CAVEOLIN-1 AND MEMBRANE DOMAIN REGULATION OF FOCAL ADHESIONS AND TUMOR CELL MIGRATION

by

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B.Sc., Nankai University, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Cell and Developmental Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2016

ABSTRACT

Caveolin-1 (Cav1), a key protein component of cell surface invagination caveolae and a major substrate of Src kinase, has been shown to be associated with cancer malignancy. Galectin-3 (Gal3), a galactose-specific lectin, forms oligomers and crosslinks N-glycans on cell surface to form the galectin lattice. Gal3 and Cav1 function together to regulate focal adhesion dynamics and tumor cell migration. In this thesis we hypothesize that the galectin lattice, Cav1 membrane domain organization (caveolae, Cav1 scaffolds) and Cav1 molecular motifs (tyrosine 14 phosphorylation (pY14), the caveolin scaffolding domain (CSD)) are all involved in Cav1 promotion of focal adhesion dynamics and tumor cell motility.

Firstly, we found a synergistic expression of Cav1 and Gal3 in malignant thyroid cancer cells, which was required for focal adhesion kinase (FAK) stabilization in focal adhesions (a measure of focal adhesion dynamics), RhoA activation and cell migration. Co-overexpression of Cav1 and Gal3, but not either alone, in an anaplastic thyroid cancer cell line stabilized FAK within focal adhesions. Therefore, co-function of Cav1 and Gal3 is required to promote focal adhesion dynamics and cell migration in thyroid cancer.

Next we found that overexpression of PTRF/cavin-1 in PC3 prostate cancer cells, and consequent formation of caveolae, decreased cell motility by destabilizing FAK in focal adhesions. The impaired focal adhesion stabilization of FAK in PTRF/cavin-1-expressing PC3 cells was rescued by exogenous Gal3 in a Cav1-dependent manner. Hence the alteration of Cav1 microdomains by PTRF/cavin-1 overexpression decreases cell motility through affecting focal adhesion dynamics, which is overridden by reinforced Cav1-Gal3/galectin lattice co-function.

Finally, using Cav1-positive but tyrosine 14-phosphorylated Cav1 (pY14Cav1)-negative DU145 prostate cancer cells, various Cav1 Y14 and CSD mutants and a CSD

mimicking/competing peptide, we found a CSD-dependent pY14Cav1 regulation of focal adhesion dynamics and cell motility. Vinculin, a mechano-sensor at focal adhesions that was previously shown to recruit and stabilize other focal adhesion components, preferentially bound pY14Cav1 and was stabilized in focal adhesions by pY14Cav1 in a CSD-dependent manner. Vinculin tension was induced by pY14Cav1 in a CSD-dependent manner. Therefore, a novel interplay between pY14 and the CSD of Cav1 regulates focal adhesion dynamics and tension favouring cell migration.

PREFACE

In Chapter 1, all figures are used with permission from applicable sources.

Chapter 2 is based in part on a published paper titled “Coordinated Expression of Galectin-3 and Caveolin-1 in Thyroid Cancer” (Shankar J, Wiseman SM, Meng F, Kasaian K, Strugnell S, Mofid A, Gown A, Jones SJ, Nabi IR. Journal of Pathology. 2012). I contributed the immuno-labeling, confocal imaging and FRAP analysis of Figures 2-4 and 2-5, for which Dr. Jay Shankar conducted the siRNA knockdown. Dr. Jay Shankar provided Figures 2-1, 2-2 and 2-3 with Alireza Mofid helping with the western blots (Figure 2-1). I also participated in the discussion and revision of the manuscript. The published material is reprinted with permission from the publisher John Wiley and Sons.

Chapter 3 is based on a paper published in PLoS One titled “Galectin-3 Overrides PTRF/Cavin-1 Reduction of PC3 Prostate Cancer Cell Migration” (Meng F, Joshi B, Nabi IR. 2015). I conducted all the presented experiments with Dr. Bharat Joshi assisting with the Transwell cell migration assay (Figure 3-1). Both Dr. Ivan Robert Nabi and I contributed to the working model schematics (Figure 3-6). I wrote the manuscript under supervision of Dr. Ivan Robert Nabi and Dr. Bharat Joshi participated in the discussion and revision of the manuscript. The published material is reprinted under CC-BY license.

Chapter 4 is based on a work in preparation for submission. I did all the confocal imaging, FRAP assay, FRET assay, transwell cell migration assay, cell viability assay, and data analysis (Figures 4-1C, D, E, 4-2, 4-3C, 4-4 and 4-5). Dr. Bharat Joshi designed and constructed the tagged Cav1 mutants, which I helped to construct (Figure 4-1A). Dr. Bharat Joshi and I worked together to generate the stably transfected DU145 cell lines and perform the Cav1 and pY14Cav1 western blots (Figure 4-1B). Dr. Bharat Joshi and Sandeep Saxena generated the GST-Cav1(1-101) constructs. Sandeep Saxena conducted the GST pull-down,

elution, proteomic analysis and western-blot validation with help from Dr. Bharat Joshi and Dr. Jay Shankar (Figure 4-3A). Kyung-Mee Moon from laboratory of Dr. Leonard J. Foster (Department of Biochemistry & Molecular Biology and Michael Smith Labs, Centre for High-Throughput Biology, University of British Columbia, Vancouver) conducted the mass spectrometry analysis of the GST pull-downs (Figure 4-3A). The AP and AP-Cav peptides were gifts from Dr. Pascal Bernatchez (The James Hogg Research Centre, Institute for Heart + Lung Health, St Paul's Hospital, and Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver).

Unless specially mentioned, all people involved in the presented works were from the laboratory of Dr. Ivan Robert Nabi.

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ACKNOWLEDGEMENTS

I first would like to thank my supervisor, Dr. Ivan Robert Nabi. Dr. Nabi's professionalism and passion in scientific research has greatly inspired and guided me in my work. I appreciate him very much for his supervisory work that leads to the accomplishments and the experience that I have gained over the past few years.

I would like to extend my thanks to my supervisory committee: Dr. Edwin Moore, Dr. Pascal Bernatchez and Dr. Tim O'Connor. Their comments came from different scientific backgrounds and were always helpful. I appreciate the time and effort that they spent working with me.

I thank my lab mates, past and present, the wonderful and talented people I spent the past few years with in the lab. Especially, I would like to thank Dr. Bharat Joshi for his genuine help from my first day in the lab. He has made working in the lab an enjoyable process. I thank him for being such a nice colleague in work and a great friend in life.

I appreciate my girlfriend Wei Xiao, who is also working toward her Ph.D. degree in cell biology, for being here with me. She has brightened my life and made the years of my Ph.D. study full of happy memories and for that I truly thank her.

Finally, I would like to thank my parents and the rest of my family for their love and support. Without them I could not have come so far.

CHAPTER 1 Introduction

1.1 Tumor cell migration

1.1.1 Tumor metastasis

Tumor metastasis, also called the invasion-metastasis cascade, is a multistep process involving the invasion of tumor cells through surrounding extracellular matrix (ECM) and tissues and into circulatory vessels, survival and arrest in the circulation, exit from circulatory vessels, and survival and proliferation at a secondary site, resulting in metastatic colonization (Steeg, 2006; Valastyan and Weinberg, 2011). It is defined as one of the six primary hall-marks of cancer (Hanahan and Weinberg, 2000). Tumor metastasis is responsible for the spread of cancer and thus makes cancer hard to target and treat. Metastasis has been evaluated as the most lethal event during cancer progression and contributes to 90% of tumor-caused deaths (Gupta and Massague, 2006). Around half of the patients diagnosed with cancer have already developed metastases (Hernandez-Alcoceba et al., 2000). Therefore it is important to understand the tumor metastasis process in order to prevent it.

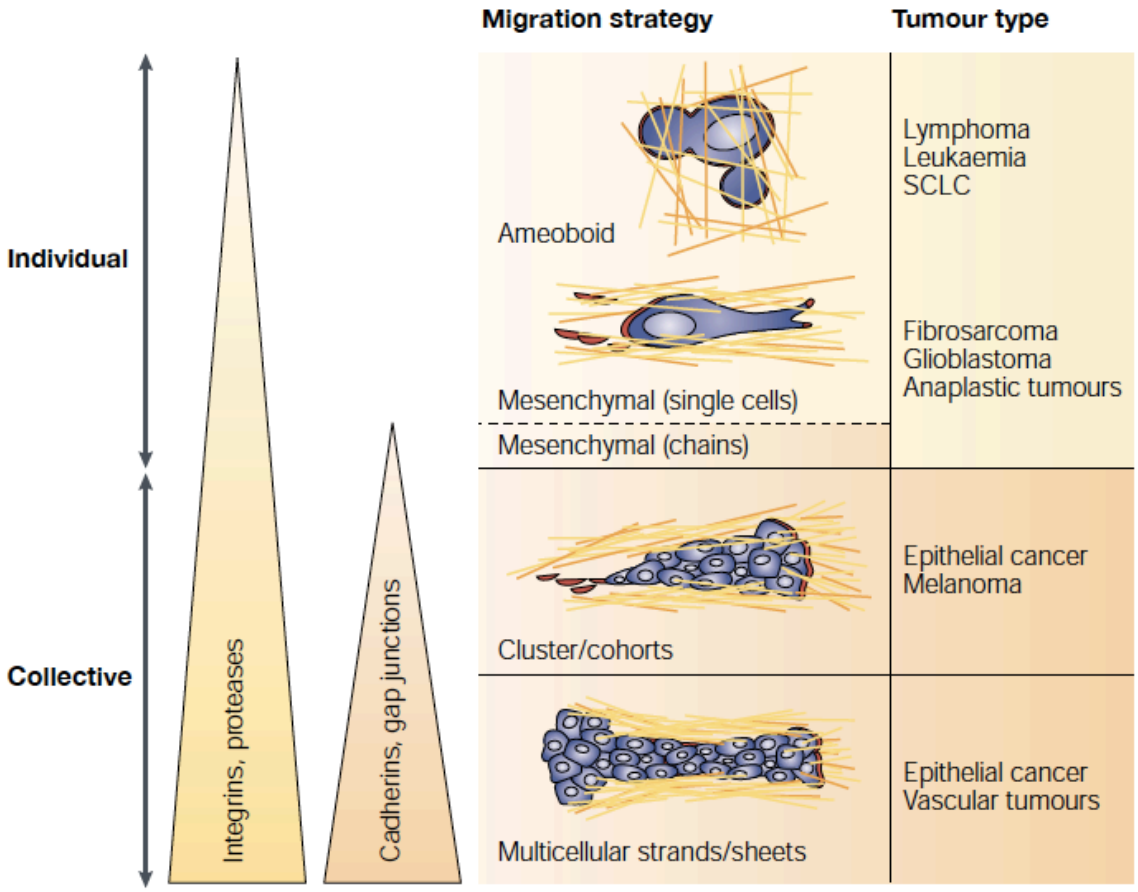
1.1.2 Molecular mechanism of cell migration

As the basis for tumor metastasis, cell migration is a dynamic cellular activity that depends on the spatiotemporal feedback between the actomyosin cytoskeleton and cell adhesions (Gupton and Waterman-Storer, 2006). It consists of multiple steps including polarization and protrusion of cell leading edge, formation of new cell adhesions, transmission of traction forces and locomotion, and disassembly of cell adhesions at the trailing edge (Lauffenburger and Horwitz, 1996). Multiple cells can migrate collectively in cohesive units, e.g. in strands or clusters, dependent on intercellular junctions; alternatively, individual cells migrate in either a “mesenchymal migration” manner that presents cortical actin, stress fibers and

focalized integrins and proteases, or in an “amoeboid migration” manner that is mediated only by cortical actin without stress fibers or focalized integrins and proteases (Figure 1-1) (Friedl and Wolf, 2003; Valastyan and Weinberg, 2011). Tumor cells can switch between these migration methods to compensate for loss of certain required factors, e.g. intercellular junctions, adhesions, ECM proteolysis, etc. (Friedl and Wolf, 2003; Valastyan and Weinberg, 2011).

Figure 1-1. Tumor invasion mechanisms.

Triangles in the left side indicate the requirement for different molecular programs that determine individual or collective tumor cell migration models. Different migration strategies and selective tumor types that apply such migration strategies are listed on the right side. From individual (top) to collective (bottom) migration, integrins and ECM proteases provide increased control of cell–ECM interaction. Cadherins and gap junctions facilitate cell–cell adhesion for collective migration in strands or clusters but they are not required for single cell migration, i.e. mesenchymal and amoeboid migration. Adapted from (Friedl and Wolf, 2003).



