogenous FAP in SUM1315 cells to detect FNDC3B. The result showed that FNDC3B interacts with FAP only under chemical-crosslinking condition half the time, suggesting the interaction may be transient and possibly indirect (Figure 3-7). We also performed the IP on HEK293T cells overexpressing MH-FAP and GFP-FNDC3B, but did not detect any interaction (not shown). Since HEK293T cells do not form invadopodia or degrade the ECM, this suggests the FAP-FNDC3B interaction requires the presence of invadopodia.

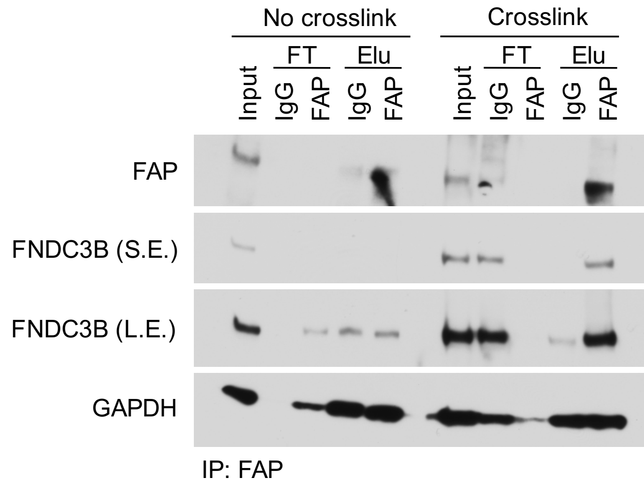

Figure 3-7: FAP and FNDC3B interaction requires chemical crosslinking. Immunoprecipitation of endogenous FAP in SUM1315 cells under non-crosslinking and crosslinking conditions. S.E.: shorter exposure; L.E.: longer exposure.

3.5 FNDC3B Localization and Orientation in Cancer Cells

Previous reports associated FNDC3B localization to the ER and Golgi network (Cai et al., 2012; Lin et al., 2016). These experiments were performed on cells overexpressing tagged FNDC3B or on endogenous FNDC3B imaged at low magnifications. We imaged SCC61 and MDA-MB-231-Src cells at high magnification, focusing on the plasma membrane, and found endogenous FNDC3B colocalized with F-actin and Cortactin positive puncta, demonstrating that FNDC3B is found in invadopodia of these cells (Figure 3-8).

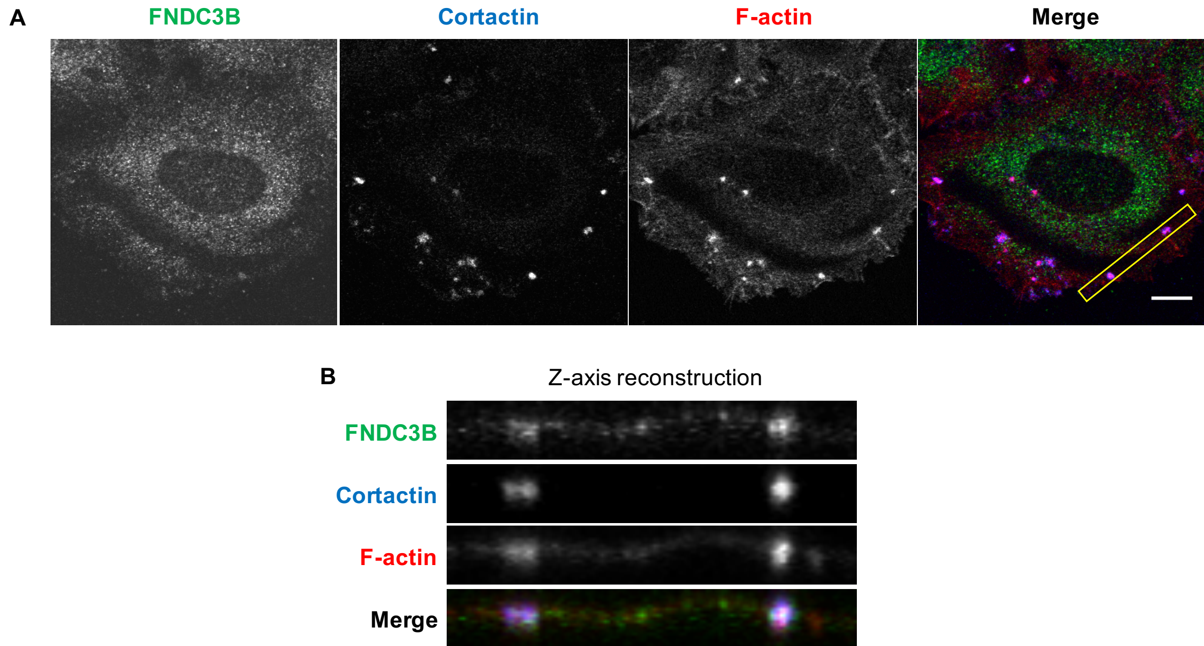

Figure 3-8: FNDC3B localizes to invadopodia in SCC61 cells. Cells were stained using antibodies against FNDC3B, Cortactin, and Phalloidin-546 (for F-actin). A) Whole image, (scale bar: 10μm). B) Z-axis reconstruction of selected area in A) by yellow rectangle.

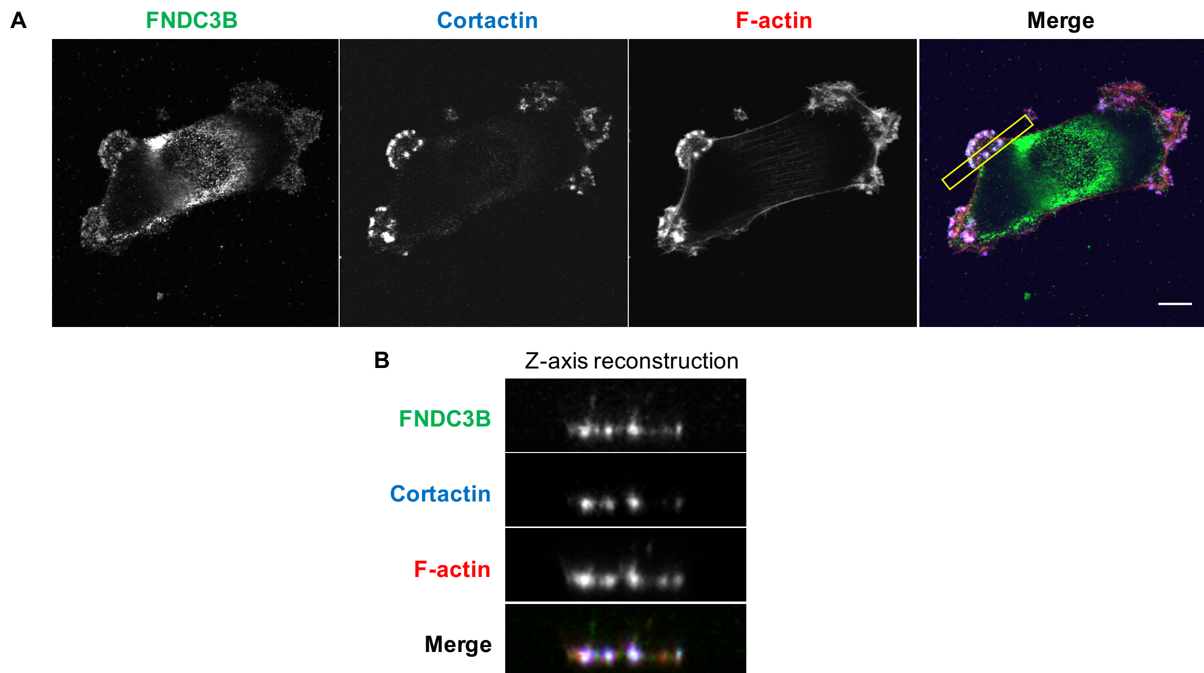

Figure 3-9: FNDC3B localizes to invadopodia in MDA-MB-231-Src cells. Cells were stained using antibodies against FNDC3B, Cortactin, and Phalloidin-546 (for F-actin). A) Whole image, (scale bar: 10μm). B) Z-axis reconstruction of selected area in A) by yellow rectangle.

Given FAP is an extracellular membrane protein that has only 6 aa. in the cytoplasmic domain, its interaction with FNDC3B is likely at the extracellular region. However, the FNDC3B protein orientation is unknown. FNDC3B contains a transmembrane domain in the C-terminus, suggesting the protein is either completely extracellular- or intracellular (Figure 3-10). To determine FNDC3B's protein orientation at the membrane, we used an antibody that recognizes the C-terminal region of FNDC3B in a non-permeabilizing condition. Under this condition, FNDC3B antibody is unable to enter the cell, therefore can only recognize the epitope if it is presented on the outside of the cell. We saw diffused FNDC3B staining and loss of FNDC3B staining at invadopodia under non-permeabilizing conditions, suggesting the epitope is found inside the cell (Figure 3-11). Since FNDC3B's transmembrane domain is in the C-terminus, this suggests the majority of the protein is intracellular. This observation begs the question: how does FAP interact with FNDC3B? This interaction could be mediate by the 6aa. intracellular domain on FAP or through intermediate proteins.

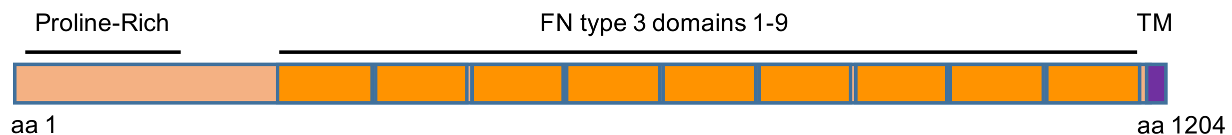

Figure 3-10: Structural schematic of FNDC3B protein. FNDC3B has an N-terminal proline-rich domain, followed by nine Fibronectin (FN) type 3 domains, then a C-terminal transmembrane (TM) domain.

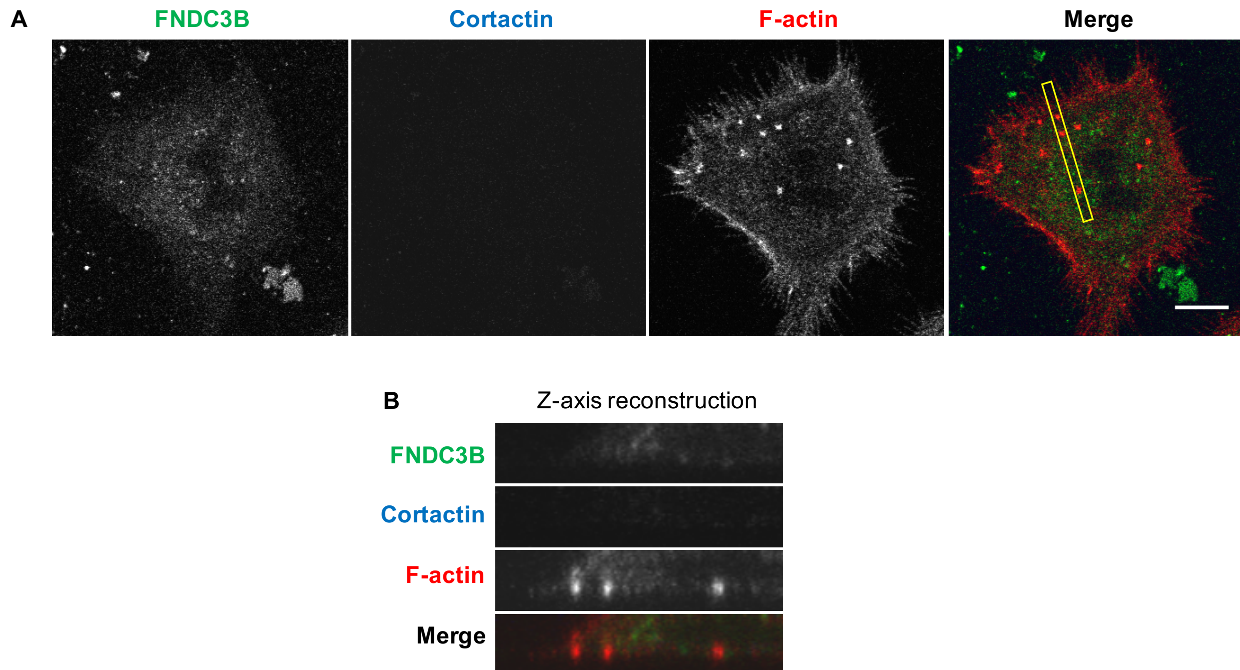

Figure 3-11: FNDC3B is an intracellular protein. SCC61 cells were stained under non-permeabilizing conditions using antibodies against FNDC3B, Cortactin, and Phalloidin-546 (for F-actin). A) Whole image, (scale bar: 10μm). B) Z-axis reconstruction of selected area in A) by yellow rectangle.

3.6 FNDC3B is Required for ECM Degradation *In Vitro*

FNDC3B is reported to promote cell migration and invasion in esophageal squamous cell carcinoma and hepatocellular carcinoma cells (Lin et al., 2016; Yang et al., 2017). To test whether FNDC3B regulates cell migration and invasion via regulating the formation and/or function of invadopodia, gelatin degradation assay was performed using the SCC61 and MDA-MB-231-Src shRNA-mediated FNDC3B knockdown cell lines (Figure 3-12).

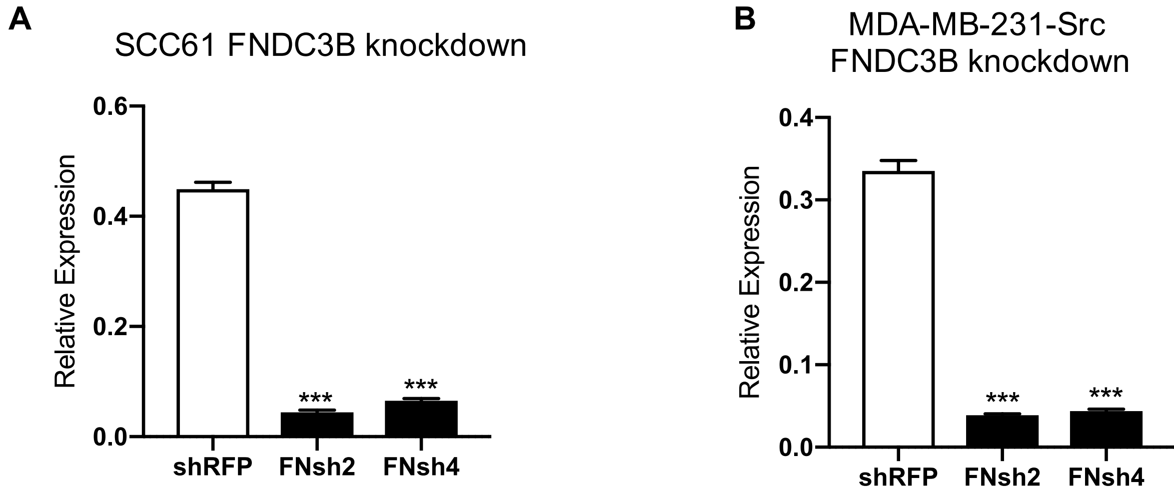

Figure 3-12: FNDC3B knockdown cells were generated using shRNAs. A) qPCR was performed to measure FNDC3B mRNA expression SCC61 cells. B) qPCR was performed to measure FNDC3B mRNA expression MDA-MB-231-Src cells. ***, $P \leq 0.001$

Gelatin degradation assays performed using FNDC3B knockdown cells showed significantly reduced ECM degradation in SCC61 cells, suggesting that FNDC3B plays an important role in regulating invadopodia function in these cells (Figure 3-13).

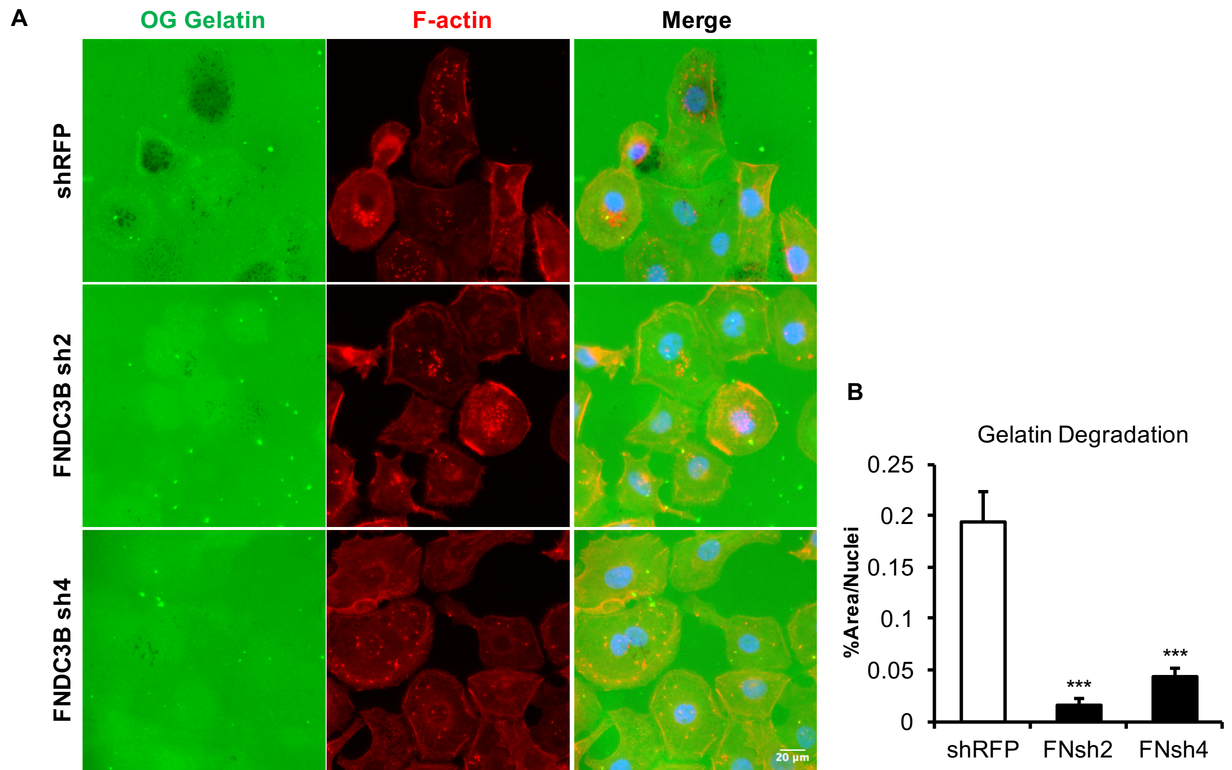

Figure 3-13: FNDC3B is required for invadopodia-mediated ECM degradation in SCC61 cells. A) Sample images of gelatin degradation assay using SCC61 knockdown control and FNDC3B knockdown cells (scale bar: 20μm). B) Quantification of gelatin degradation images (30 images per group). ***, P ≤ 0.001

FNDC3B knockdown in MDA-MB-231-Src cells also exhibited similar defect in ECM degradation, this shows FNDC3B's role in invadopodia function is not SCC61 specific (Figure 3-14). Since the loss of FNDC3B has a similar effect as the loss of FAP as described in chapter 2, the two proteins may cooperate together to regulate invadopodia function. The question remains: How does FNDC3B regulate invadopodia function? What is the relationship between FAP and FNDC3B? How do they work together to recruit proteins to invadopodia?