1.1.2.1 Actin turnover and mechanics

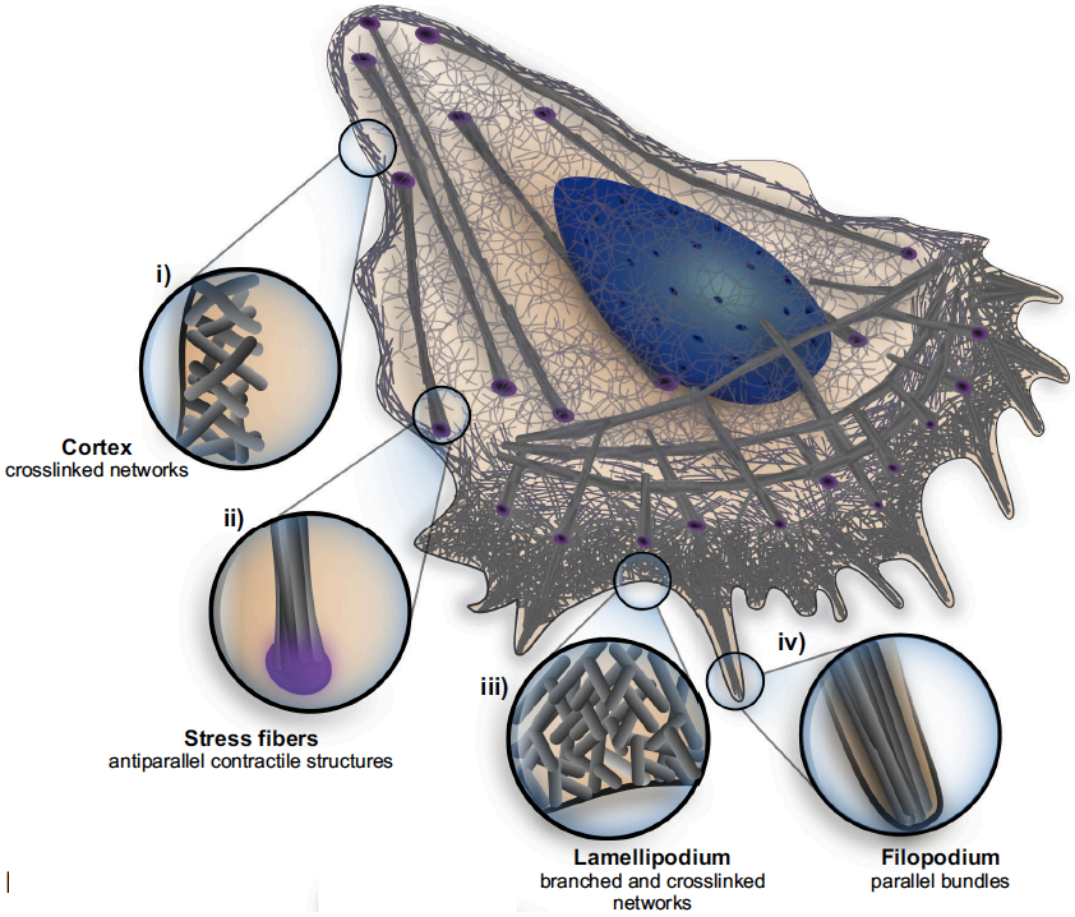
The dynamic assembly and disassembly of actin filaments promote the remodeling of the actin cytoskeleton, which facilitate cellular morphological changes and locomotion during cell migration (Blanchoin et al., 2014; Revenu et al., 2004). Actin filaments are formed by basic units of the actin monomer, a 42-kDa globular protein (G-actin), that nucleates into trimers to initiate actin filament elongation (Holmes et al., 1990; Kabsch et al., 1990; Sept et al., 1999). Once actin nucleation is initiated with trimers, elongation can happen with a fast rate depending on the concentration of available actin monomers (Wegner and Engel, 1975). Actin filaments are polarized structures with a barbed end and a pointed end: the barbed end has the capability of elongating the filaments 10 times faster than the pointed end (Pollard, 1986). Disassembly of actin filaments can happen when they interact with actin depolymerizing factor (ADF)/cofilin proteins that sever the actin filaments and dissociate Arp2/3 complex from the branched actin filaments, promoting the turnover of actin filaments (Carlier et al., 1997; Chan et al., 2009; Ingerman et al., 2013; Mahaffy and Pollard, 2006; Reymann et al., 2011). ADF/cofilin has a preference of binding to ADP-actin that prevents it from interacting with the barbed end of actin filaments where dynamic recruitment of ATP-actin is happening (Blanchoin and Pollard, 1999; Suarez et al., 2011). Myosin has also been found to induce actin disassembly by an “orientation selection” mechanism that disassembles anti-parallel actin filaments without affecting parallel actin filaments (Reymann et al., 2012). The disassembly is proposed to happen through the buckling of actin filaments induced by myosin contraction, suggesting a tension-based regulation of actin dynamics (Murrell and Gardel, 2012; Vogel et al., 2013).

Actin forms the cortical cytoskeleton, actin networks at cortical region of a cell adjacent to plasma membrane, and stress fibers, contractile bundles of actin filaments with myosin and crosslinking proteins. Both the cortical cytoskeleton and stress fibers facilitate cell protrusion,

traction force transmission and release of cell rear (Friedl and Wolf, 2003; Lauffenburger and Horwitz, 1996). Cellular contraction is produced by the motor protein myosin that assembles with filaments in the actin network and develops actin filament gliding and thus cellular contraction and focal-adhesion tension (Blanchoin et al., 2014). Filamentous actin (F-actin) forms diverse structures that facilitate various cell protrusions (Figure 1-2), i.e. bundled actin filaments for filopodium, branched actin filaments for lamellipodium, etc. (Blanchoin et al., 2014; Revenu et al., 2004). The activated Arp2/3 complex is required for both bundled and branched actin filaments (Mullins et al., 1998; Reymann et al., 2010; Robinson et al., 2001). When capping proteins are absent from the branched actin network, the freely elongating actin filaments may bundle as parallel filaments by electrostatic interactions and are further stabilized by crosslinking proteins such as facsin (Achard et al., 2010; Haviv et al., 2006; Reymann et al., 2010; Svitkina et al., 2003; Vignjevic et al., 2003; Zheng et al., 2011). Such conversion of F-actin structures contributes to changes in cell protrusion and mechano-signaling transduction (Blanchoin et al., 2014).

Figure 1-2. Various actin architectures within a cell.

With the dark lines denoting actin filaments, multiple types of actin architectures within a cell are shown here, including: i) the cell cortex; ii) a contractile actin fiber, i.e. the stress fiber attached to the cell adhesion complex (purple spots); iii) the lamellipodium; and iv) the filopodium. The zoomed regions show architectural features of different regions within the cell. Adapted from (Blanchoin et al., 2014).

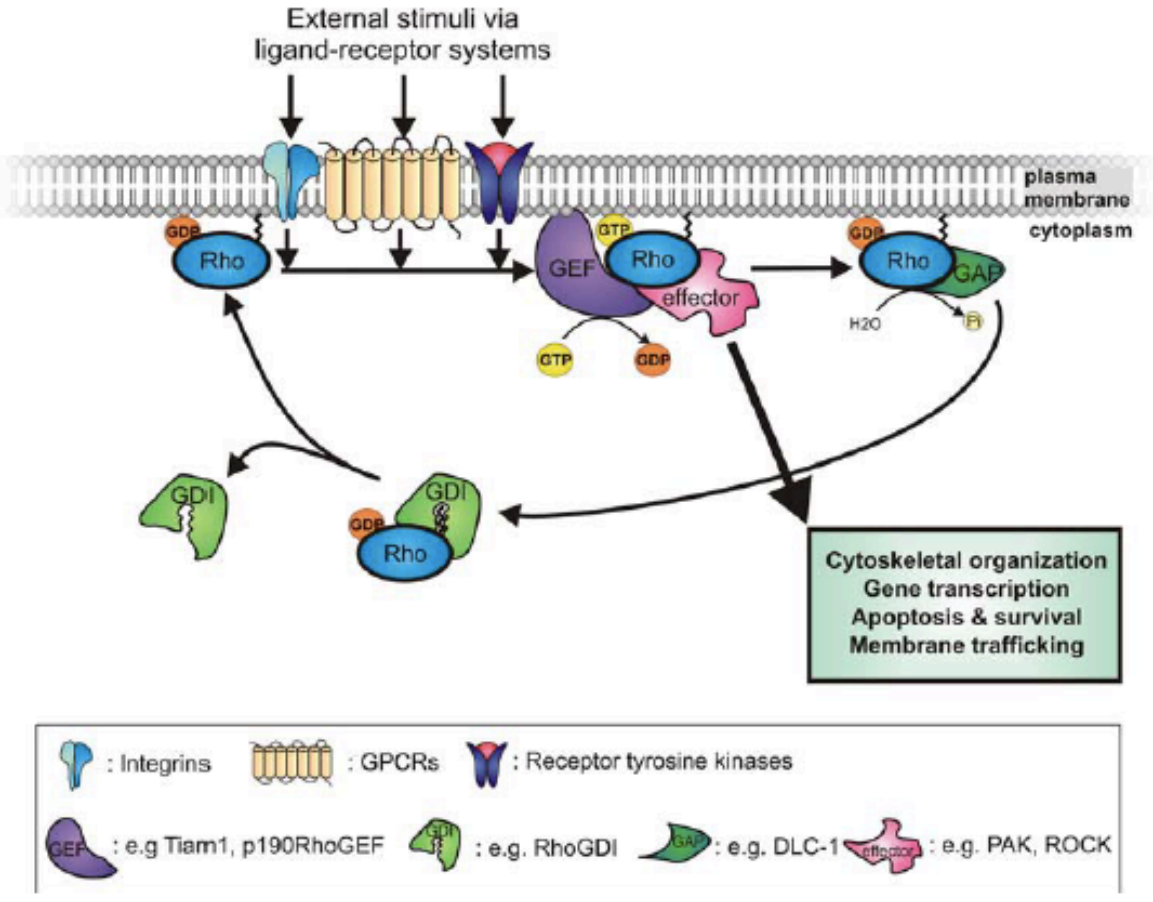


1.1.2.2 Rho GTPase family

The Rho GTPase family is a subfamily of the Ras superfamily proteins and acts as the most important intracellular regulators of actin cytoskeleton dynamics and cell migration. Rho, Rac and Cdc42 are the most studied members of the Rho family. These GTPases are bound to GTP in an active state, or GDP in an inactive state, and function as molecular switches by cycling between these two states. GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs) associate with the Rho family proteins to regulate the activity levels of the latter (Figure 1-3) (Sit and Manser, 2011; Spiering and Hodgson, 2011). GAPs promote the intrinsic hydrolysis of GTP to GDP while GEFs interact with GTPases to assist the exchange of GDP to GTP. GDIs down-regulate Rho GTPase activity not only by binding to GTPases and preventing the exchange of GDP to GTP, but also by relocating Rho GTPases from their action site at the cell membrane to the cytoplasm (Spiering and Hodgson, 2011).

Figure 1-3. Regulation of the GTPase cycle.

GDP-bound inactive GTPases are maintained by GDIs binding and preventing the guanine exchange. Once the GDI dissociates, GTPases can translocate to the plasma membrane and be activated by GEFs upon external stimuli from cell surface receptors such as adhesion receptors, G-protein coupled receptors (GPCRs) and receptor tyrosine kinases. Consequently, active Rho GTPases bind various effectors to induce downstream signaling pathways, which can be mediated by GEFs. GAPs inactivate the Rho GTPases by hydrolyzing the GTP to GDP, and thus switch off the downstream signaling. Adapted from (Ellenbroek and Collard, 2007).



These small GTPases, when activated, bind to various protein kinases and actin-related proteins that lead to signaling pathways mediating actin cytoskeleton assembly and disassembly (Jaffe and Hall, 2005; Sit and Manser, 2011; Zhao and Manser, 2005). Studies on specific Rho GTPases have found that RhoA promotes formation of stress fibers and focal adhesions, Rac1 facilitates lamellipodial protrusion and Cdc42 induces filopodial protrusion (Nobes and Hall, 1995a; Nobes and Hall, 1995b; Ridley and Hall, 1992; Ridley et al., 1992). Various downstream effectors are involved in these signaling pathways. For example, Rho proteins, namely RhoA, B and C, activate Rho-associated coiled-coil kinase1/2 (ROCK) that phosphorylates several actin-related proteins (Narumiya et al., 2009; Spiering and Hodgson, 2011). ROCK phosphorylates and activates myosin light chain while it phosphorylates and inactivates myosin light chain phosphatase, which both contribute to actomyosin contractility (Riento and Ridley, 2003; Wang et al., 2009b). ROCK also activates LIM kinase (LIMK), which further phosphorylates ADF/cofilin inhibiting its ability to bind and sever the actin cytoskeleton (Lin et al., 2003; Maekawa et al., 1999; Ohashi et al., 2000).