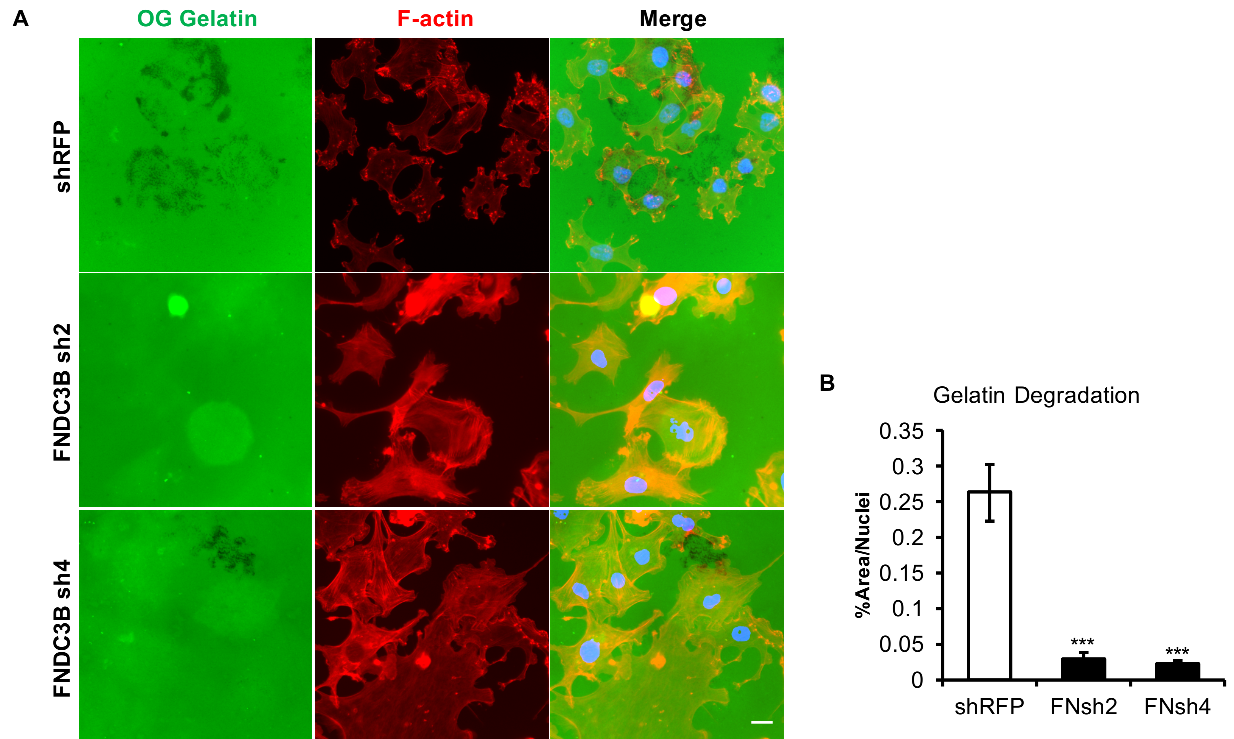

Figure 3-14: FNDC3B is required for invadopodia-mediated ECM degradation in MDA-MB-231-Src cells. A) Sample images of gelatin degradation assay using MDA-MB-231-Src knockdown control and FNDC3B knockdown cells (scale bar: 20µm). B) Quantification of gelatin degradation images (30 images per group). ***, P≤ 0.001

3.7 FNDC3B is Not Required for Invadopodia Formation

Given that FNDC3B deficient cells exhibit similar phenotype as FAP deficient cells so far, we next wanted to test whether FNDC3B was also involved in invadopodia formation. Invadopodia formation was measured in both control and FAP depleted SCC61 cells using Alexa 546-Phalloidin, a fluorescent probe for F-actin. In SCC61 cells, loss of FNDC3B expression had no significant effect on invadopodia formation, which was quantified as percentage of cells that could form 5 or more invadopodia (Figure 3-15). This suggests that FNDC3B is not an important player during invadopodia initiation, as evidenced by the F-actin puncta formation. FNDC3B is most likely involved in later steps of invadopodia formation.

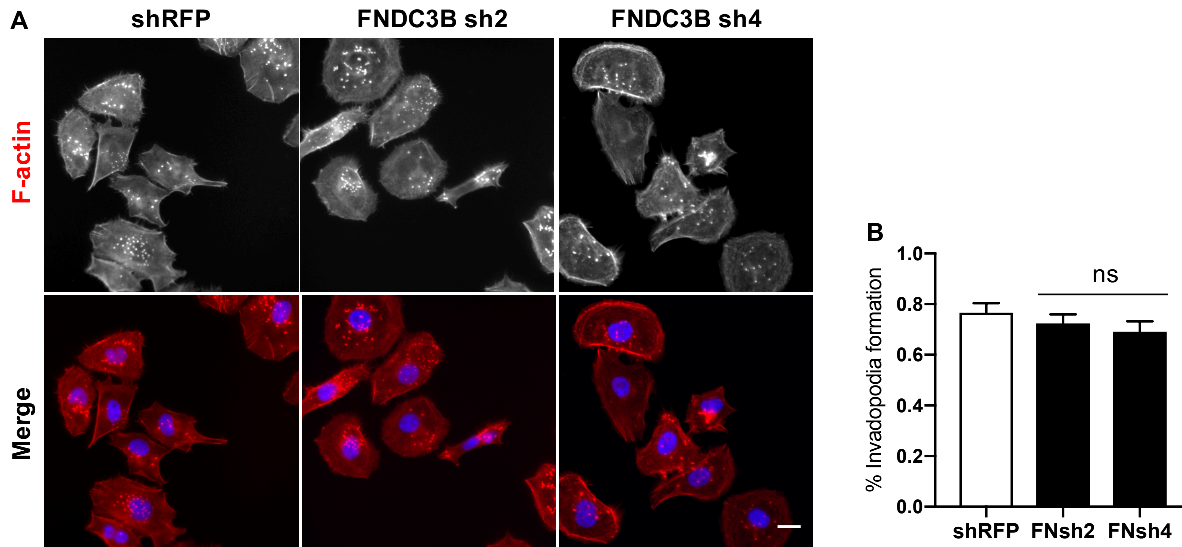

Figure 3-15: FNDC3B is not required for invadopodia formation A) Sample images of F-actin staining by Phalloidin in SCC61 control and FNDC3B knockdown cells (scale bar: 20µm). B) Quantification of gelatin degradation images (20 images per group).

3.8 FNDC3B is Required for FAP Presentation at the Plasma Membrane

Given that FNDC3B and FAP transiently interact via IP experiments and their defects exhibit similar effects on matrix degradation, we wanted to further characterize the dependencies between the two. Due to the limitations of FAP and FNDC3B antibodies, it was challenging to measure FAP or FNDC3B localization at invadopodia by immunostaining. Instead, we performed an enzymatic assay to indirectly measure the amount of FAP on the plasma membrane in control and FNDC3B knockdown cells. C95 is a membrane impermeable probe that fluoresces upon cleavage by both FAP and another protease called POP. In order to distinguish between the two enzymatic activities, two additional inhibitors were used. J94 is a POP specific inhibitor while M83 is a dual POP and FAP inhibitor, and comparing the enzymatic activity between the two would show FAP specific activity. SUM1315 cells expressing control and FNDC3B shRNAs were treated with J94, M83, and PBS as negative control. All cell lines treated

with M83 had no enzymatic activity, as proof of concept that the C95 probe was specific to only FAP and POP (Figure 3-16-A, -B). In J94 treated cells, which only exhibits FAP enzymatic activity, there were two groups of activity. Control knockdown cells had robust enzymatic activity, whereas cells with FNDC3B knockdown had their enzymatic activity reduced by half (Figure 3-16-A, -B). These findings show that there is less FAP membrane presentation in FNDC3B knockdown cells, suggesting FNDC3B is involved with FAP recruitment to the membrane and possibly invadopodia. Additionally, the decrease in FAP membrane presentation is not due to an overall loss of FAP expression in FNDC3B knockdown cells (Figure 3-16-C).

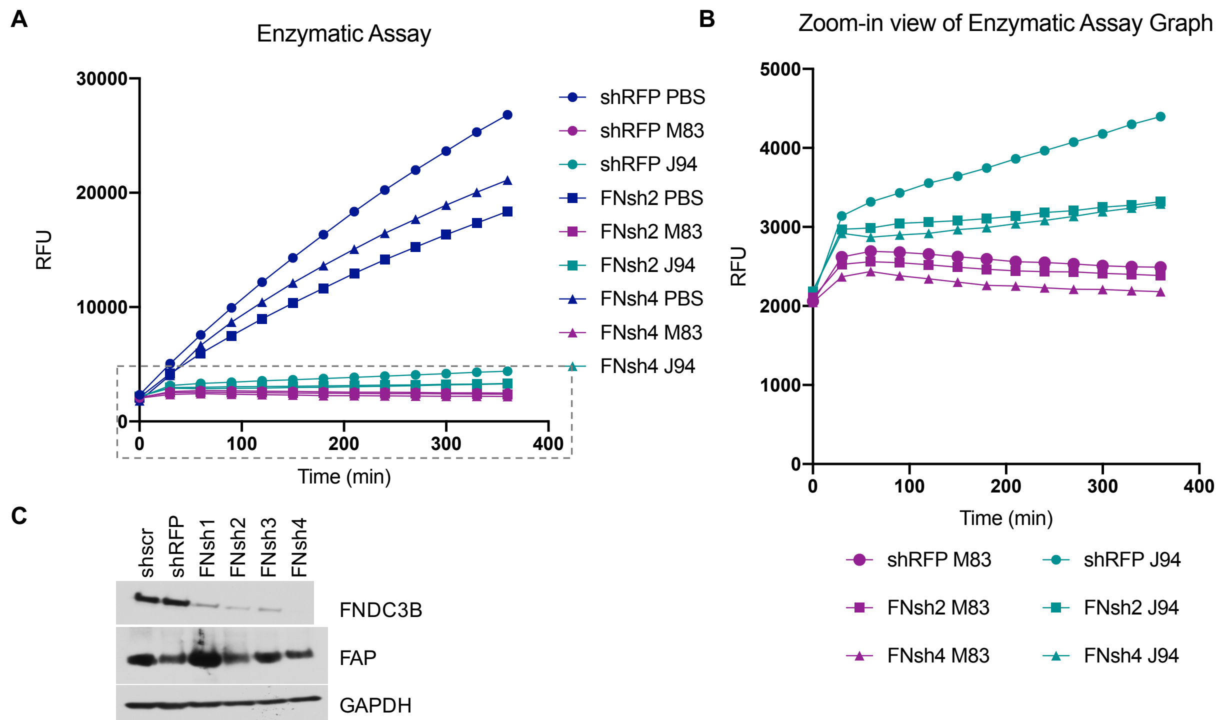

Figure 3-16: FNDC3B is required for FAP membrane presentation. A) Enzymatic assay was performed on SUM1315 control and FNDC3B knockdown cells. Fluorescent intensity was collect every 10 minutes for 6 hours. M83: POP and FAP inhibitor; J94: POP inhibitor. B) Zoom-in view of boxed region in A) shows J94 and M83 treatment results. C) Western blot showing FAP expression in FNDC3B knockdown cells.

3.9 Conclusion

In this chapter, we identified a novel regulator of invadopodia function, Fibronectin Type III Domain Containing 3B (FNDC3B). As we demonstrated in the previous chapter, FAP is an important regulator of invadopodia-mediated ECM degradation and metastasis *in vivo*. Using immunoprecipitation (IP) followed by mass spectrometry to identify novel FAP interacting proteins, we identified FNDC3B to be an interesting target. Characterization of FNDC3B demonstrated that it is a cytoplasmic membrane protein that localizes to invadopodia. Furthermore, FNDC3B is required for matrix degradation *in vitro*.

Beside our study identifying FNDC3B as an intracellular membrane protein localized to invadopodia, other researchers have also found tagged-FNDC3B at the ER and Golgi (Cai et al., 2012; Lin et al., 2016). Our results also showed that in addition to localizing to invadopodia, endogenous FNDC3B also exhibit perinuclear staining that could be at the ER or Golgi (Figure 3-8 & 3-9). Co-staining experiments of FNDC3B with ER or Golgi markers will be performed to further characterize its localization. The function of FNDC3B at the ER and Golgi remains unclear. However, given that FNDC3B is also at invadopodia, it might function in facilitating transport of other proteins to invadopodia. Since the defect of FNDC3B knockdown in ECM degradation is similar to that of MT1-MMP knockdown and FNDC3B is not required for invadopodia initiation, we hypothesized that FNDC3B may help facilitate MT1-MMP transport to invadopodia. To test this hypothesis, time-lapse live imaging experiments can be formed on cells expressing fluorescently tagged FNDC3B, MT1-MMP, and ER or Golgi marker. Several vesicle proteins, including v-SNARE, Sec1p and Rab family proteins, have been shown

to participate in MT1-MMP membrane. To test whether FNDC3B interacts with secretory vesicle coat proteins to facilitate transport to invadopodia, cells can be stained for FNDC3B and vesicle coat proteins including v-SNARE, Sec1p and Rab family proteins. Time-lapse live imaging can also be performed using fluorescently tagged versions of these proteins to better understand the dynamics FNDC3B-mediated transport. If FNDC3B is indeed involved with vesicle trafficking to invadopodia, this will help explain its transient interaction with FAP. The fact that the proteolytic activity of FAP is not required for ECM degradation supports a role for FAP as a scaffolding protein at invadopodia. FAP may help recruit MT1-MMP-containing vesicles to invadopodia by interacting with FNDC3B. This interaction would only occur during MT1-MMP transport to invadopodia and therefore would be brief and difficult to capture by IP. To test this hypothesis, time-lapse live imaging experiments will be formed on cells expressing fluorescently tagged FAP, FNDC3B, MT1-MMP, and vesicle coat proteins including v-SNARE, Sec1p and Rab family proteins. These characterizations will help us better understand both FNDC3B and FAP's role at invadopodia.

FNDC3B contains 9 Fibronectin type III (FNIII) domains with high structural homology. Lin et al. demonstrated that FNIII domains 1-4 are required for cell migration. They also reported that the first four FNIII domains interacts with phosphorylated Annexin A2 (ANXA2), which is a phospholipid binding protein that can regulate membrane-membrane and membrane-cytoskeletal interactions (Caldieri et al., 2012; Lin et al., 2016). Additionally, ANXA2 have been implicated in exocytosis, endocytosis, and vesicle transport and can be phosphorylated by Src kinase (Sharma & Sharma, 2007). Given these information, ANXA2 and FNDC3B interactions might be important for

invadopodia function. ANXA2 displays calcium-enhanced affinity for PI(3,4)P2, which is a phospholipid found at invadopodia. Upon invadopodia initiation, Src kinase can phosphorylated ANXA2 and the phospholipid tethered ANXA2 recruit FNDC3B bound vesicles to invadopodia. Further studies to characterize ANXA2 localization to and function in invadopodia as well as interactions with FNDC3B at invadopodia will contribute to a better understanding of FNDC3B's role at invadopodia.

While FNDC3B's role in metastasis *in vivo* remains to be characterized, other studies have also implicated FNDC3B in cancer. FNDC3B can induce EMT in epithelial cells, suppress invasion and metastasis in melanoma cells in a STAT3-dependent manner, and promote cell migration and metastasis in hepatocellular carcinoma (Cai et al., 2012; Katoh et al., 2015; Lin et al., 2016; Yang et al., 2017). These conflicting discoveries suggests that FNDC3B may be differentially involved in tumorigenesis depending on tissue type. One thing is certain, FNDC3B is important in cancer and further characterization of this protein will have therapeutic value.

3.10 Materials and Methods

3.10.1 Cell Lines and Cell Culture

The SCC61 cell line was cultured in DMEM with 10% FBS and 0.5ug/ml hydrocortisone. The MDA-MB-231 cell line was cultured in DMEM with 10% FBS. All cell lines also had 1% penicillin and streptomycin in the culture media. Viral production and infection to produce stable knockdown cell lines was carried out as previously described (Eckert et al., 2011).

3.10.2 Plasmids

Control shRNAs in the pLKO.1 vector were obtained from the Sabatini lab (Addgene) and purchased from Sigma. ShRNAs targeting FAP were purchased from Sigma and have the following targeting sequences: ShRNAs against FNDC3B where purchased from Sigma and have the following targeting sequences: FNDC3B sh2: GATGCAGCTCAGCAGGTTATTC; FNDC3B sh4: GGACAGAAACAAGAGGTTTAT.

3.10.3 Generation of C-Terminal Myc-His Tagged FAP

In order to perform the IP/Mass spectrometry, a C-terminally Myc-His tagged version of FAP was generated via PCR and restriction enzyme cloning. Primers used to amplify FAP are: Fwd: TACCTGGTACCATGAAGACTTGGGTAAAAATCG Rev: CGACGTCTCGAGCTGTCTGACAAAGAGAAACAC. The PCR fragment was inserted into the pCDNA4.B plasmid via XhoI and KpnI restriction sites. The sequence was verified by EtonBio sequencing services.

3.10.4 Western Blotting

Cells at 80-90% confluency were washed with cold PBS and lysed in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10 mM NaF, 1% Triton X-100), with 100 µM Na_3VO_4, PMSF, and the Protease Inhibitor Cocktail Set III (Calbiochem) diluted 1:200. Protein lysates were boiled in 50mM DTT LDS sample buffer at 70°C for 10 minutes and then ran on 4-12% pre-cast gels (PAGEgel). Antibodies used for western blotting were diluted in 1% BSA in PBST (1X PBS+1% Tween 20) + Sodium Azide.

The following antibodies were used for western blotting:

FAP (1:500, D8, Vitatex), FNDC3B (1:500, HPA007859, Sigma), Twist1 (1:500), GAPDH (1:2500, GeneTex).

3.10.5 Immunoprecipitation (IP) with DSP Crosslinking

30 plates of SUM1315 cells on 15cm dishes were transiently transfected with the FAP-Myc-His construct. A transfection control plate containing both FAP-Myc-His and pRRL-GFP construct was used to measure transfection efficiency. 48 hours following transfection, 30 transfected plates and 30 untransfected control plates were washed twice with warm PBS. The cells were then treated with DSP to crosslink protein-protein interactions and allow for more stringent IP washes. 5ml of warm 0.5mM DSP were added to each plate and incubated for 30mins at 37°C. The crosslinking reaction is then quenched with 10min incubation of 20mM Tris-Cl pH7.4 at room temperature. Cells were lysed in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10 mM NaF, 1% Triton X-100), with 100 μM Na_3VO_4, PMSF, and the Protease Inhibitor Cocktail Set III (Calbiochem) diluted 1:200, scraped off the culture dish, and sonicated. Ni-NTA Agarose beads (Invitrogen) were washed per manufacturer's protocol, incubated with lysates overnight at 4°C, washed three times with IP lysis buffer supplemented with 500 mM NaCl and then washed twice with 250mM NaCl. Sample was eluted using 50mM DTT LDS sample buffer at 70°C for 10 minutes. Western blot was performed using 1:120 of elute to quantify IP efficiency.

3.10.6 Quantitative Real-Time PCR

Total RNA from cells at 80-90% confluency using the NucleoSpin RNA II kit (Macherey-Nagel), and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNAs were analyzed in triplicate using the SYBR-Green PCR Mix. Relative mRNA levels were determined by $2^{-(Ct-Cc)}$,

where Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH values. Primers used for qRT-PCR are as follows:

HPRT:
Forward: ACGTCTTGCTCGAGATGTGA
Reverse: TCCCCTGTTGACTGGTCATT

FAP:
Forward: AATGAGAGCACTCACACTGAAG
Reverse: CCGATCAGGTGATAAGCCGTAAT

FNDC3B:
Forward: ACGGCTTACTACCCACCTGTTACC
Reverse: CCTCCGCCACTTCCACCACT

3.10.7 ECM Degradation Assays

ECM degradation assays were carried out as previously described (Artym et al., 2009; Eckert et al., 2011).

3.10.8 Immunofluorescence

Prior to cell seeding, coverslips were coated with 100ug/ml rat tail collagen (Corning) diluted in DPBS for 30mins at 37°C, then washed once in 1X DPBS. Cells were seeded on the coated coverslips for at least 48 hours and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at 37°C. Cells were washed 3 times with 1X PBS, permeabilized with 0.25% Triton X-100 in PBS for 5 minutes, and then blocked with 1% BSA in PBS for 1 hour. Samples were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 hour at room temperature. Samples were then washed 3 times with 1X PBS, then mounted with VECTASHIELD Anti-fade Mounting Medium with DAPI (Vector Laboratories) prior to imaging. Confocal images were acquired using an Olympus FV1000 with 405, 488, 555, and 647 laser lines. Images were linearly analyzed and pseudo-colored using ImageJ analysis

software. Primary antibodies used for ICC include Cortactin (1:200, 4F11, Millipore), FAP (1:500, D28, Vitatex), FNDC3B (1:25, HPA007859, Sigma). Secondary antibodies used include Alexa Fluor 488 and 546 (Invitrogen). Alexa 546-Phalloidin was used to label F-actin (Invitrogen).

3.10.9 FAP Enzymatic Activity Assay

SUM1315 cells were plated in 96-well plates 48 hours prior to assay, and washed 3 times with 1X PBS. The wash solution was replaced with fresh PBS, and either additional PBS or 10 μM M83 inhibitor was added. 125 μM C95 substrate was added to all wells, and then fluorescence emission was measured at 360/460 nm excitation/emission wavelengths. The M83 inhibitor and the C95 substrate were generously provided by Dr. Patrick McKee.