Despite the distinct functions of Rho GTPases, Rho, Rac and Cdc42 are antagonistically regulated in complex cascades that lead to cytoskeleton remodeling and cell migration (Spiering and Hodgson, 2011). The antagonistic regulation of RhoA and Rac1 has been well studied. It has been proposed, and observed in some cell systems, that active Rac1 enriches at the leading edge to drive cell protrusions while active RhoA facilitates cell retraction at the rear end (Kraynov et al., 2000; Rottner et al., 1999; Van Keymeulen et al., 2006; Wong et al., 2006). Some other studies also found in cancer cells and fibroblasts that active RhoA locates at the leading edge, which suggests a more complex spatiotemporal regulation of RhoA/Rac1 coordination at very fine time-scales in regions of dynamic cytoskeletal changes, e.g. protrusion, retraction and endocytosis (Machacek et al., 2009; Nalbant et al., 2004; Pertz et al., 2006; Shen et al., 2006).

Altered Rho GTPase signaling contributes to the increased mobile capability of cancerous cells. Although near one-third of Ras super family proteins have been found to be mutated in human cancers, very few Rho family members have been found to be genetically altered (Ellenbroek and Collard, 2007). Instead, overexpression of multiple Rho GTPases, including RhoA, Rac1 and Cdc42, has been found in multiple cancer types; also, regulators of Rho GTPases, including various of GEFs, GDIs and GAPs, are aberrantly expressed or mutated in many types of cancer (Ellenbroek and Collard, 2007).

1.1.2.3 Focal adhesions

Focal adhesions are macromolecular complexes formed by transmembrane integrin family proteins, which interact with ECM components, and various cytoplasmic proteins such as vinculin, talin, α-actinin, etc. that form complexes with F-actin (Burridge and Fath, 1989; Burridge et al., 1988). Focal adhesions connect the ECM to the cytoskeletal F-actin, which makes these structures an important transmitter for cell-ECM signalling and mechanical force (Burridge and Fath, 1989; Burridge et al., 1988). Mechanical force caused by both ROCK-dependent actomyosin contraction and ECM rigidity requires cell adhesions and contributes to the regulation of focal adhesion development (Burridge and Guilluy, 2015; Hoffman, 2014). An optimal model for cell migration also requires the continuous formation and disassembly of cell adhesions including focal adhesions (Gupton and Waterman-Storer, 2006; Ridley et al., 2003).

The formation of focal adhesions happens along with the protrusive changes of cell morphology at the leading edge (Gardel et al., 2010). Microclusters of transmembrane integrin proteins are activated through association with the cytoplasmic protein talin, a focal adhesion component that binds to vinculin and actin (Tadokoro et al., 2003; Wiseman et al., 2004). Activated integrins have high affinity for the ECM; they engage with ECM or

associate with the dynamic actin at leading edge for directed movement in search for permissive sites for adhesion development (Galbraith et al., 2007; Tadokoro et al., 2003). Paxillin is then recruited to integrin clusters, which form nascent adhesions and recruit more integrins (Laukaitis et al., 2001; Webb et al., 2004; Wiseman et al., 2004). Nascent adhesions disassemble quickly unless they undergo tension-mediated transition to focal complexes and focal adhesions (Balaban et al., 2001; Choi et al., 2008; Chrzanowska-Wodnicka and Burridge, 1996). Mechanical tension induces small adhesion-associated, α-actinin-mediated actomyosin bundles where the cytoskeleton-binding focal adhesion components vinculin and zyxin can be recruited (Choi et al., 2008). Such force is also proposed to facilitate unfolding and conformational changes of focal adhesion proteins that open their protein binding sites to recruit further focal adhesion proteins, and thus promote the maturation of nascent adhesions to focal adhesions (Vogel and Sheetz, 2006).

1.1.2.3.1 Focal adhesion proteins

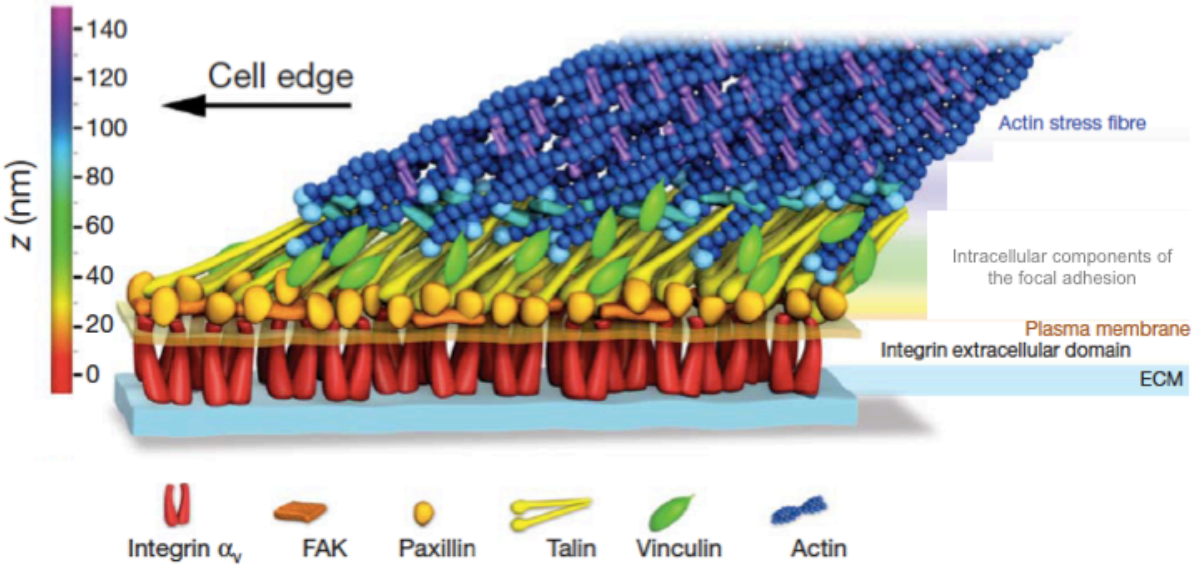
An extensive proteomics study on isolated focal adhesions found 905 focal adhesion proteins, suggesting that focal adhesions are a macromolecular complex that involves massive protein components and signaling networks (Kuo et al., 2011). As a cornerstone of the focal adhesion architecture, transmembrane integrins connect ECM and intracellular components of focal adhesions and initiate the formation of focal adhesions (Gardel et al., 2010). Heterodimers are formed from one α and one β subunits of integrins. There are 18 α and 8 β integrin subunits and different combinations correlate to their recognition of distinct substrates in ECM (Hynes, 2002; van der Flier and Sonnenberg, 2001). For example, α_4-, α_5-, α_8-, α_{IIb}-, and α_V-subunit-containing integrins bind to RGD (Arginine-Glycine-Aspartic Acid)-containing components in ECM, commonly fibronectin and vitronectin (van der Flier and Sonnenberg, 2001).

Paxillin is a signal-transducing adaptor protein in focal adhesions identified in 1990s (Turner et al., 1990) and has a N-terminal region enriched with protein interaction sites that bind with diverse binding partners including multiple other components of focal adhesions, such as focal adhesion kinase (FAK), vinculin and integrins (Brown and Turner, 2004). It binds with integrin clusters upon initiation of nascent adhesions and helps the growth of nascent adhesions to focal adhesions (Laukaitis et al., 2001; Wiseman et al., 2004). Paxillin is tyrosine-phosphorylated by Src and FAK, which subsequently interacts with the adaptor protein Crk and promotes cell migration (Bellis et al., 1995; Brown and Turner, 2004). Talin, an essential protein for focal adhesion formation, interacts with and activates integrins for their engagement with ECM and links focal adhesions to the actin cytoskeleton (Tadokoro et al., 2003; Yan et al., 2015). Talin has strong binding affinity for vinculin, another actin-binding protein that is recruited to focal adhesions to strengthen the linkage to the cytoskeleton (Yan et al., 2015). Both talin and vinculin have been evaluated as mechanical sensors that transmit mechanical force and signaling at focal adhesions (Atherton et al., 2016; Yan et al., 2015).

FAK is the major kinase in focal adhesions that undergoes phosphorylation in response to integrin-ECM engagement; phosphorylated FAK (pFAK) functions as an important signal transducer and regulator of focal adhesion development (Burridge et al., 1992; Mierke, 2013). Talin was found to mediate integrin-induced FAK phosphorylation while a more recent study found that FAK recruits talin to nascent adhesions to mediate cell motility (Chen et al., 1995; Lawson et al., 2012), suggesting a complex spatiotemporal signaling network within focal adhesion components. Particle-localization super-resolution microscopy analysis has demonstrated a distribution of key focal adhesion proteins in height relative to ECM (Kanchanawong et al., 2010), providing insights into the organization and interactions of focal adhesion proteins (Figure 1-4).

Figure 1-4. Nanoscale architecture of focal adhesions.

Schematic model showing the molecular architecture of focal adhesions, as determined by particle-localization super-resolution microscopy. Focal adhesion proteins are distributed at different heights relative to ECM (indicated by the color-coding height bar on the left side). Integrin extracellular domain interacts with ECM. Within the intracellular components of the focal adhesion, FAK, paxillin and talin locate closely to integrins and are related with integrin-mediated signaling. Vinculin interacts with both talin and the actin stress fiber to transduce mechanical force. This model does not represent protein stoichiometry. Adapted and modified from (Kanchanawong et al., 2010).



1.1.2.3.2 FAK and focal adhesion dynamics

FAK is the major kinase involved in focal adhesion signaling and regulates focal adhesion dynamics through its kinase domain and tyrosine 397 (Y397) autophosphorylation. Reduced FAK Y397 phosphorylation increases FAK exchange between focal adhesions and cytosol, which slows focal adhesion disassembly and cell migration (Giannone et al., 2004; Hamadi et al., 2005), suggesting a correlation between the FAK dwelling/stabilization in focal adhesions and its activation and function. Indeed, the integrin-dependent autophosphorylation of FAK Y397 creates a high-affinity binding site for Src homology 2 (SH2) domain-containing proteins, such as Src kinase (Burridge et al., 1992; Mitra and Schlaepfer, 2006). Such FAK-Src interaction forms a transient signaling complex that further phosphorylates FAK on other tyrosine sites (Y576, Y577, Y861 and Y925) and recruits adaptor proteins including paxillin and Crk, which in turn promote focal adhesion dynamics and cell motility (Mitra and Schlaepfer, 2006; Webb et al., 2004).

1.1.2.3.3 Vinculin and force transmission

Vinculin is a cytoplasmic component of focal adhesions that consists of a head portion (Vh, domains D1-D4) and a tail portion (Vt, domain D5), which binds various focal adhesion and cytoskeleton proteins distinctly (Ziegler et al., 2006). Upon activation at sites of cell adhesions, vinculin switches from a closed globular conformation to an extended conformation allowing binding of specific partners to the Vh and Vt domains (Bakolitsa et al., 2004; Cohen et al., 2006; Ziegler et al., 2006). Interaction with both the talin-integrin complex (with Vh) and the actin cytoskeleton (with Vt) places vinculin at an ideal position to transmit tension-induced signalling in focal adhesions (Carisey et al., 2013; Cohen et al., 2006; del Rio et al., 2009; Kanchanawong et al., 2010; Ziegler et al., 2006). Indeed, recruitment of vinculin to focal adhesions correlates with increased contractile force at focal

adhesion sites which was monitored with a fluorescence resonance energy transfer (FRET)-based vinculin tension sensor (VinTS) construct (Balaban et al., 2001; Grashoff et al., 2010). The tension is also considered to facilitate the recruitment and stabilization of vinculin at focal adhesions (Atherton et al., 2016). Intriguingly, active extended vinculin stabilizes various focal adhesion proteins, including FAK, within focal adhesions and activates integrins in an actin- and talin-dependent manner (Carisey et al., 2013). As a key component of cell adhesions, vinculin is essential for mammalian embryonic development; in mice, vinculin knockout is lethal and leads to defects in heart and nervous system and retarded somites and limbs (Xu et al., 1998).

1.2 Caveolin-1 (Cav1)

Caveolin-1 (Cav1) is a member of the caveolin protein family and the key component of caveolae, flask-shaped invaginations on the cell surface involved in many cellular processes such as vesicular transport, intracellular signaling and mechanical transduction (Parton and Del Pozo, 2013). The 178-amino acid protein Cav1 is also associated with the regulation of lipid rafts and of multiple cancer-associated processes including cell death and survival, cell migration and invasion, and tumor growth and metastasis, such that Cav1 is a diagnostic marker and treatment target for various cancers (Goetz et al., 2008b; Navarro et al., 2004; Parton and Del Pozo, 2013; Williams and Lisanti, 2005).

1.2.1 Caveolin family

Caveolin homologues are found in wide variety of vertebrates, such as human, mouse, dog, cow, *Xenopus*, etc., and even in invertebrates such as *C. elegans* (Tang et al., 1997; Williams and Lisanti, 2004b). The caveolin protein family has three members, caveolin-1 (Cav1), caveolin-2 (Cav2) and caveolin-3 (Cav3) (Williams and Lisanti, 2004a). They are predominantly expressed at the plasma membrane due to three palmitoylation sites at the

C-terminal region, but can also be found in the Golgi apparatus, endoplasmic reticulum and cellular vesicles; caveolins may also exist in soluble cytoplasmic and secreted forms (Williams and Lisanti, 2004b). All three caveolin family members have both their N- and C-termini in the cytoplasm and a long hairpin-like membrane-embedded domain (Dietzen et al., 1995; Monier et al., 1996; Williams and Lisanti, 2004b).