3.10.10 Statistical Analysis

All statistical analysis was carried out using Prism8 software. Student t-test was performed to determine statistical significant between two test groups. One-way ANOVA test was carried out to determine statistical significance of three or more test groups.

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Chapter 4

Conclusion and Future Perspectives

4.1 Cellular View of Invadopodia

4.1.1 Visualizing Invadopodia *In Vivo*

Invadopodia-mediated ECM degradation and invasion *in vitro* has been well studied. Given its ability to degrade the matrix and that knockdown of invadopodia regulators, such as Src, TWIST1, and Tks5, significantly reduces tumor metastasis *in vivo*, invadopodia is suspected to play an important role during metastasis. Xenograft models involving the injection of cancer cell lines depleted of invadopodia regulators, including Src, TWIST1, or Tks5, into mice demonstrated indirectly that invadopodia formation is important for tumor metastasis. However, as many of these proteins serve other functions during tumorigenesis and metastasis, it is difficult to attribute the effects of these proteins on metastasis to their roles in invadopodia function. One way to study invadopodia *in vivo* is to image and capture invadopodia in the individual steps during metastasis: invasion into the surround stroma, intravasation into the circulature, and extravasation out of it. Additionally, many characterizations of invadopodia formation has been carried out in 2D as opposed to the 3D tumor environment *in vivo*. It is unknown whether invadopodia forms and functions similarly in 3D. With recent advances, researchers began to image invadopodia during cell invasion and metastasis *in vivo* to help answer that question.

Using intravital imaging, a technology that allows the imaging of live animals through an implanted imaging window, researchers were able to observe the formation

of invadopodia in 3D over time. MTLn3 rat mammary adenocarcinoma cells injected into the mammary fat pad of SCID mice formed invadopodia like protrusions (Gligorijevic et al., 2012). Additionally, tumor sections were then stained positive for Cortactin and proteolytic activity, by the presence of cleaved collagen 3/4 (Gligorijevic et al., 2012). This finding demonstrates that primary tumor forms invadopodia in order to invade the surrounding stroma. Additionally, invadopodia formation in GFP-labeled MTLn3 cells were also observed during the intravasation of these cells into a blood vessel (W. Wang et al., 2002). The study showed that invadopodia formation is dependent on cell types, as nonmetastatic MTC tumors do not form invadopodia (W. Wang et al., 2002). Using ex ovo chicken embryos, researchers were also able to observe invadopodia formation during tumor extravasation. The chorioallantoic membrane (CAM) of chicken embryo form highly organized capillary bed, which provides an ideal model for the visualization of human tumor cells *in vivo*. GFP-labeled human epidermoid carcinoma cells (HEp3-GFP) were injected into the vitelline vein of chicken embryos. Using intravital imaging, invadopodia formation was observed to form prior to extravasation (Leong et al., 2014). Higher resolution of the intravital imaging revealed that the majority of the invasive protrusions project through slightly separated endothelial junctions (Leong et al., 2014). Histological sections of CAM containing extravasating cells exhibited F-actin and Cortactin staining, indicating the presence of invadopodia presence.

Xenograft models combined with intravital imaging is an excellent combination to studying the dynamics of invadopodia *in vivo*. Intravital imaging can also be performed in conjunction with xenograft models using cells devoid of Tks5 or other invadopodia proteins identified in my study. Imaging these cells would help further demonstrate

whether the defect in metastasis is due to the loss of invadopodia formation *in vivo*. Additionally, tumor cells with fluorescently-tagged invadopodia markers including Tks5, Cortactin, FAP, or FNDC3B can be used in the xenograft model to further demonstrate the presence of invadopodia *in vivo*. Since current xenograft models typically involve injection of immortalized and invasive cell types that are predisposed to form invadopodia. A next step for the assay is to use transgenic mouse models that induce tumor formation over time in order to study whether invasive tumor cells naturally form invadopodia *in vivo*. The model can include a promoter specific GFP transgene to label tumor cells that are invasive or form invadopodia.

4.1.2 Invadopodia and Other Protrusive Structures

When tumor cells become more invasive during metastasis, the transition is typically accompanied by the switch from the formation of focal adhesions to invadopodia. Focal adhesions serves to connect the actin cytoskeleton to the ECM and contain large clusters of transmembrane receptors, integrins, and cytosolic proteins (Murphy & Courtneidge, 2011). Invadopodia and focal adhesions share a number of molecular components, with some major differences. Despite the fact that both are actin-rich structures, focal adhesions link to unbranched actin-based stress fibers and have minimal to no ECM degradation ability. On the other hand, invadopodia primarily associate with branched actin and carry out ECM degradation.

Noninvasive cancer cells tend to form more focal adhesions and less invadopodia, and vise versa for more invasive cancer cells. How is the transition from focal adhesion to invadopodia formation mediated in invasive cells? One way tumor cells regulate the transition is through the Focal Adhesion Kinase (FAK). FAK is a

nonreceptor tyrosine kinase that is involved in cell migration (Mitra et al., 2005). FAK is involved in lamellipodia formation, focal adhesion turnover, and invadopodia function (Chan et al., 2009). Chan et al, demonstrated that active FAK sequesters Src at focal adhesions to facilitate tyrosine phosphorylation of focal adhesion proteins such as Paxillin and p130Cas. The sequestration of Src at focal adhesions prevents it from partaking in invadopodia initiation. This is evident by the observation that loss of FAK leads to increased invadopodia formation (Chan et al., 2009). Loss of FAK causes Src release from focal adhesions and allows Src to localize to invadopodia and promote invadopodia formation. It is not known what conditions would lead to loss of FAK in cells under physiological conditions, interestingly enough, FAK expression has been associated with human tumor invasiveness and implicated in breast cancer progression and invasion in mouse models (Mitra et al., 2005; Owens et al., 1995; Zhao & Guan, 2011). In additional to FAK, our lab has also demonstrated ADAM12 can regulate invadopodia and focal adhesion formation through different protein domains. The disintegrin domain of ADAM12 is required for focal adhesion turnover whereas the both the MMP and disintegrin domain are required for invadopodia formation and function (Eckert et al., 2017). Furthermore, ADAM12 knockdown induced FAK Y397 phosphorylation by at least 4-fold. Since FAK phosprylation at Y397 is an early event in focal adhesion formation and is required downstream signaling, this suggests a potential link between FAK and ADAM12 (Schlaepfer & Hunter, 1997). Therefore, FAK and ADAM12 may communicate with each other to ensure the proper balance between focal adhesion and invadopodia formation.

FAK has also been reported to play a role in nascent integrin adhesions (NAs) and lamellipodia formation. During cell migration, leading edge protrusion via lamellipodia and ECM adhesion via NAs are spatially and temporally coupled events (Giannone et al., 2004; Gupton & Waterman-Storer, 2006). FAK regulates lamellipodia formation in a precisely timed manner by interacting with actin nucleator Arp2/3 complex (Swaminathan et al., 2016). FAK also stabilizes lamellipodia dynamics by regulating the formation and turnover of NAs and therefore preventing its maturation into focal adhesions (Swaminathan et al., 2016). Since metastasis requires both tumor cell migration and invasion, the exact mechanism of how the two abilities are coordinated is not known. The above findings show that FAK is the molecular switch between focal adhesions and invadopodia and is involved in lamellipodia formation. It would be interesting to see whether FAK can also mediate the switch between lamellipodia and invadopodia to regulate coupled tumor cell migration and invasion. The study would also shed light on how tumor cells balance between cell migration and ECM invasion, two processes important for metastasis. It is possible that the switch is so transient that it would be difficult to capture or that cells do not undergo a complete switch from lamellipodia formation to invadopodia. These structures may co-exist but coordinate their activities in order to facilitate migration and invasion. In order to better understand this process, time-lapse live cell imaging can be performed on migrating tumor cells to study the dynamics between invadopodia and other cellular protrusions such as lamellipodia. Fluorescently labeled proteins shared amongst the structures can also be over-expressed or CRISPRed into cells to allow live visualization of these protein under various conditions.

4.1.3 Invadopodia and Cell Migration

Studying the dynamics between invadopodia and other protrusions helps answer the questions - what is the mechanism that spatially coordinate tumor cell invasion and migration, and what is invadopodia's role in coordinating this? There are limited answers to these questions. Planar cell migration requires the coordination of focal adhesion assembly, maturation, and turnover of lamellipodia at the leading cell edge. During collective cell migration, MT1-MMP-mediated collagen degradation is important to generate tracks for cells to move (Wolf et al., 2007). Mechano-cues from the ECM are recognized to be important for the formation of focal adhesions and growth of adhesion-anchored stress fibers (Murphy et al., 2011). Additionally, podosomes and invadopodia have been shown to act as mechanosensors and exert similar amount of force as focal adhesions on the matrix (Collin et al., 2008). Podosome assembly does not depend on the intracellular tension, but podosome lifespan, distance between podosomes, podosome rosette organization, and rosette size all depend on flexibility of the substrate (Collin et al., 2008). Additionally, increase in ECM rigidity has been shown to cause increase in invadopodia-mediated matrix degradation in breast carcinoma cells. This increase in invadopodia activity seems to be mediated via mechanosensing proteins p130Cas and FAK (Alexander et al., 2008). These findings suggest invadopodia facilitate cell migration upon activation by mechanical cues to create a matrix track for the cell to migrate on and to help the cells generate enough traction to move forward. Additional studies on the dynamics between cell migration and invadopodia would help the field better understand how the process is coupled, specifically- are invadopodia required for cell migration?

The role of the actin network on invadopodia formation and function as been very well characterized, however it is unclear how other cytoskeletal systems contribute to invadopodia formation. Certain studies report an important role for intact microtubules in invadopodia and podosome function (Linder, 2007). However, in breast cancer cells, intact microtubules are not required for invadopodia formation but does regulate invadopodia elongation and maturation (Kikuchi & Takahashi, 2008; Linder, 2007; Schoumacher et al., 2010). Microtubule disruption by inhibitory drugs in melanoma cells prevents their *de novo* formation and ECM degradation of already formed invadopodia (Caldieri et al., 2012). Microtubules are involved in polarized transport, but not overall protein transport in polarized cells, which suggests that microtubules might be essential for the delivery of MMPs to invadopodia (Caldieri et al., 2012). Intermediate filaments have been reported to interact with actin filaments through fimbrin during podosome formation in macrophages (Correia et al., 1999). Vimentin intermediate filament localized to newly formed podosomes on vascular smooth muscle cells, however its involvement is unclear. In cancer cells, Vimentin is not required for invadopodia initiation, but required for elongation of mature invadopodia (Schoumacher et al., 2010). Vimentin expression is upregulated during EMT of increased tumor cell invasion, it is possible intermediate filaments are involved with invadopodia stabilization.

4.2 Molecular View of Invadopodia

4.2.1 Model of Invadopodia Formation

Works of many researchers helped contribute to the current working model of invadopodia formation, which includes 3 stages: invadopodia precursor core initiation, precursor elongation, and invadopodia maturation (Beaty & Condeelis, 2014, Murphy &

Courtneidge, 2011). The initial core formation involves recruitment of central actin polymerization machinery and support proteins, Arp2/3, N-WASP, Cortactin, and Cofilin. This core formation occurs very rapidly and is unstable until Tks5 recruitment. Tks5 mediates anchoring of the precursor core to PI(3,4)P2 on the plasma membrane and stabilized the precursor. Tks5 also recruits Mena, which in turn forms a Mena-Arg-SHIP2 complex. SHIP2 produces additional PI(3,4)P2 in the membrane, allowing further Tks5-mediated precursor tethering to the membrane. During invadopodia elongation, additional actin polymerization is mediated through phosphorylation of Cortactin to enhance Arp2/3-mediated actin polymerization. Cortactin phosphorylation also further activates Cofilin's actin severing ability to stimulate additional actin polymerization. The maturation step is represented by the recruitment of MMPs, especially MT1-MMP, to the tip of the protrusions and this involves a number of proteins, Cortactin, Tks4, and β1 integrin. Finally, Cortactin dephosphorylation blocks Cofilin activity, and actin structure is stabilized (Eddy et al., 2017; Murphy et al., 2011). The initial triggers for invadopodia formation are well characterized and will be discussed later in this chapter. However, it is unclear whether proteins at the mature invadopodia can impact the structure's function and dynamics. For example, how does MMP enzymatic activity affect invadopodia formation, adhesion, and mechanics? Additionally, what are specific markers of invadopodia that can be used as targets for prognosis of distant metastasis and therapeutic inhibition of tumor cell dissemination?

4.2.2 FAP and MT1-MMP at Invadopodia

MT1-MMP is essential for tumor cell invasion and is well established as the power engine for invadopodia-mediated ECM degradation. Growing research

suggests that MT1-MMP not only serves a role in proteolysis, but is also important for overall invadopodia maturation. MT1-MMP activity inhibition resembles similar phenotype as that of Cortactin knockdown. Inhibitor treated cells have abolished ECM degradation, fewer number of cells forming invadopodia, as well as fewer number of invadopodia per cell (Clark et al., 2007). This suggests that degradation products of MT1-MMP provides positive feedback to promote new invadopodial formation. Interestingly enough, our studies of FAP saw that cells deficient of FAP had significantly reduced ECM degradation, fewer number of cells forming invadopodia. Preliminary studies show that invadopodia number per cells is also reduced in FAP knockdown cells (data not shown). The fact that MT1-MMP and FAP knockdown cells exhibit similar phenotypes suggest that the two proteins may regulate invadopodia formation and function in similar mechanisms. However, the major difference between the two proteins is that the proteolytic activity of MT1-MMP is required for these functions where as FAP's is not. This suggest that although MT1-MMP and FAP may regulate invadopodia formation and function through similar mechanisms, their roles are different. Given our hypothesis that FAP may act as a scaffolding protein to recruit MT1-MMP to invadopodia, these correlations further support the hypothesis. Preliminary studies performed using sucrose gradient fractionation to isolate invadopodia-containing lipid rafts showed that less MT1-MMP was found in the lipid rafts of FAP knockdown cells (not shown). This finding suggests that FAP is required for proper localization of MT1-MMP to invadopodia. Through IP experiments against endogenous FAP, we demonstrated that MT1-MMP and FAP do not interact with each other, suggesting that FAP may interact with other proteins to facilitate MT1-MMP

transport (not shown). It is known that Cortactin is an important regulator of MT1-MMP secretion and loss of Cortactin has similar phenotype as MT1-MMP inhibition (Clark et al., 2007). FAP may regulate invadopodia formation and function by helping Cortactin recruit MT1-MMP to invadopodia. To test this, MT1-MMP localization in control and FAP-deficient cells will be measured by immunofluorescent imaging. Additionally, MT1-MMP will be overexpressed in FAP knockdown cells to test whether MT1-MMP alone can rescue the ECM degradation defect of FAP knockdown cells. Finally, to characterize the dynamics between FAP, MT1-MMP, and Cortactin, time lapse live imaging will be performed using SCC61 cells overpressing fluorescently labeled-FAP, -MT1-MMP, and -Cortactin.

4.2.3 Protease-Independent Role of MT1-MMP

Besides our discoveries of the protease-independent role of FAP at invadopodia, numerous studies now suggest MMPs, including MT1-MMP, MMP3, MMP9, and MMP12, can also play proteolysis-independent roles in cancer. In physiology, MMPs are significant mediators of cellular differentiation and morphology. For example, MT1-MMP was identified to have DNA binding abilities that effects reprogramming of macrophage function. It can also play a role in osteoclast differentiation by modulating Rac1 and p130Cas activity in a non-proteolytic mechanism (Gonzalo et al., 2010). In developing mammary gland, MT1-MMP catalytic activity is required for epithelial cell invasion and branching in dense collagen gels, however the transmembrane and cytoplasmic domain also plays a role in mammary branch morphogenesis via interactions with integrin β1 (Galvez et al., 2002).

In cancer, MT1-MMP can also effect tumor cell survival, regulate proinflammatory signaling, and increase tumor grade. The intracellular, non-catalytic domain of MT1-MMP is required for transducing the intracellular signals that leads to glioblastoma (GBM) cell apoptosis. Although it is not known which specific region on MT1-MMP is required for this signaling, MT1-MMP can negatively regulate glucose-6-phosphate transporter(G6PT) expression to control cell survival (Belkaid et al., 2007). A study also demonstrated that silencing of the MT1-MMP/ G6PT pathway decreases sphinosine-1-phosphate-mediated Ca2+ mobilization, an interaction known to increase GBM cell proliferation (Fortier et al., 2008). MT1-MMP is also involved in an IKK/NF-κB-dependent pathway that induces COX2 expression, which is correlated with increasing histological grade of gliomas (Sina et al., 2010). At invaopodia, TIMP2 is a known inhibitor of MT1-MMP activity. Their interaction seems to be more than that, for together they can also rapidly activate ERK1/2 and Akt signaling to promote tumor survival in a dosage-dependent manner (Valacca et al., 2015).

These studies clearly demonstrate MT1-MMP's involvement in many proteolysis independent roles in physiology and tumorigenesis. In additional to MT1-MMP, other MMPs also play non-proteolytic roles in physiology and tumorigenesis (Itoh, 2015). This may help explain why blocking MMP activity has failed in clinical trials as an anti-metastasis therapy. Blocking ECM remodeling alone is not sufficient to preventing tumor invasion and metastasis as the MMPs are also involved in other "cancer hallmarks" that promote tumor growth and invasion. Inhibitors that target MMPs activity in addition to their interaction and/or localization to specific membrane areas for signaling might be of therapeutic value.

4.2.4 Protease-independent Role of FAP

In chapter 2, it was demonstrated that Fibroblast Activation Protein (FAP) is important for invadopodia-mediated ECM degradation. FAP expression has been correlated with invasiveness of human melanoma and carcinoma cells. Given the fact that FAP has both exo- and endopeptidase activity, it is logical to assume FAP participates in ECM degradation as a protease. Although the role of FAP at invadopodia remains to be defined, we demonstrate that the proteolytic activity of FAP is not required for this function. In chapter 2 and this chapter, we have discussed potential roles FAP may play at invadopodia and experiments to test them.

Other studies have also suggested FAP can function independent of its protease function. *In vivo* experiments demonstrated that MDA-MB-231 cells overexpressing FAP have greater tumorigenic potential and gave rise to faster growing tumors than wild type MDA-MB-231 cells (Huang et al., 2011). Additionally, mice overexpressing FAP catalytically dead point mutant also exhibited rapid tumor growth rate, and mice receiving FAP inhibitor, talabostat, did not show significantly slowed tumor growth (Huang et al., 2011). However, given the role of FAP in invadopodia, it is would be interesting to know whether lung metastasis is affected in the conditions above. Chung et al. demonstrated that FAP is important for bone marrow-mesenchymal stem cells (BM-MSCs) migration by showing a transwell migration defect in BM-MSCs with shRNA-mediated FAP knockdown. They further demonstrated that inhibition of FAP peptidase activity using two different inhibitors, 2F01 and 2F09, had no effect on cell migration (Chung et al., 2014).

If the catalytic activity of FAP is not required for invadopodia function, tumor growth, and cell migration, then what is the involvement of FAP in these functions? Our hypothesis agrees with that of other researchers: FAP may function as a regulatory receptor or scaffold adaptor, which can be mediated through its large extracellular domain to the short 6 aa. cytoplasmic region. Despite the short length of the cytoplasmic region, a FAP homologous protein, DPPIV, have been shown to interact with CARMA-1 via the cytoplasmic region for T-cell activation (Ohnuma et al., 2007). FAP also can interact with integrin β1, including α3β1 complex (Artym et al., 2002; Mueller et al., 1999). Integrins are known to signal and increase the expression of several MMPs. Integrin αvβ3 can upregulate MMP2 secretion and MMP9 expression is indirectly mediated by integrin signaling via FAK and Src kinase (Brooks et al., 1996; Hsia et al., 2003). Integrin α3β1 in particular has been linked to elevated MMP9 secretion in MDA-MB-231 cells (Lichtner et al., 1998; Morini et al., 2000).

In addition to increasing evidence demonstrating the proteolytic-independent role for FAP, there may be cellular contexts where the FAP protease activity is more important over non-proteolytic signaling. In one study, HEK293, a human embryonic kidney cell line, overexpressing wild type FAP gave rise to rapidly growing tumor in mice (Cheng et al., 2005). However, overexpression of FAPS624A exhibited significantly slower growth than wild type FAP did *in vivo*, suggesting the protease activity of FAP is required for tumor growth promotion (Cheng et al., 2005). Similar experiments using FAP catalytic activity inhibitors also reported similar effects on tumor growth of lung and colon cancers (Santos et al., 2009). The difference in the reported requirement for FAP proteolytic activity might be explained by the different cell contexts. Further

characterization of FAP's proteolytic activity in disease may help improve our understanding.