Cav1, a 178-amino acid protein, was the first identified member of the caveolin family (Glenney, 1989; Rothberg et al., 1992). It exists in two isoforms *in vivo* – the full-length α isoform containing residues 1-178, and the truncated β isoform containing residues 32-178 (Scherer et al., 1995). The truncation results in the differential phosphorylation of the two isoforms of Cav1 since only Cav1α can be phosphorylated at the tyrosine 14 (Y14) residue by Src kinases (Li et al., 1996b). The truncation is also considered important in differential compartmentalization of the two isoforms of Cav1 (Li et al., 2001b).

The other two members of the caveolin family, Cav2 and Cav3, differ in expression and cellular distribution. Cav2, although not essential for caveolae formation, forms a stable hetero-oligomeric complex with Cav1 and requires Cav1 for its targeting to the plasma membrane (Lahtinen et al., 2003; Silva et al., 1999; Williams and Lisanti, 2004a). Cav3 has high sequence similarity to Cav1 but is primarily located in muscle and glia where it is essential for caveolae biogenesis (Galbiati et al., 2001; Way and Parton, 1995; Williams and Lisanti, 2004a).

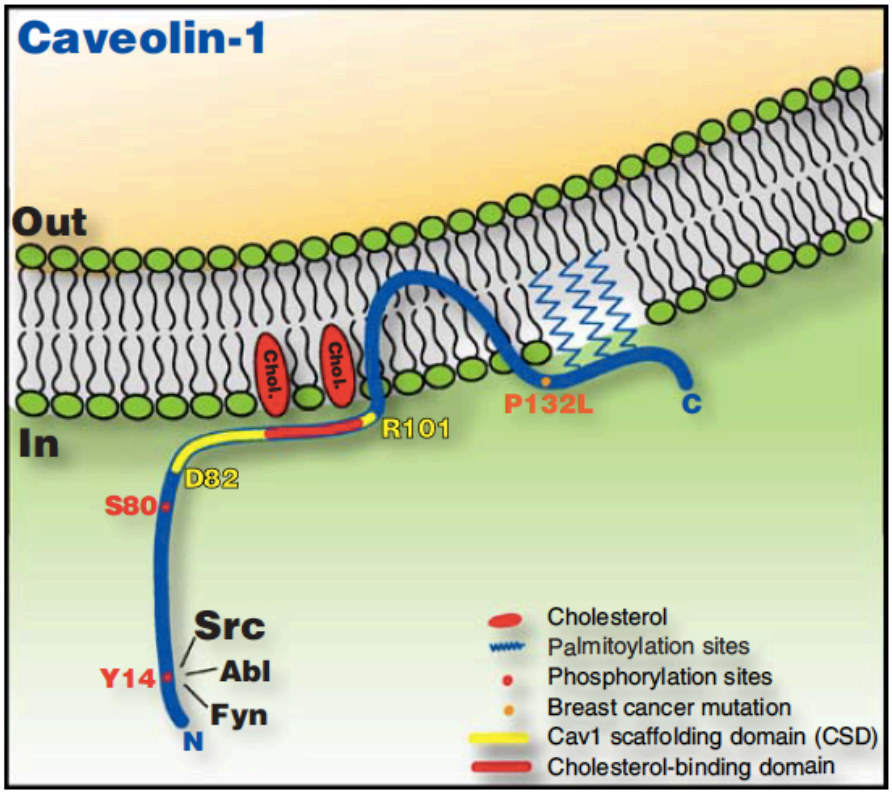
1.2.2 Cav1 structure

Cav1 is a 178-amino acid integral membrane protein (Figure 1-5), with both the N and C termini facing the cytoplasm and a putative “hairpin” intramembrane domain embedded within the membrane bilayer (Glenney and Soppet, 1992; Rothberg et al., 1992; Schlegel and Lisanti, 2000). Cav1 can be phosphorylated on tyrosine 14 (Y14) by the Src, Abl and

Fyn kinases, which links Cav1 to cellular regulation of focal adhesions and migration, etc., and on serine 80 (S80), which converts Cav1 to a soluble secreted protein that can bind to endoplasmic reticulum membranes and enter the secretory pathway (Goetz et al., 2008b; Li et al., 1996b; Schlegel et al., 2001). Cav1 forms oligomers via the oligomerization domain, which consists of residues 61 to 101 (Sargiacomo et al., 1995). The caveolin scaffolding domain (the CSD) is defined as residues 82 to 101, and mediates Cav1 interaction with multiple signaling molecules (Li et al., 1996a). Within the CSD, residues 94 to 101 comprise the cholesterol binding domain and the cholesterol recognition/interaction amino acid consensus (CRAC) motif, which assists Cav1 binding to cholesterol (Epand et al., 2005; Murata et al., 1995). Cav1 can also be palmitoylated on cysteine 133 (C133), cysteine 143 (C143) and cysteine 156 (C156). This is not required for Cav1 localization to caveolae but is important for Cav1 oligomerization (Dietzen et al., 1995; Monier et al., 1996). Palmitoylation on C156, but not C133 or C143, has been shown to be required for Cav1 Y14 phosphorylation by Src kinase in the cell (Lee et al., 2001). A more recent study has found that Cav1 palmitoylation is required for Cav1 interaction with fatty acid synthase (FASN) and the maintenance of active Src and Akt levels in prostate cancer cells (Di Vizio et al., 2008).

Figure 1-5. Structure of Cav1 molecule.

Both N and C termini of Cav1 face the inside of the cell. A 'hairpin' intramembrane domain and three palmitoyl groups in the Cav1 C-terminal region orchestrate Cav1 insertion into the cell membrane. A mutation discovered in breast cancers (P132L) is localized close to the palmitoylation sites. Both S80 and Y14 can be phosphorylated. S80 phosphorylation has been implicated in Cav1 secretion. Y14 phosphorylation, by Src, Fyn or Abl tyrosine kinases, has been associated with various cellular activities including mechanotransduction, signal transduction, endocytosis, cell migration and focal adhesion dynamics. The Cav1 scaffolding domain (CSD) comprises amino acids D82 to R101 (yellow) is a highly conserved region that contains a sequence that interacts with cholesterol via conserved basic and bulky hydrophobic residues. Adapted from (Goetz et al., 2008b).



1.2.2.1 The caveolin scaffolding domain (CSD) and its regulation of cell signaling

The CSD of Cav1 comprises residues 82-101 and is related to regulation of various cellular signalling pathways. The CSD mediates Cav1 interaction with and compartmentalization of many signaling molecules, including both upstream receptors and downstream effectors, such as growth factor receptors, Src family kinases, endothelial nitric oxide synthase (eNOS), G proteins and G protein-coupled receptors (GPCRs) (Couet et al., 1997a; Garcia-Cardena et al., 1997; Li et al., 1996a; Li et al., 1995; Ostrom and Insel, 2004). A CSD-containing region in Cav1 has been shown to interact with G protein α subunit and a polypeptide of the CSD sequence suppresses the basal activity of heterotrimeric G proteins (Li et al., 1995). Subsequent studies have implicated the CSD of Cav1 in signaling by numerous proteins including human epidermal growth factor receptor 2 (ErbB2/Neu), H-Ras, endothelial nitric oxide synthase (eNOS), epidermal growth factor receptor (EGFR) and inhibitor of differentiation/DNA synthesis ID-1 (Couet et al., 1997b; Engelman et al., 1998; Engelman et al., 1997; Garcia-Cardena et al., 1997; Zhang et al., 2007b). A more recent study on Cav1-eNOS interaction mapped the interaction to residues 90 – 99 of Cav1, a sub-sequence within the CSD (Trane et al., 2014), suggesting a critical role of this CSD subsequence in mediating the Cav1 interactions. Indeed, the F92TVT95 motif was shown to be the most critical for Cav1 interaction with G protein α subunits (Couet et al., 1997a). A mutation within this subsequence, F92A/V94A, blocks the function of the CSD in regulating insulin receptor (IR) and EGFR signaling (Lajoie et al., 2007a; Nystrom et al., 1999). A polypeptide of the CSD sequence fused to an internalization sequence from antennapedia (AP-Cav) mimics CSD function, inhibiting eNOS and blocking NO release from endothelial cells *in vitro* (Bernatchez et al., 2005; Bucci et al., 2000; Gratton et al., 2003). AP-Cav inhibition of eNOS decreases vasodilation, inflammation and hyperpermeability of tumor microvasculature thereby blocking tumor angiogenesis and delaying tumor progression *in*

vivo (Bernatchez et al., 2005; Bucci et al., 2000; Gratton et al., 2003). This demonstrates the important role for the CSD in endothelial cell signaling and tumor progression, and suggests that the CSD mimicking peptides may represent an effective anti-cancer therapy (Williams and Lisanti, 2005).

1.2.2.2 Cav1 tyrosine 14 (Y14) phosphorylation

Cav1 is a major substrate of the tyrosine kinase Src and is phosphorylated on Y14 (Li et al., 1996b). The dephosphorylation of Y14-phosphorylated Cav1 (pY14Cav1), on the other hand, is conducted by protein tyrosine phosphatase 1B (PTP1B) (Lee et al., 2006). Therefore, the expression and activities of both Src and PTP1B control the levels of pY14Cav1. For example, prostate cancer cell lines DU145 and PC3 have similar levels of Src activities (Xu et al., 2012) while PC3, compared to DU145, exhibits significantly higher levels of reactive oxygen species (ROS) that inhibit PTP1B (Boivin and Tonks, 2015; Jayakumar et al., 2014). This may contribute to the higher pY14Cav1 levels in PC3 cells than in DU145 cells (Joshi et al., 2008a). It has been shown that pY14Cav1 is involved in regulation of multiple Cav1-related processes ranging from caveolae biogenesis, cell proliferation and transformation, cell death and survival to cell migration and metastasis (Goetz et al., 2008b; Senetta et al., 2013). Indeed, v-Src phosphorylated Cav1 has been found to induce and colocalize with caveolae in normal rat fibroblasts and kidney cells (Nomura and Fujimoto, 1999). EGF-induced pY14Cav1 is linked with increased formation of cytoplasmic caveolae and development of cell-surface caveolae, suggesting that pY14Cav1 is a key factor involved in caveolae internalization (Aoki et al., 1999; Orlichenko et al., 2006). Expression of the insulin receptor substrate-1 (IRS-1), an important protein in cell proliferation and differentiation, correlates with Cav1 in multiple cell lines; IRS-1 binds to Cav1 pY14 through its phosphotyrosine binding (PTB) domain to avoid proteasomal degradation, which suggests a role for pY14Cav1 in cell proliferation (Chen et al., 2008). pY14Cav1 has also been found to

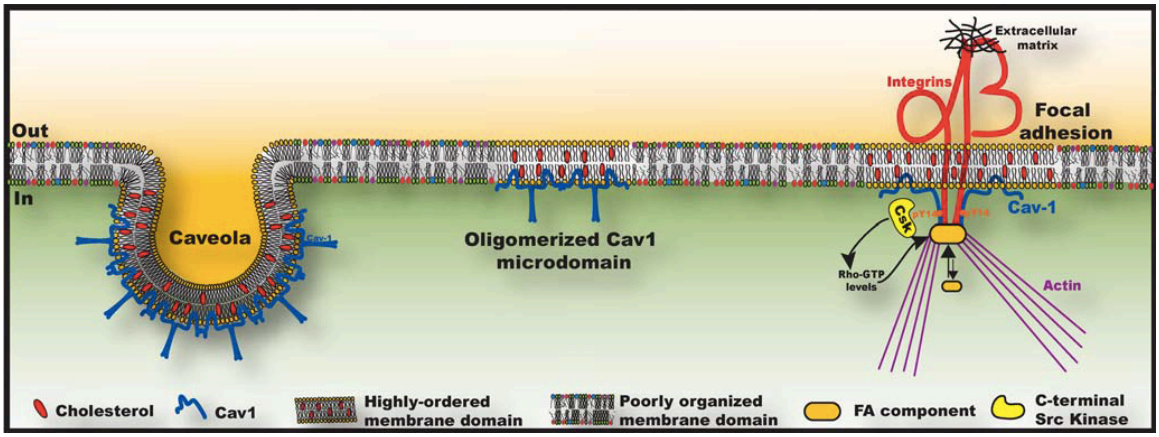
sensitize MCF-7 breast cancer cells to paclitaxel-mediated cell death (Shajahan et al., 2012; Shajahan et al., 2007). In addition, pY14Cav1 mediates focal adhesion dynamics and cell migration (Goetz et al., 2008a; Joshi et al., 2008a), and plays a role in the cellular response to mechanical stress (Parton and Del Pozo, 2013).