4.2.5 FAP and DPPIV

DPPIV is in the same family of serine proteases as FAP and have also been associated with invadopodia previously (Ghersi et al., 2006). Whereas FAP is uniquely expressed during embryogenesis and diseased adults, DPPIV is ubiquitously expressed in epithelial and endothelial cells and has been reported to cleave a number of important peptides in circulation (Chen et al., 2003). FAP and DPPIV has been shown to dimerize at invadopodia protrusions of endothelial cells to regulate matrix degradation, cell migration, and invasion (Ghersi et al., 2006). The study further showed inhibition of DPPIV gelatin-binding domain blocked matrix degradation, cell migration, and invasion. They suggested that DPPIV and FAP work together to facilitate ECM degradation, where DPPIV binds the gelatin and FAP degrades it. However, it is unclear whether blocking FAP enzymatic activity would block ECM degradation. In our studies, we found that unlike FAP, knockdown of DPPIV does not affect ECM degradation, suggesting that either DPPIV is not required for matrix degradation or FAP can substitute the loss of DPPIV, but not vice versa. It remains unknown if DPPIV-FAP complexes at invadopodia in our studies. As it has also been previously reported, DPPIV-FAP complexes during vascular morphogenesis and angiogenesis (Aimes et al., 2003). This study and many others report DPPIV-FAP complex formation in endothelial cells. Perhaps this complex formation and function is specific for endothelial cells, whether the complex form in tumor cells and regulates matrix degradation needs to be studied.

4.2.6 FAP is a Tumor suppressor and Tumor promoter

FAP expression have been reported in mesenchymal and epithelial cells of tumors. In mesenchymal cells, FAP is expressed in mesenchymal stem cells (MSCs), cancer associated fibroblasts (CAFs), sarcoma, and melanoma cells (O'Brien & O'Connor, 2008). In epithelial tumor cells, FAP is also found in primary breast infiltrating ductal carcinoma (IDC), color adenocarcinoma, lung adenocarcinoma, and metastatic colon adenocarcinoma (O'Brien et al., 2008). It is unclear what roles stromal and tumor FAP play in tumorigenesis.

FAP expression has been correlate with both worse or better patient outcomes. Stromal FAP expression in breast IDC is associated with longer survival of patients in a number of studies (Ariga et al., 2001). FAP expression in cervical carcinoma and cervical intraepithelial neoplasm is positively correlated with the malignant phenotype (Jin et al., 2003). FAP expression is higher in colorectal cancer tissue than normal tissue and there is a significant correlation between FAP expression and lymph node metastasis (Iwasa et al., 2003). On the other hand, patients with epithelial ovarian carcinoma had better survival with decreased FAP expression (Zhang et al., 2007). It is unclear what contributes to the differing observations, one possibility may be the use the different FAP antibodies that recognize different epitopes on the protein.

These observations suggest that FAP may play a suppressive or promoting role in tumorigenesis. Ramirez-Montagut et al. reported that FAP or catalytic mutant FAPS624A overexpression in B78H1 melanoma cells suppressed tumor formation *in vivo* where as wild type cells gave rise to tumors (Ramirez-Montagut et al., 2004). The overexpression of FAP or FAPS624A also restore contact inhibition and growth factor dependence in these cells (Ramirez-Montagut et al., 2004). Since DPPIV, a protease in

the same family as FAP, have also been reported to have tumor suppressive roles, the researchers also demonstrated that overexpression of FAP or FAPS624A does not induce DPPIV expression (Ramirez-Montagut et al., 2004).

As for the tumor promoting role of FAP, knockdown of FAP in MDA-MB-435 and MDA-MB436 cells rendered these cells sensitive to serum starvation while overexpression of FAP in the same cells did not (Goodman et al., 2003). Overexpression of FAP in MDA-MB-231 cells led to more rapid tumor growth and tumor vascularization compared to control cells without FAP (Huang et al., 2004). Additionally, overexpression of FAP in human hepatic stellate cells LX-2 increased cell adhesion, migration, and invasion (X. M. Wang et al., 2005). Interesting enough, as it was discussed earlier in this chapter, the proteolytic activity of FAP was not necessary for those functions (X. M. Wang et al., 2005). These opposing findings of FAP having both tumor suppressing and promoting roles suggest that the effect of FAP in tumorigenesis may depend on the tumor tissue type. Different tumor cell types may have different signaling molecules present, contributing to the duality of FAP function in tumor growth. We must better understand the role of FAP in different cancer types before considering FAP as a therapeutic target in that disease.

4.2.7 Targeting FAP in cancer

Given the unique expression pattern of FAP and its implications in cancer described in earlier sections, researchers have considered FAP as a therapeutic target for cancer treatment for quite some time. Several different strategies to target FAP have been explored. Low molecular weight inhibitors of FAP enzymatic activity typically target both FAP and DPPIV activity. Some of these inhibitors have an effect on cell migration

and invasion *in vitro* only at high concentrations (Waster et al., 2011). Other inhibitors showed conflicting results in tumor proliferation depending on the *in vivo* model utilized for the study (Busek et al., 2018). Talabostat is the only FAP inhibitor that has been tested in clinical trials, but failed at phase II due to lack of clinical effect (Narra et al., 2007). Combination therapy of talabostat and docetaxel or cisplatin also failed to show improvement in metastatic outcome (Eager et al., 2009a; Eager et al., 2009b).

The unique proline-specific proteolytic activity of FAP has also been utilized in the design of FAP pro-drugs. Upon cleavage by FAP, the harmless pro-drugs become highly cytotoxic and will kill FAP-positive and neighboring FAP-negative cells (Busek et al., 2018). This technology would allow highly cytotoxic drugs to only target the tumor and tumor stroma and avoid off-target side effects that many cancer patients experience. Some of the challenges with this method are solubility issues, proper delivery to the tumor, and uptake by cells (Busek et al., 2018). Through many rounds of modifications and improvements, a few pro-drugs successfully showed reduced toxic side effects, similar anti-tumor effects in mouse models or significantly improved anti-tumor effect in vitro (Akinboye et al., 2016; Brennen et al., 2012; J. Wang et al., 2017). However, in tumors or stromal regions with low FAP expression, the pro-drugs can still be activated by blood plasma FAP and lead to a partial deposition of the toxic drug in healthy organs (Brennen et al., 2014). It may seem that the use the pro-drugs is not ready for clinical testing. However, further modifications of the pro-drug to allow for more effective tumor homing and tumor-specific activation may be useful.

Another approach to target FAP is through FAP antibodies. The F19 antibody was the first FAP antibody used to identify the protein as a cancer-associated antigen

(Garin-Chesa et al., 1990). One version of the antibody, Sibrotuzumab, was tested in Phase I clinical trials in metastatic cancer patients and showed therapeutic response (Hofheinz et al., 2003). The effect of the F19 antibody targeting and whether it effects FAP enzymatic activity or scaffolding abilities is unknown. Despite the use of FAP antibody having limited anti-tumor effect, they can be conjugated to toxin, radioisotopes, or immunomodulatory cytokines for other treatment or applications in cancer. Other therapeutic methods that have been explored include liposomes containing FAP antibodies for tissue specific delivery, FAP vaccines to eliminate FAP-positive cells, chimeric antigen receptor T cells with specificity for FAP for targeted immunotherapy (Busek et al., 2018).

Based on our finding that the protease activity of FAP is not required for invadopodia formation and function, this may explain the failures of FAP catalytic inhibitors to prevent metastasis. Since FAP may play a scaffolding role at invadopodia, using the technologies described above to target FAP-expressing cells overall or prevent FAP interactions might be more effective. As most of these technologies are in development, it takes some time to test them in preclinical studies and their efficacy in clinical settings remains to be tested.

4.3 Regulation of Invadopodia by Tumor Microenvironment

Tumorigenesis is partially driven by oncogenic activation in important genes such as Src (Src mutation is very rare) and Ras. These mutations can also induce invadopodia formation in epithelial cells. However, growing evidence suggests that while genetic drivers provides tumor cells the competency to invade *in vivo*, and the tumor microenvironment plays an important role in driving cell invasion. As such,

invadopodia formation can be regulated by a number of microenvironmental cues, including the well characterized growth factor stimulation by PDGF, TGFβ, and EGF. These growth factors activate downstream signaling pathways involving Src and PKC (Murphy et al., 2011). Additional stimuli for invadopodia formation includes EMT, hypoxia, integrin signaling, stromal cells, and cell-cell contact. Understanding how individual microenvironmental cues regulate the invadopodia assembly machinery to promote invadopodia formation and function could provide additional targets of tumor invasion.

4.3.1 Hypoxia and Invadopodia

When tumor growth exceeds their available blood supply, hypoxia occurs within the tumor microenvironment and is regulated by the transcription factor HIF-1α. HIF-1α is required for hypoxia-induced invadopodia formation in head and neck, lung, and pancreas cancer cells (Diaz et al., 2013). HIF-1α activates Notch signaling to stimulate ADAM12 sheddase activity to increase HB-EGF levels for invadopodia formation (Diaz et al., 2013). Hypoxia can stimulate invadopodia formation by increasing metabolic stress via the production of reactive oxygen species (ROS) by NADPH oxidase (Nox) (Choi & Lim, 2013). More importantly, HIF-1α can induce TWIST1 expression by directly binding to the hypoxia-response element in the TWIST1 proximal promoter (Yang et al., 2008). This finding provides another mechanism for hypoxia-induced invadopodia formation, as our lab have demonstrated that TWIST1 induces invadopodia formation (Eckert et al., 2011). As different environmental cues can trigger different invadopodia complex to form, it would be interesting to study whether hypoxia can also induce FAP localization to invadopodia since FAP is a downstream target of TWIST1.