1.2.3 Caveolae and Cav1 scaffolds

Cav1 forms various membrane domains such as caveolae and non-caveolar Cav1 scaffolds (Figure 1-6). Caveolae were identified as uniform plasma membrane invaginations more than 60 years ago by their relatively constant diameter of 50-80 nm in mouse gall bladder epithelial cells and have since been defined as sterol- and sphingolipid-enriched heterogeneous membrane domains involved in the compartmentalization of various cellular processes (Pike, 2006; Yamada, 1955). Diverse functional roles of caveolae in multiple physiological processes have been reported and could be explained by multiple models - scaffolding for signaling activities, forming intracellular transport vesicles, regulating plasma membrane lipid composition and fatty acid incorporation, and serving as membrane buffers to protect cell membrane from tension (Hansen and Nichols, 2010; Sinha et al., 2011). Apart from caveolae, Cav1 also exists and functions in non-caveolar Cav1 oligomers in cholesterol-rich raft areas of cell surface, termed Cav1 scaffolds (Hill et al., 2008; Lajoie et al., 2009a). Cav1 scaffolds were described as immobile Cav1 membrane microdomains that limited EGFR signaling and proposed to correlate with the smallest SDS-stable Cav1 oligomers composed of ~15 molecules (Lajoie et al., 2007a; Monier et al., 1995). However, further studies found that induction of caveolae in caveolae-null prostate cancer cells significantly impaired the growth and metastasis of tumors formed by these cells, suggesting important roles for the non-caveolar Cav1 scaffolds in prostate cancer progression (Moon et al., 2013).

Figure 1-6. Cav1-related membrane domain organization.

Oligomerized Cav1 at the plasma membrane forms: 1) caveolae, through membrane curvature and ordering; 2) Cav1 scaffolds, by non-caveolar Cav1 oligomerization at ordered plasma membrane microdomains; and 3) although debatable, ordered domains within focal adhesions, via pY14Cav1 activation of Rho-GTPase and regulation of focal adhesion dynamics. Adapted from (Goetz et al., 2008b).



1.2.3.1 The biogenesis of caveolae

Two major protein components are required for caveolae formation – caveolins and cavins. Stepwise study on the formation of caveolin and cavin complexes has found that Cav1 forms 70S complexes in the Golgi apparatus that are transported to the plasma membrane and recruit cavin-1, which forms 60S complexes independently, to the cell membrane (Hayer et al., 2010).

The caveolin family members, Cav1, 2 and 3, are all caveolar coat proteins while only Cav1 and muscle-specific Cav3 are capable to induce caveolae formation (Capozza et al., 2005; Fra et al., 1995). Cav1 serves as the key driver and regulator of caveolae formation and dynamics. Genetic disruption of Cav1 causes loss of caveolae in non-muscle tissues, and expression of Cav1 leads to *de novo* formation of caveolae in cells lacking endogenous caveolins or caveolae (Drab et al., 2001; Fra et al., 1995; Razani et al., 2002); in contrast, reduced Cav1 expression is associated with the formation of non-caveolar scaffolds instead of caveolae (Lajoie et al., 2007a). Cav1 has also been found to regulate the dynamics and organization of lipid rafts by its ability to interact with cholesterol and modulate lipid raft expression (Parton and Del Pozo, 2013; Parton and Simons, 2007). In addition, pY14Cav1 has been related to caveolae formation and endocytosis. EGF-induced pY14Cav1 is linked with increased formation of cytoplasmic caveolae and development of cell-surface caveolae, indicating pY14Cav1 as a key factor involved in caveolae internalization (Aoki et al., 1999; Orlichenko et al., 2006). Indeed, a previous study revealed that pY14Cav1 functions as a mechano-sensor and promotes expression of Cav1 and PTRF/Cavin-1 through the transcription factor Egr-1, and thereby increases the formation of caveolae (Joshi et al., 2012). Another recent study found that pY14Cav1 promotes caveolae swelling and detachment from the cell membrane (Zimnicka et al., 2016), suggesting that pY14Cav1 mediates not only caveolae biogenesis, but also their recycling from the plasma membrane.

The cavin family includes polymerase I and transcript release factor (PTRF or cavin-1), serum deprivation protein response (SDPR, SDR or cavin-2), sdr-related gene product that binds to C-kinase (SRBC or cavin-3) and muscle restricted coiled-coil protein (MURC or cavin-4) (Bastiani et al., 2009; Hansen et al., 2009; McMahon et al., 2009; Vinten et al., 2005). Cavin-1 and 2 are required for caveolae morphogenesis. Cavin-2 interacts with cavin-1 and recruits it to the membrane, and might be involved in generation of membrane curvature; cavin-3 is localized on caveolae vesicles, required for intracellular Cav1 traffic and hence may be associated with caveolae in the intracellular transport machinery; cavin-4 might be required for caveolae formation in muscle due to its distribution to striated muscle with the exact role unknown (Hansen and Nichols, 2010). Cholesterol, a key component of lipid rafts, is also essential for caveolae formation, since it binds Cav1 and facilitates Cav1 oligomerization (Murata et al., 1995), is involved in regulation of Cav1 transcription (Fielding et al., 1997), and its depletion results in caveolae flattening and perturbation of caveolae formation (Rothberg et al., 1992).

More recently, another two proteins have been found to assist the formation of caveolae - Eps-15 homology domain-containing protein 2 (EHD2) and PKC and casein kinase substrate in neurons 2 (PACSIN2) (Parton and Del Pozo, 2013). EHD2 forms large complexes that bind to caveolae at the caveolar opening in an ATP-dependent manner (Ludwig et al., 2013; Stoeber et al., 2012). Although EHD2 is not essential for caveolae formation, it plays a role in maintaining the balance between stationary cell-surface caveolae and caveolae endocytosis, by linking caveolae to the actin cytoskeleton and thus down-regulating their mobility and endocytosis (Simone et al., 2014; Stoeber et al., 2012). PACSIN2 is an important mediator for caveolae maintenance since depletion of PACSIN2 causes loss of caveolae; PACSIN2 detachment from caveolae by its phosphorylation also leads to endocytosis and flattening of caveolae (Hansen et al., 2011; Senju and Suetsugu,

2015). Indeed, PACSIN2 directly interacts with the N-terminus of Cav1 and F-actin, suggesting a possible role for PACSIN2 in mediating caveolae morphology and dynamics by bridging between caveolae and the cytoskeleton (Kostan et al., 2014; Senju et al., 2011).

1.2.3.2 Cellular functions of caveolae

Caveolae have been proposed as a docking site for various signaling proteins and have been related to signaling by diverse molecules including growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), and small GTPases such as H-Ras (Patel et al., 2008). Caveolae are involved in signaling pathways through either direct caveolin interaction or compartmentalization of signaling molecules in caveolae (Parton and Del Pozo, 2013).

Caveolae can bud from the cell surface, traffic and fuse with intracellular organelles, or fuse back to plasma membrane, which is important for transportation of ligands and membrane components, and for endocytosis of some viruses like SV40 (Hansen and Nichols, 2010; Oh et al., 2007; Pelkmans et al., 2001; Sharma et al., 2004). Caveolae also functions in the regulation of lipids and cholesterol, especially the trafficking and accumulation of fatty acids (Pilch et al., 2007). In vivo studies have confirmed a role for caveolae in regulating lipid and cholesterol metabolism (Bastiani and Parton, 2010).

In addition, caveolae play a role in mechano-sensing and respond to mechanical stress by caveolae flattening and disassembly in various cell types including skeletal muscle, cardio-myocytes, fibroblasts and endothelia (Dulhunty and Franzini-Armstrong, 1975; Lee and Schmid-Schonbein, 1995; Parton and Del Pozo, 2013; Sinha et al., 2011). Upon acute mechanical stress, cavin complexes dissociate from Cav1 and caveolae flatten to increase the cell surface area, which protect the cell from membrane damage or cell lysis (Parton and Del Pozo, 2013; Sinha et al., 2011), suggesting a strategy for cells to protect themselves

from mechanical stress. Indeed, the tyrosine-14 phosphorylated form of Cav1, a key coat protein of caveolae, acts as a mechano-sensor that drives expression of Cav1 and cavin1 and also the formation of caveolae in response to mechanical stress (Joshi et al., 2012; Zhang et al., 2007a).

1.2.3.3 Non-caveolar Cav1 scaffolds

Apart from caveolae, Cav1 also exists in non-caveolar Cav1 microdomains (Cav1 scaffolds) on the cell membrane that exhibit significantly smaller sizes and different distribution compared to caveolae; Cav1 scaffolds also have a distinct impact on the molecular composition of lipid rafts compared to caveolae (Zheng et al., 2011). In mouse mammary tumor cells, reduced Cav1 expression causes formation of non-caveolar scaffolds that negatively regulate EGFR signaling as well as the dynamics of the GM1-ganglioside (as marked by raft marker cholera toxin b-subunit (CT-b)) in a caveolae-independent manner (Lajoie et al., 2009b; Lajoie et al., 2007a). The PC3 prostate cancer cell line is an excellent cellular model to study non-caveolar functions of Cav1 as they lack PTRF/cavin-1 and caveolae but show elevated levels of Cav1 (Gould et al., 2010). Indeed, expression of PTRF/cavin-1 in PC3 cells is sufficient to induce caveolae formation (Hill et al., 2008) and thereby reduces the non-caveolar Cav1 microdomains. This alteration of Cav1 membrane domains decreases the motility and anchorage-independent growth of the cell, as well as to down-regulate tumor growth and metastasis *in vivo* as shown using a orthotopic prostate cancer xenograft model in mouse (Aung et al., 2011; Hill et al., 2012; Moon et al., 2014). These studies indicate a role for the Cav1 scaffolds in promoting cell migration and tumor progression. A further study found that the overexpression of PTRF/cavin-1 in PC3 cells reduces their ability to promote angiogenesis and lymphangiogenesis (Nassar et al., 2013b), suggesting a role for Cav1 scaffolds in facilitating tumor vascularization and dissemination. Expression of PTRF/cavin-1 also dramatically changes the secretome of PC3 cells through

affecting cholesterol dynamics and cytoskeleton (Inder et al., 2012; Zheng et al., 2011). This suggests distinct secretion pathways mediated by Cav1 scaffolds that may also contribute to the roles of Cav1 scaffolds in tumor progression.

1.2.4 Cav1 regulation of focal adhesion dynamics and cell migration

Cav1 has been closely linked to regulation of focal adhesions and cell migration. Cav1 has been shown to directly interact with the key focal adhesion component integrin and to regulate Src- and Rho-dependent cell polarization and migration (Beardsley et al., 2005; Grande-Garcia et al., 2007; Wary et al., 1998). Depletion of Cav1 dramatically decreases focal adhesion sites and cell adhesion by disrupting Src-integrin association and affecting FAK activation (Wei et al., 1999). pY14Cav1 mediates the integrin-driven internalization of membrane domains (del Pozo et al., 2005) and also regulates the organization of lipid domains within focal adhesions (Gaus et al., 2006). pY14Cav1 has further been shown to stabilize focal adhesion components, stimulating focal adhesion dynamics and promoting tumor cell migration (Goetz et al., 2008a; Joshi et al., 2008a). Early studies, using a monoclonal anti-pY14Cav1, localized pY14Cav1 to the leading edge and focal adhesions and found it in association with focal adhesion components such as FAK, paxillin and vinculin (Beardsley et al., 2005; Lee et al., 2000; Swaney et al., 2006). However, the specificity of the commonly used monoclonal anti-pY14Cav1 antibody was doubted due to cross-reaction with phosphorylated paxillin (Hill et al., 2007). Nevertheless, use of a phosphotyrosine-absorbed polyclonal anti-pY14Cav1 antibody and live-cell imaging with a phospho-mimetic Cav1Y14D construct confirmed the localization of pY14Cav1 to the cell leading edge (Joshi et al., 2008a; Nomura and Fujimoto, 1999). Also, Cav1 interaction with integrins, pY14Cav1 promotion of focal adhesion dynamics and pY14Cav1 regulation of focal adhesion membrane order (Gaus et al., 2006; Goetz et al., 2008a; Joshi et al., 2008a; Wary et al., 1998) all support a close proximity of Cav1, especially pY14Cav1, to focal

adhesions, and a role for Cav1 and pY14Cav1 in regulation of focal adhesion activities and cell migration.

1.2.5 Cav1 and cell tension

Cav1 is closely associated with cellular mechano-signaling. Cav1 undergoes tyrosine phosphorylation at Y14 upon mechanical tension and functions as a mechano-sensor and mechano-signaling transducer (Joshi et al., 2012; Radel and Rizzo, 2005; Zhang et al., 2007a). Caveolae and Src-phosphorylated Cav1 are required for mechanical force-induced EGFR and Akt activation (Zhang et al., 2007a). Stretch-induced pY14Cav1 promotes expression of Cav1 and cavin-1 through transcription factor early growth response protein 1 (Egr1), and thus the formation of caveolae (Joshi et al., 2012). As a regulator of cell tension, Cav1 has been found to be involved in integrin-dependent cell adhesion and signaling (Wary et al., 1998; Wei et al., 1999), focal adhesion stability and dynamics (Goetz et al., 2008a; Shankar et al., 2012), Rho/ROCK signaling (Grande-Garcia et al., 2007; Joshi et al., 2008a) and ECM proteolysis and remodeling (Du et al., 2009; Goetz et al., 2011; Sainz-Jaspeado et al., 2010), which together contribute to development of actin filament contractility and cell tension. Moreover, Cav1 promotes cell elongation and Rho-dependent contraction through regulation of the Rho activity inhibitor p190RhoGAP (Goetz et al., 2011; Yang et al., 2011). Tyrosine-phosphorylated Cav1, pY14Cav1, has also been shown to interact with a GEF member, Vav2, which activates Rho family GTPases (Boettcher et al., 2010). All these studies demonstrate a role for Cav1 in both mechano-sensing and development of cell tension, possibly defining a positive feedback loop between Cav1 expression and cell tension.

1.3 Galectin-3 (Gal3) and the galectin lattice

Galectin-3 (Gal3) is a member of the galectin family of proteins, which are galactose-specific lectins. Gal3 preferentially binds cell surface GlcNAc-transferase V (Mgat5)-modified N-glycans and forms complexes that crosslink the glycosylated cell surface proteins to a dynamic galectin lattice (Nabi et al., 2015). Gal3 and the galectin lattice have been shown to mediate epithelial cell lamellipodial formation and focal adhesion dynamics and regulate tumor metastasis by activating FAK and phosphatidylinositol 3-kinase (PI3K), promoting integrin recruitment to fibrillar adhesions, and facilitating F-actin turnover and ECM remodeling (Dumic et al., 2006; Granovsky et al., 2000; Lagana et al., 2006; Saravanan et al., 2009).

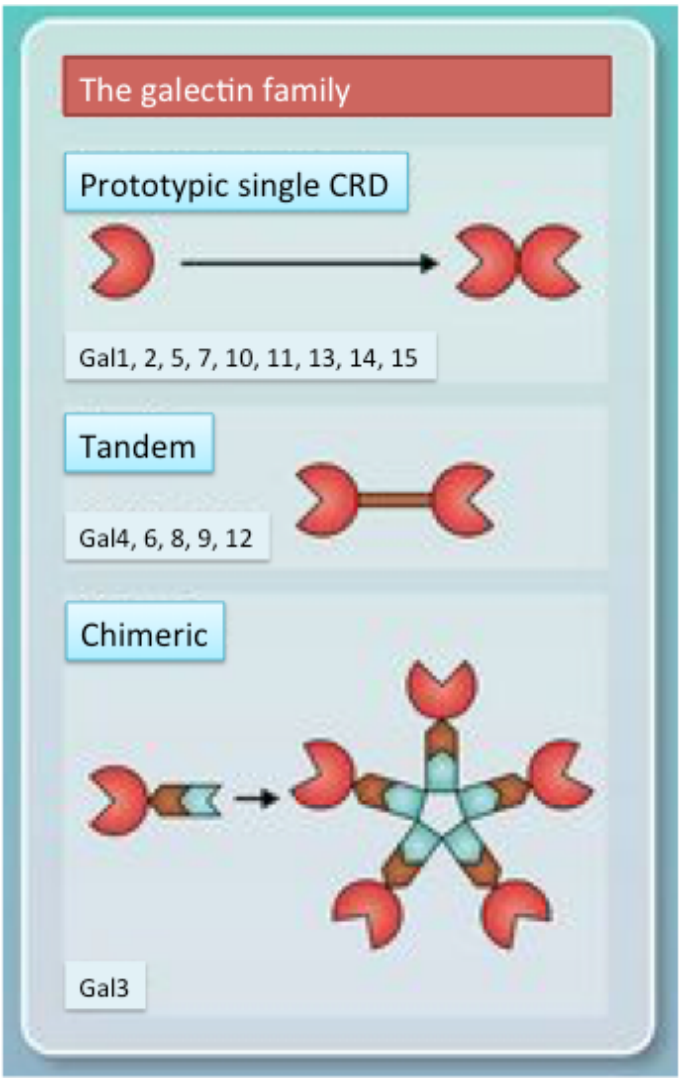
1.3.1 Galectin family

Galectins were first systematically described in 1994 as a phylogenetically conserved family of animal lectins; all the members share primary structural homology in an approximately 130 amino-acid region called the carbohydrate-recognition domain (CRD) (Barondes et al., 1994a; Barondes et al., 1994b; Cooper, 2002). Also termed as S-type lectins, the galectin family is characterized by the ability to bind β-galactose-containing glycoconjugates. Galectins are ubiquitously distributed in multiple cell compartments such as nucleus, cytoplasm, plasma membrane, and secreted into the extracellular space (Houzelstein et al., 2004). They are present in diverse organisms including animal, sponges and some fungi (Cooper, 2002; Hirabayashi and Kasai, 1993). So far a total of 15 mammalian galectins have been identified and further divided into three subfamilies as prototypic, tandem-repeat and chimera-type based on their protein structures (Hirabayashi and Kasai, 1993). Galectin-1, -2, -5, -7, -10, -11, -13, -14, -15, are described as prototypical galectins, consisting of a single CRD that may associate to form homodimers. The tandem-repeat galectins like galectin-4, -

6, -8, -9 and -12, on the other hand, have as least two CRDs linked by a short peptide. Only one member in vertebrates, Gal3, is chimera-type galectin composed of one CRD and an N-terminal domain promoting the formation of multivalent oligomers, up to a pentamer (Figure 1-7) (Nabi et al., 2015).

Figure 1-7. Subfamilies of galectins.

Prototypic galectins have a single CRD and often form homodimers. Tandem-repeat galectins feature at least two CRDs. Chimera-type galectin, namely Gal3, has a CRD and a disordered N-terminal domain that promotes oligomeric formation, up to pentamers. Adapted from (Nabi et al., 2015).



1.3.1.1 Galectin-3 (Gal3)

Of all the members of the galectin family, galectin-3 (Gal3) (formerly known as Mac2, CBP35, εBP, L-29, L-34, and LBP) (Cerra et al., 1985; Liu and Orida, 1984; Raz et al., 1987; Roff and Wang, 1983; Sparrow et al., 1987; Woo et al., 1990) is structurally unique. This 31-kDa galectin has one C-terminal CRD connected to an N-terminal domain rich in proline, glycine and tyrosine residues (Ahmad et al., 2004; Herrmann et al., 1993). Although the CRD is responsible for carbohydrate binding, the N-terminus domain interacts with both carbohydrate and non-carbohydrate ligands and allows the formation of oligomers and ultimately, the galectin lattice (Ahmad et al., 2004; Birdsall et al., 2001; Hsu et al., 1992; Massa et al., 1993; Nieminen et al., 2007; Shekhar et al., 2004). Due to their intrinsic binding affinity for the CRD, glycans with repeating units of [Galβ1-4GlcNAcβ1-]n or poly-N-acetyllactosamine are the preferential ligands of Gal3 (Agrwal et al., 1993; Knibbs et al., 1993; Sato and Hughes, 1992). Intriguingly, although with a lower affinity, Gal3 also recognizes and binds glycosphingolipids (GSLs) for clustering and membrane curvature as found in the process of Gal3-driven clathrin-independent endocytosis (Lakshminarayan et al., 2014). The Gal3-glycocarbohydrate binding strength is significantly reduced by phosphorylation of N-terminal Ser6 (Gong et al., 1999; Huflejt et al., 1993; Mazurek et al., 2000; Yoshii et al., 2002), suggesting that phosphorylation may serve as an “on/off” switch for the downstream biological effects of Gal3.

Gal3 can be cleaved by several proteases such as metalloproteinase-1 (collagenase-1), metalloproteinases-2, –9 (also known as gelatinase A and B respectively), metalloproteinase-7 (matrilysin-1) and metalloproteinase-13 (collagenase-3) (Guevremont et al., 2004; Ochieng et al., 1994; Ortega et al., 2005; Puthenedam et al., 2011). The main cleavage position is at the A62-Y63 bond (Ochieng et al., 1994). Upon proteolytic cleavage of the N-terminal domain, truncated Gal3 prevents intact Gal3 from binding glycans,

presumably due to competition by the cleaved Gal3 that is unable to oligomerize (Nieminen et al., 2007; Ochieng et al., 1998a; Shekhar et al., 2004).

1.3.2 Mgat5, protein glycosylation and the galectin lattice

Glycosylation is one of the common post-translational modifications (PTMs) of proteins, with crucial biological and physiological functions such as protein conformational change, distribution, stability and activity (Spiro, 2002; Varki and Lowe, 2009). At the present stage of our knowledge, an impressive diversity of glycoprotein linkages has been described in virtually all living organisms from eubacteria to eukaryotes (Apweiler et al., 1999; Lechner and Wieland, 1989; Messner, 1997; Upreti et al., 2003).

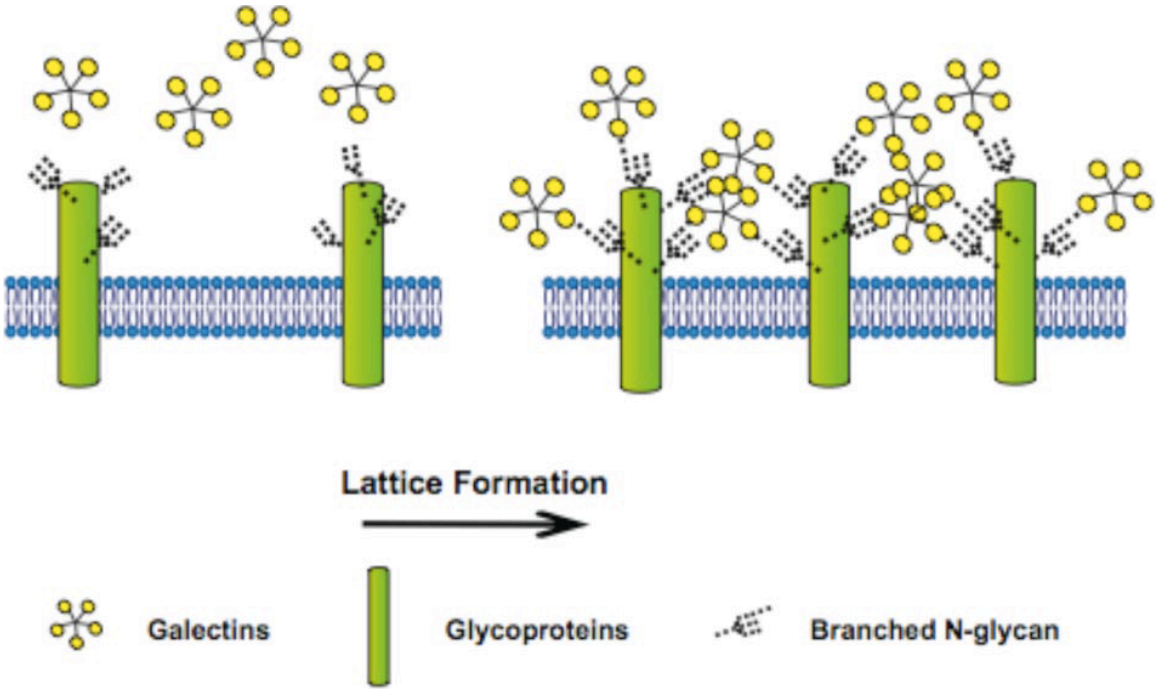
The glycans can be divided into five distinct classes by their core structures proximal to the peptide (Spiro, 2002). N-linked or O-linked glycans are two major classes amongst many membrane-bound and secreted proteins. The N-linked glycan, usually represented by the β-glycosylamine linkage of GlcNAc to the nitrogen atom of asparagine, is the most prevalent form of protein glycosylation and has demonstrated importance in protein folding, secretion and cellular attachment (Varki, 1993). On the cell surface, N-glycans provide the sites of ligand attachment for galectins to regulate receptor clustering and dynamics (Drickamer and Fadden, 2002). N-linked glycosylation occurs in eukaryotic endoplasmic reticulum and archaea sometimes, but very rarely in bacteria. O-linked glycans, on the other hand, usually involve N-Acetyl-galactosamine covalently attached to an oxygen atom of serine or threonine. They are important in protein localization and trafficking, recognition and cell-cell interactions. Besides archaea and bacteria, O-linked glycosylation mainly occurs in the eukaryotic Golgi apparatus. Biosynthesis of N-linked glycosylation is a co-translational event beginning in the endoplasmic reticulum and continuing in the Golgi, while O-glycans are built

up in a stepwise fashion with sugars added incrementally by Golgi glycosyltransferases (Spiro, 2002; Van den Steen et al., 1998).