4.3.2 Extracellular Matrix (ECM) and invadopodia

ECM serves as a structural substrate and signaling surface in the tumor microenvironment. As the primary function of invadopodia is to recruit proteases for ECM degradation, are there special features of the ECM such as composition, rigidity, and topography that contribute to the recruitment and activation of the different formation of invadopodia? In order to answer this question, one must study the cell-surface adhesion proteins that convey ECM signals to the cell, such as integrins and CD44. Many integrins, α2, α3, α5, α6 and β1 have been shown to localize to invadopodia. Four specific combination of integrin receptors (α2β1, α3β1, α5β1 and α6β1) localize to invadopodia, suggesting the different combination of integrin receptors mediate invadopodia response to specific ECM substrates, including fibronectin, type I collagen, and laminin (Eddy et al., 2017). For example, interaction between fibronectin and α5β1 is associated with increased tumor invasion while laminin and α3β1 suppresses invadopodia formation (Bae et al., 2013; Beaty et al., 2013; Murphy et al., 2011). An indirect way matrix stiffness can induce invadopodia formation is through transcription factors. Our lab reported that stiffer matrix induces transcription factor TWIST1 translocation from the cytoplasm to the nucleus to induce EMT and promote tumor invasion and metastasis (Wei et al., 2015). Since TWIST1 is also known as an inducer of invadopodia, this suggests a change in matrix stiffness can also induce invadopodia formation and function. These findings suggest that the microenvironment, through cues like different matrix composition, can regulate invadopodia formation through multiple pathways. Do different ECM molecules or other environmental cues

promote the formation of specific protrusive structures? If so, what signaling molecules, such as integrins, are involved? These questions remain unanswered.

In addition to integrins, CD44 is involved with tumor cell adhesion to type I and IV collagen, lamini, fibronectin (McFarlane et al., 2015). CD44 can promote α5β1 activation in response to fibronectin to promote invadopodia maturation (McFarlane et al., 2015). CD44 can also complex with MMP9 at invadopodia due to its involvement in MMP9 recruitment to cell surface (Lagarrigue et al., 2010). In addition to localizing to invadopodia, CD44 is also involved with MT1-MMP recruitment to lamellipodia, this interaction is mediated via its hemopexin-like domain (Mori et al., 2002). Is CD44 involved in the switch from MT1-MMP localization from lamellipodia to invadopodia and vice versa? Since CD44 is a hyaluronan receptor and interacts with different ECM materials, do different ECM composition trigger directed localization of CD44-MT1-MMP complex to lamellipodia or invadopodia? The answer to these questions will help researchers better understand the interplay between invadopodia function and other protrusive structures as well as how ECM cues may trigger the formation of specific protrusions.

Following stimulating invadopodia formation, the ECM can continue to contribute to invadopodia plasticity. Even after invadopodia dissembly, MT1-MMP has been observed near the sites of ECM degradation (Hoshino et al., 2013). The protease may remain there to deposit other membrane bound MMPs, allowing them to adhere to the exposed ECM substrate to allow for quicker invadopodia formation and ECM degradation in the future (El Azzouzi et al., 2016). Another potential mechanism of ECM-enforced invadopodia activation is through the generation of bioactive ECM

proteins post MMP activity. One byproduct of MMP-mediated degradation is laminin-111-derived peptides AG73 and C16, and they have been shown to increase invadopodia formation in an integrin β1-dependent mechanism (Siqueira et al., 2016). These byproducts may also help mark specific areas in the ECM that have been visited by other tumor cells as less resistant to migration and there are more hospital routes to promoting metastasis.

4.3.3 EMT-Inducing Factors and Invadopodia

Epithelial-Mesenchymal Transition (EMT) is a developmental program that is also activated by tumor cells to promote cell migration and invasion for metastasis. Recent studies also indicate EMT as an inducer of invadopodia-mediated ECM degradation. Transcription factor TWIST1, a master regulator of EMT, regulates ECM degradation via induction of invadopodia formation and function (Eckert et al., 2011). Furthermore, TWIST1 directly promotes PDGFRα expression, which then activates Src kinase to promote invadopodia formation (Eckert et al., 2011). TGFβ is also a potent inducer of EMT and invadopodia formation. TWIST1 knockdown prevented TGFβ induction of PDGFRα and invadopodia formation, showing a link between TWIST1 and TGFβ (Eckert et al., 2011). Additionally, TGFβ promotes Hic-5, a focal adhesion adaptor protein, localization to invadopodia in MCF-10A cells. Hic-5 phosphorylation by Src kinase is required for TGFβ-induced invadopodia formation. These findings demonstrate that EMT-inducing factors play important role in invadopodia formation and function.

In addition to the tumor-microenvironment interactions explored above, additional tumor and stroma interactions characterized include with immune cells and fibroblasts. Interaction between tumor cells and proangiogenic macrophages can stimulate

invadopodia formation on tumor cells in the presence of endothelial cells (Pignatelli et al., 2014). Invadopodia promote tumor cell intravasation by helping increase vascular permeability and promote transendothelial migration by breaking down endothelial cell-cell junctions (Harney et al., 2015). High resolution imaging reveals that the tumor-macrophage complex induces Notch1 signaling and subsequence RhoA and Mena dependent invadopodia formation by the tumor cell (Eddy et al., 2017). However, since many stromal cells such as macrophages, fibroblasts, and endothelial cells can also form invadopodia, it would be interesting to determine what role their invadopodia may have in tumor progression.

There is no doubt the tumor microenvironment plays important roles in invadopodia formation, maturation, and function. Some of these stimuli are better characterized than others. However, some important questions remain: which aspect of invadopodium assembly, maturation, and function is regulated by each of the tumor microenvironment cues? Do different microenvironmental signals lead to invadopodia with different lifetimes and ECM degradation activities? Using time lapse imaging on live cells treated with different stimuli in 3D may help answer some of these questions. The challenge lies in how to best recapitulate the tumor microenvironment *in vivo*. Often times, the same cell may receive multiple stimuli from the stroma at the same time. Do cells incorporate multiple stimuli for more robust and active invadopodia formation and function? Or do the multiple stimuli converge to activate the same machinery and once that is activated, and additional stimuli have little to no effect on the already established structure?

4.4 Concluding Remarks

In this dissertation, I have discussed the molecular machinery that mediates ECM degradation during metastasis. I, along with the help of others, have identified novel regulators of invadopodia-mediated ECM degradation and metastasis *in vivo*. FAP is expressed upon TWIST1 induction, therefore providing a link between EMT and invadopodia formation. More interestingly, we demonstrated that the enzymatic activity of FAP is not required for invadopodia function, suggesting that FAP may play a structural role in protease recruitment. FNDC3B was identified via a proteomic screen to identify novel FAP interacting proteins and was also found to regulate invadopodia function.

Upon identifying FNDC3B as a potential target for further studies, we first performed endogenous IPs to test whether FNDC3B interacts with FAP. The two proteins only interact with each other in some occasions under crosslinking conditions, suggesting that their interaction may be transient and indirect. To further validate this interaction, *in vitro* translated FNDC3B and FAP can be used for the IP. Since only those two proteins will be present in the IP, it can clarify whether their interaction is direct. If the interaction between FNDC3B and FAP is direct, it is likely weak unless enhanced by other binding partners. Non-permeabilized immunofluorescent experiments demonstrated that FNDC3B is an intracellular membrane protein with no extracellular region. On the other hand, FAP is an extracellular membrane protein with a 6aa. cytoplasmic region. Is it unclear whether FAP can interact with other intracellular proteins using its cytoplasmic region, but homologue of FAP, DPPIV, have been shown to interact with CARMA-1 via a similar region (Ohnuma et al., 2007). Immuno-precipitation experiments performed using FAP truncation mutants will help better

understand how FAP and FNDC3B interact. If the interaction between FNDC3B and FAP is indirect, then what are the intermediate proteins and how and why are they recruited to invadopodia?

Regardless of how FAP and FNDC3B interacts with each other, our enzymatic assay findings suggest FNDC3B mediates FAP localization at invadopodia. Further characterization of the relationship between FNDC3B and FAP is needed. In lieu of FAP antibody, fluorescently-tagged FAP can be overexpressed in FNDC3B knockdown cells to directly measure the effect of FNDC3B knockdown on FAP recruitment, and vice versa. Additionally, sucrose gradient fractionation can be performed on control and FNDC3B knockdown cells to measure the amount of FAP localized to invadopodia found in lipid rafts. As chapter 2 explained, the phenotype of FAP knockdown cells suggests FAP may function in invadopodia initiation to maturation. On the other hand, FNDC3B knockdown had no effect on invadopodia initiation, suggesting FNDC3B function at invadopodia later temporally. Based on the timing of when FAP and FNDC3B might be required at invadopodia, how can FAP's dependency on FNDC3B be explained? One possible explanation is that FAP localization and function during invadopodia initiation is independent of FNDC3B. However, during later steps of invadopodia formation, FNDC3B is required for FAP to remain at invadopodia and contribute to its stabilization. To test this hypothesis, live imaging of SCC61-Lifeact cells expressing fluorescently-labeled FAP and FNDC3B will be performed. Characterizing the timing of FAP and FNDC3B recruitment relative invadopodia formation will help clarify the roles of these proteins at invadopodia.

In addition to understanding the relationship between FAP and FNDC3B, It would be of great importance to understand how FAP and FNDC3B regulates invadopodia function. The fact that FAP and FNDC3B knockdown cell have matrix degradation defect suggests they may function in MT1-MMP recruitment to invadopodia through direct or indirect mechanisms. They might facilitate MT1-MMP transport from the TGN or endosomal recycling compartment to the plasma membrane or direct already delivered MT1-MMP to sites of matrix contact points for degradation. Given that FNDC3B have reportedly been localized to both the ER and Golgi, it is possible that FNDC3B functions in directing MT1-MMP transport near the Golgi and FAP facilitates MT1-MMP degradation at focal sites. Further studies to characterize the dynamics of FAP- MT1-MMP and FNDC3B-MT1-MMP interactions as well as the recruitment of MT1-MMP in FAP or FNDC3B knockdown cells might help improve our understanding of their functions.

A different approach to understanding the role of FAP and FNDC3B at invadopodia is to perform domain studies. We hypothesized that FAP functions as a scaffolding protein at invadopodia, given that the proteolytic activity is not required. Which region in FAP is required for mediating protein-protein interactions? Is the entirety of the protein required or is a modular domain of FAP sufficient to rescue degradation? In order to facilitate protein transport, FAP and FNDC3B would need to interact with vesicle coat proteins or SNAREs. Does FAP or FNDC3B interact with transport proteins and if so, which region is responsible for facilitating the interaction? Understanding specific domain requirements might facilitate the generation of effective targeted therapy against FAP or FNDC3B. We believe that there is a lot of therapeutic

potential in FAP and FNDC3B. Metastasis is a devastating disease, but there is hope in invadopodia-targeting therapies. Through a combined effort of researchers, we hope to advance the understanding of metastasis on a molecular level and move closer toward viable treatments in the near future.

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