The Mgat5 gene encodes N-acetyl-glucosaminyltransferase V (GlcNAc-TV), a medial Golgi enzyme that transfers β1,6GlcNAc from UDP-GlcNAc to N-glycans, leading to elongation of 4th branch with poly N-acetyl-lactosamine (Gal-β1,4-GlcNAcn) (Barboni et al., 2000; Henrick et al., 1998; Perillo et al., 1998; Yu et al., 2002). The branched N-glycans formed by the activity of Mgat5 are preferred ligands for Gal3 (Demetriou et al., 2001). In the presence of Mgat5-modified N-glycans, the N-terminal domain of Gal3 mediates rapid conversion into oligomers, thereby crosslinking glycoproteins and forming the galectin lattice in a concentration dependent manner (Figure 1-8) (Ahmad et al., 2004; Nieminen et al., 2007). Mgat5 and the galectin lattice are related to tumor progression – indeed, Mgat5 has been found to be upregulated in various cancer models (Dennis et al., 1999) and a close association between increased N-glycan branching and tumor malignancy has been revealed (Dennis et al., 1987; Dennis et al., 2009b). Consistently, Mgat5-deficient mouse models exhibit reduced mammary tumor growth and metastasis (Cheung and Dennis, 2007; Granovsky et al., 2000), and impaired sensitivity to growth factors (Partridge et al., 2004b). In addition, the Gal3- and Mgat5-dependent galectin lattice affects cell–cell adhesions of mammary tumor cells by destabilizing neuronal (N)-cadherin, a key molecule that mediates cell-cell junctions, thereby promoting cell motility (Boscher et al., 2012; Guo et al., 2003).

Figure 1-8. Formation of the galectin lattice microdomains on plasma membrane.

Oligomerized Gal3 binds N-glycans on cell surface proteins and crosslinks to form the galectin-glycan complex: the galectin lattice. Adapted from (Chiu et al., 2010).



1.3.3 Functions of the Gal3/galectin lattice

In the extracellular environment, Gal3 can interact with either ECM glycoproteins or cell membrane-associated glycoproteins. For instance, ECM proteins (Kuwabara and Liu, 1996; Massa et al., 1993; van den Brule et al., 1995), fibronectin (Sato and Hughes, 1992), hensin (Hikita et al., 2000), elastin (Ochieng et al., 1999), collagen IV (Ochieng et al., 1998b) and tenascin (Probstmeier et al., 1995) have all been found to bind Gal3 in biochemical assays. On the cell surface of the macrophages, specific types of integrins ($\alpha_1\beta_1$ and $\alpha_M\beta_1$ integrin), the primary cell adhesion molecules, bind Gal3, enhancing cell adhesion (Dong and Hughes, 1997; Ochieng et al., 1998b). In addition, cell surface receptors, e.g. EGFR, also bind Gal3 (Partridge et al., 2004b). Another membrane-bound protein recognized by Gal3 is lysosome associated membrane glycoprotein 1 (LAMP-1), which is known to carry several N-glycans (Inohara and Raz, 1994; Sarafian et al., 1998).

To date, it has been well accepted that with Mgat5-modified N-glycans, Gal3 oligomerizes and forms a dynamic complex called the galectin lattice to organize glycoprotein assembly on the cell surface (Brewer et al., 2002; Lajoie et al., 2007a; Partridge et al., 2004b). The ability to create homotypic or heterotypic complexes with numerous glycoproteins allows Gal3 to regulate a variety of cell functions, including cellular homeostasis, metabolism and migration (Brewer et al., 2002; Garner and Baum, 2008; Rabinovich et al., 2007). In particular, the key roles that galectin lattices play in regulating cell functions include: 1) organizing membrane diffusion, compartmentalization and endocytosis of membrane glycoproteins and glycolipids; 2) regulating receptor kinase signaling; and 3) impacting receptor signaling thresholds by inhibiting endocytosis (Chiu et al., 2010; Dumic et al., 2006).

1.3.3.1 Gal3 regulation of focal adhesion dynamics and cell motility

Like other aspects of galectin-glycoprotein lattices, the function of Gal3 as a modulator of cell adhesion and cell motility relies on its multivalent properties and its ability to bind glycoproteins of the ECM and on the cell surface. Depending on the cell type and the glycoproteins to which Gal3 binds, Gal3 can either potentiate or hinder cell adhesion and cell migration (Chiu et al., 2010; Dumic et al., 2006). For example, Gal3 promotes adhesion of neutrophils to laminin, which is dependent on both the CRD and the N-terminal oligomerization domain (Kuwabara and Liu, 1996). Gal3 also promotes neutrophil adhesion to endothelial cells in an oligomerization-dependent manner (Sato et al., 2002), suggesting a role for the crosslinking Gal3/galectin lattice in regulation of cell adhesion. Furthermore, Gal3 enhances binding of L-selectin-triggered lymphocytes to dendritic cells (Swarte et al., 1998). Breast carcinoma cell lines with stable Gal3 overexpression exhibited remarkably enhanced surface expression of $\alpha_4\beta_7$ integrins, laminin, fibronectin and vitronectin resulting in increased cell adhesion (Matarrese et al., 2000). Recently, a compelling study demonstrated a role for Gal3 in facilitating neuroblast migration from the subventricular zone to the olfactory bulb (Comte et al., 2011). Notably, Gal3 can also act as an inhibitor of cell adhesion in different environments. In the thymus, Gal3 disrupts thymocyte-thymic microenvironment interactions (Villa-Verde et al., 2002). *In vitro*, Gal3 incubation prior to plating leads to reduced cell attachment on ECM possibly through blocking $\alpha_1\beta_1$ integrins from binding ECM (Ochieng et al., 1998b).

In studies of cancer cells, Gal3 has been shown to be upregulated and promote cancer metastasis and cell motility in multiple cancer types such as breast cancer and colon cancer (Bresalier et al., 1998; Le Marer and Hughes, 1996). Gal3 knockdown in human PC3 prostate cancer cells reduces cell migration, invasion, cell proliferation, anchorage-independent colony formation, and tumor growth (Wang et al., 2009a). On the cell surface of

mammary carcinoma cells, Gal3 interacts with Mgat5-modified N-glycans, resulting in the activation of FAK, PI3K, and the recruitment of $\alpha_5\beta_1$ integrin to fibrillar adhesions, thereby regulating fibronectin-dependent cell spreading and motility (Lagana et al., 2006). Such Mgat5-dependent galectin lattices also function in concert with pY14Cav1 to facilitate RhoA activation and ECM remodelling, and thereby promote focal adhesion dynamics and cell migration (Boscher and Nabi, 2013; Goetz et al., 2008a).

1.4 Cav1 and Gal3 in cancer progression

Both Cav1 and Gal3 are closely associated with cancer progression and biomarkers for many cancer types (Goetz et al., 2008b; Thijssen et al., 2015). Cav1 has been shown to facilitate cancer cell ECM proteolysis, invasion and migration, and growth; while Gal3 and galectin lattices regulate tumor metastasis by mediating cell surface receptor signaling, adhesion dynamics and ECM remodeling (Goetz et al., 2008b; Song et al., 2014). Furthermore, it has been shown that Cav1, especially pY14Cav1, functions in concert with the galectin lattice to regulate tumor cell migration (Boscher and Nabi, 2013; Goetz et al., 2008a).

1.4.1 Thyroid cancer

Thyroid cancer is the most commonly diagnosed human endocrine malignancy and the incidence of thyroid cancer have been increasing rapidly in the past decade in US and Japan (Williams, 2015). Differentiated thyroid cancer (DTC) includes papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and Hurthle cell carcinoma, which all derived from follicular thyroid cells (LiVolsi and Asa, 1994). PTC is the most common type of DTC and is often associated with metastases (LiVolsi, 2011). The majority of DTC patients have a promising outlook after treatments including surgery, radioiodine therapy and Thyroid-stimulating hormone (TSH) suppression (American Thyroid Association Guidelines

Taskforce on Thyroid et al., 2009). However, a few patients develop local recurrence or cancer metastasis that leads to death for approximately 1-2% of the patients (Nilubol and Kebebew, 2011). In contrast, another follicular cell-derived thyroid cancer, anaplastic thyroid cancer (ATC) is considered as one of the most lethal human solid tumors (O'Neill et al., 2010). ATC transforms from DTC and possibly represents the dedifferentiated form of thyroid cancer and the end point of thyroid cancer progression (Wiseman et al., 2003).

1.4.1.1 Cav1 in thyroid cancer progression

The involvement of Cav1 in thyroid tumors has been studied and shows varied effects depending on the specific type of tumor. An extensive microarray analysis of gene expression in thyroid autonomous adenomas, a benign encapsulated thyroid tumor, found that Cav1 expression is significantly down-regulated (Wattel et al., 2005). When assaying malignant thyroid cancers, FTC and ATC show similar Cav1 expression levels as in benign thyroid tumors; in contrast, PTC shows higher Cav1 expression levels, similar to that of normal tissues, and even overexpression of Cav1 in early stages (Aldred et al., 2003; Aldred et al., 2004; Ito et al., 2002). These studies implicate Cav1 in the development of metastatic PTC cells compared to FTC and ATC cells. Comparison between FTC and a subtype of PTC, follicular variant of papillary carcinoma (FVPC) that structurally mimics FTC, also confirms the positive Cav1 expression in FVPC and negative Cav1 expression in FTC (Ito et al., 2005). Using Cav1 knockout mice, an *in vivo* study found that Cav1 serves to maintain proper synthesis of thyroid hormone and the homeostasis of thyroid cell number in the thyroid gland (Senou et al., 2009). Together with the association of Cav1 expression with malignancy and aggressiveness of thyroid tumors, from benign thyroid tumors to FTC to PTC (Jankovic et al., 2012), it suggests that the reduced Cav1 in relatively non-aggressive thyroid tumors may facilitate local tumor growth whereas regaining Cav1 expression in high-aggressiveness tumors may favor tumor aggressiveness, e.g. tumor metastasis. Curiously,

another member of the caveolin family usually found in muscle cells, Cav3, is found expressed in ATC, but not in PTC, possibly due to the anaplastic transformation of the ATC cells (Kim et al., 2012). Specific roles of Cav1 in malignant thyroid cancer cells remain to be examined.

1.4.1.2 Gal3 in thyroid cancer progression

Gal3 is highly expressed in malignant local FTC and PTC and in metastases of PTC, but not in benign tumors or normal tissues (Xu et al., 1995). It has been classified as the single most accurate diagnostic marker for DTC (Bartolazzi et al., 2001; Bartolazzi et al., 2008b; Chiu et al., 2010; Inohara et al., 1999). Gal3 is a regulator of thyroid follicular cell transformation to malignancy, since knockdown of Gal3 in PTC cells dramatically reduces their malignant phenotype while overexpression of Gal3 in normal follicular cells enhances their malignant phenotype (Takenaka et al., 2003; Yoshii et al., 2001). Overexpression and nuclear localization of Gal3 promotes the proliferation of malignant thyroid cancer cells of PTC cells, where it up-regulates the transcriptional activity of thyroid-specific transcription factor 1, which leads to the highly proliferative state of the PTC cells (Paron et al., 2003). Gal3 knockdown in PTC cells causes cellular apoptosis (Lin et al., 2009a), suggesting a role for Gal3 in regulating the survival of malignant thyroid cancer cells. Finally, given the findings that Gal3 regulates cancer cell invasion and migration and metastasis of multiple cancer types (Bresalier et al., 1998; Nabi et al., 2015; Song et al., 2014), it is possible that Gal3 also facilitates the metastasis of malignant thyroid cancers.

1.4.2 Prostate cancer

Among all the cancer types, prostate cancer is one of the most diagnosed cancers for men and is ranked the first in newly diagnosed cases and the third in mortality for male Canadians in 2015 (Canadian Cancer Society. Canadian Cancer Statistics 2015. Available

at: http://www.cancer.ca. Accessed May 29, 2016). Initial treatments for prostate cancer include surgical removal of the prostate gland (prostatectomy) and radiation to remove and destroy the cancer cells. However, these treatments provide limited outlook for patients because of recurrence or late diagnosis after spread of the cancer (Feldman and Feldman, 2001). The initial growth of the prostate tumor is dependent on androgen and a basic treatment for prostate cancer is androgen ablation that leads to regression of androgen-dependent tumors (Huggins, 1967). However, there is a high probability of prostate cancer cells gaining androgen-independency and becoming androgen-independent prostate cancers (AIPC), a progressive and metastatic form of prostate cancer that accounts for most of prostate cancer deaths (Feldman and Feldman, 2001). Androgen, upon cell entry, is converted to dihydrotestosterone (DHT) that binds to the androgen receptor (AR) in the nucleus and activates it for regulation of target gene transcription (Brinkmann et al., 1999; McKenna et al., 1999; Quigley et al., 1995). Very often, prostate cancer cells undergo various genetic mutations to acquire hypersensitive/aberrantly activated AR and/or to alter/bypass AR pathways to become androgen-independent, leading to AIPC; no effective therapy has been developed for AIPC (Feldman and Feldman, 2001). Cav1 has been evaluated as a novel biomarker for prostate cancer, especially associated with aggressive AIPC cells (Gould et al., 2010). The roles of Cav1 and Gal3 in prostate cancer progression are studied to determine their significance as biomarkers for the prostate cancer and to identify potential therapeutic targets.

1.4.2.1 Cav1 in prostate cancer progression

Cav1 exhibits increased expression levels in prostate cancer and is a poor prognostic marker for prostate cancer (Yang et al., 1998; Yang et al., 1999). Consistently, a study of 61 prostate cancer patients found a correlation between increased Cav1 expression and disease stage, from normal epithelial tissue to primary adenocarcinoma to cancer

metastases in lymph node (Tahir et al., 2001). *In vitro* studies have shown that Cav1 promotes prostate cancer cell viability and clonal growth (Li et al., 2001a; Timme et al., 2000), invasion and migration (Li et al., 2009), angiogenic potential (Tahir et al., 2009) and maintenance of androgen-insensitivity (Li et al., 2001a; Nasu et al., 1998). It also regulates prostate cancer cell metabolism by interacting with lipoprotein receptor-related protein 6 (LRP6) (Tahir et al., 2013). *In vivo* suppression of Cav1 using an anti-sense cDNA confirmed the role of Cav1 in metastasis and androgen-independency (Li et al., 2001a; Nasu et al., 1998). In a transgenic adenocarcinoma of mouse prostate (TRAMP) model, inactivation of CAV1 gene decreased prostate tumor mass and metastasis (Williams et al., 2005); in contrast, prostate-specific Cav1 knock-in resulted in hyperplasia and resistance to castration-induced regression, mimicking a malignant prostate cancer phenotype (Watanabe et al., 2009).

Although Cav1 is a membrane-embedded protein, it is also found in a secreted form, which regulates prostate cancer progression through paracrine and endocrine pathways (Nassar et al., 2013a). Markedly, in the serum of prostate cancer patients, Cav1 exists in a subfraction of high-density lipoprotein 3 (Tahir et al., 2001). Cav1-negative LNCaP prostate cancer cells, when transfected with Cav1, secrete Cav1 in soluble lipoprotein particles both *in vivo* and *in vitro*, which induces growth of a distant tumor derived from Cav1-deficient LNCaP cells (Bartz et al., 2008). Indeed, prostate cancer-secreted Cav1 functions in both paracrine and endocrine pathways; that is, Cav1-negative prostate cancer cells and endothelial cells at both neighbouring sites and distant sites can take up the Cav1 secreted by Cav1 positive prostate cancer cells to facilitate prostate tumor growth and angiogenesis (Nassar et al., 2013a).

In addition, tyrosine phosphorylation of Cav1 and/or different Cav1 membrane domains may play different roles in prostate cancer progression. Cav1 positive, pY14Cav1 negative

DU145 prostate cancer cells show less motility and reduced focal adhesion dynamics compared to pY14Cav1 positive PC3 prostate cancer cells. These phenotypes are reversed upon overexpression of the phosphomimetic Cav1Y14D mutant in DU145 cells or Src inhibition to prevent Cav1 phosphorylation in PC3 cells, implicating pY14Cav1 in focal adhesion dynamics and cell migration in prostate cancer cells (Joshi et al., 2008a). PC3 cells do not express PTRF/cavin-1 leading to a lack of caveolae (Gould et al., 2010). However, Cav1 exists in Cav1 scaffolds, as non-caveolar Cav1 oligomers in lipid raft areas on the PC3 cell surface. Overexpression of PTRF/cavin-1 stabilizes the Cav1 protein and induces caveolae formation in these cells (Hill et al., 2008). Such changes in cell surface Cav1 microdomains alter raft dynamics, actin reorganization and the secretome (Inder et al., 2012), indicative of the dramatic changes in membrane domain organization and actin cytoskeleton caused by the expression PTRF/cavin-1 and the formation of caveolae. Overexpression of PTRF/cavin-1 in PC3 cells also attenuates non-caveolar Cav1 functions to down-regulate cell motility and polarization, and to decrease tumor growth and metastasis (Aung et al., 2011; Hill et al., 2012; Moon et al., 2013). Together with the study showing that tumor angiogenesis is inhibited by expression of PTRF/cavin-1 in PC3 cells (Nassar et al., 2013b), these studies demonstrate important roles for both Cav1 and PTRF/cavin-1 in regulating prostate cancer progression.

1.4.2.2 Gal3 in prostate cancer progression

Gal3 has been implicated in the progression of prostate cancer. Early studies found that decreased Gal3 expression was associated with the progression of prostate cancer based on both *in vivo* human tissue samples and *in vitro* analysis of prostate cancer cell lines (Ellerhorst et al., 1999; Pacis et al., 2000). A detailed study of extensive tumor samples found that prostate cancer cells showed exclusion of Gal3 from the nucleus and only cytoplasmic expression of Gal3, compared to both nuclear and cytoplasmic expression of

Gal3 in normal prostate glands, suggesting anti- and pro-cancer populations of Gal3 in nucleus and cytoplasm, respectively (van den Brule et al., 2000). Indeed, further studies identified the overall anti-tumor activity of nuclear Gal3 and demonstrated that cytoplasmic Gal3 promoted prostate cancer cell invasion, anchorage-independent growth, increased *in vivo* tumor growth and angiogenesis, and decreased drug-induced apoptosis by maintaining mitochondrial stability and inhibiting release of cytochrome c and caspase-3 activation (Califice et al., 2004; Fukumori et al., 2006). These studies suggest significant functions for Gal3, when excluded from the nucleus, in prostate cancer progression. Consistently, both *in vitro* knockdown of Gal3 in PC3 cells, an aggressive metastatic prostate cancer cell line that expresses cytoplasmic Gal3, and *in vivo* application of Gal3 cabohydrate-binding inhibitor lactulose-L-leucine (Lac-L-Leu), which in turn inhibits galectin lattice formation, dramatically reduced motility and tumor metastasis of the prostate cancer cells (Glinskii et al., 2012; Wang et al., 2009a). Gal3 has also been shown to regulate cell proliferation and tumor growth by stabilizing p21, a key regulator of cell cycle, DNA repair and transcription, in metastatic prostate cancer cells (Wang et al., 2013b; Wang et al., 2009a). In addition, both intact and cleaved Gal3 are found in prostasomes, prostatic epithelium-secreted exosome-like vesicles, in both normal and cancerous prostates, while cleaved Gal3 has been implicated as a regulator of bone remodeling that prevents osteoclastogenesis to facilitate prostate cancer skeletal metastasis (Kovak et al., 2013; Nakajima et al., 2016).

1.4.3 Cav1-Gal3/galectin lattice functions in cancer progression

Cav1 and Gal3 synergistically promote the progression of multiple cancer types (Goetz et al., 2008b; Thijssen et al., 2015). Indeed, it has been demonstrated that in many cases Cav1 interacts functionally with the Gal3/galectin lattice to regulate cancer cell activities. Mgat5-dependent Gal3 crosslinking of epidermal growth factor receptor (EGFR) forms a galectin lattice that competes with stabilizing microdomains of oligomerized Cav1 to enhance EGFR

signaling in mouse mammary tumor cells (Lajoie et al., 2007a). In contrast, Gal3 stimulates tyrosine-phosphorylation of Cav1 and pY14Cav1 functions together with the Gal3/galectin lattice to stabilize focal adhesion components and promote focal adhesion dynamics and cell migration (Goetz et al., 2008a). These studies contrast a competing Cav1-Gal3/galectin lattice function that limits cell surface receptor dynamics and signaling, and a concerted Cav1-Gal3/galectin lattice function that promotes cell adhesion dynamics and migration. Further study has shown that Gal3/galectin lattice-dependent EGF signaling leads to phosphorylation of Cav1 and subsequent RhoA activation, actin reorganization and ECM remodeling, promoting cell migration (Boscher and Nabi, 2013). Taken together, spatiotemporally correlated Cav1 and Gal3/galectin lattice act synergistically to coordinate cell signaling and cell movement so as to facilitate tumor growth and metastasis.

1.5 Objectives of the research

Cav1 is closely associated with malignant cancer progression and functions together with the Gal3/galectin lattice to promote focal adhesion dynamics and tumor cell migration. Cav1 forms membrane microdomains such as caveolae and Cav1 scaffolds that show differential impacts on cancer cell motility and cancer progression. The Cav1 CSD domain interacts with numerous signaling molecules while Y14 phosphorylation of Cav1 by Src promotes focal adhesion dynamics and migration. However, several questions remain to be studied including: whether both Cav1 and Gal3 are required for cancer malignancy; how do different Cav1 microdomains influence tumor cell migration; and whether the Cav1 CSD domain has an impact on the pY14-dependent functions of Cav1 and how. Hence I started my work trying to answer these questions for a better understanding of Cav1 functions on focal adhesions and tumor cell migration.

Firstly, both Cav1 and Gal3 are associated with thyroid malignancy. Cav1 plays a complex role in cancer progression in a cancer type- and progression phase-dependent manner. In contrast, the Gal3/galectin lattice has been more consistently shown to promote cancer progression in various ways, especially by promoting cell invasion and migration and cancer metastasis. Our lab showed a synergistic interaction between Cav1 and Gal3/galectin lattice in mouse mammary tumor cells, in which Cav1 functioned as a conditional tumor promoter that was switched on by the Gal3/galectin lattice to promote tumor cell migration in concert with Gal3/galectin lattice. Therefore, I investigated the possible synergistic functions of Cav1 and Gal3/galectin lattice in malignant thyroid cancer cells by monitoring Rho activation, focal adhesion dynamics and cell migration in combination with manipulation of Cav1 and Gal3 expression profiles. My hypothesis was that both Cav1 and Gal3/galectin lattice are required for focal adhesion dynamics and cell migration in malignant thyroid cancer cells.

Secondly, Cav1 was found to function through non-caveolar Cav1 scaffolds in metastatic prostate cancer cell PC3 to promote the cell migration and tumor progression. Overexpression of PTRF/cavin-1 in PC3 cells induced the formation of caveolae and a dramatic decrease of cell motility. However, it was not known whether the PTRF/cavin-1 expression and the subsequent formation of caveolae influence the Cav1-Gal3/galectin lattice regulation of focal adhesion dynamics. Therefore I compared the focal adhesion dynamics and cell migration between PC3 and PTRF/cavin-1-expressing PC3 cells and manipulated Gal3/galectin lattice by siRNA-induced knockdown or exogenous Gal3-induced reinforcement. My hypothesis was that PTRF/Cavin-1 reduces PC3 cell migration by impacting Cav1-Gal3/galectin lattice-dependent FAK stabilization in focal adhesions.

Finally, pY14Cav1 was shown to regulate focal adhesion dynamics and cell migration while the CSD domain was responsible for most Cav1-involved signaling. It had yet to be revealed whether the CSD and Y14 phosphorylation of Cav1 has any functional interaction. I

therefore used various Cav1 mutants that bear mutations on the Y14 and/or the CSD and a cell-permeable CSD competing peptide in prostate cancer cells and studied their effects on focal adhesion dynamics and tension, Cav1 interaction with focal adhesion proteins, and cell migration. My hypothesis was that the Y14 phosphorylation and the CSD are both required for Cav1 regulation of the focal adhesion dynamics and tension and the cell migration in prostate cancer cells.

CHAPTER 2 Coordinate Expression of Galectin-3 and Caveolin-1 in Thyroid Cancer Cells *

2.1 Summary

Galectin-3 (Gal3) is the single most accurate marker for diagnosis of differentiated thyroid cancer (DTC). Gal3 overrides the tumor suppressor activity of caveolin-1 (Cav1) and functions in concert with Cav1 to promote focal adhesion turnover and tumor cell migration and invasion. Coordinately elevated Gal3/Cav1 expression was observed in three DTC derived cell lines (papillary TCP1 and KTC1 and follicular FTC133) and but only one (ACT1) of five ATC derived cell lines. Using siRNA knockdown, Gal3 and Cav1 were shown to be required for Rho GTPase activation, stabilization of focal adhesion kinase (FAK), a measure of focal adhesion signaling and turnover, and increased migration of the DTC cell lines studied but not the ATC cell lines, including ACT1 that expresses elevated levels of Gal3 and Cav1. Coexpression of Gal3 and Cav1 therefore function synergistically to promote focal adhesion signaling, migration and progression of DTC and may have diagnostic and therapeutic implications for thyroid cancer treatment.

2.2 Introduction

Membrane domain organization controls the growth receptor signaling and adhesion dynamics that drive cancer cell migration. Caveolin-1 (Cav1) is a structural component of caveolae, cellular invaginations associated with cholesterol-rich lipid raft domains; caveolae and non-caveolar Cav1 scaffolds are, for the most part, associated with inhibition of cell growth and cytokine receptor signaling, leading to its proposed tumor suppressor function. Indeed, the CAV1 gene maps to a tumor suppressor locus (D7S522; 7q31.1) that is frequently deleted in human malignancies including breast cancer. However, in contrast with its apparent tumor suppressor function, Cav1 expression is associated with a poor prognosis

* A version of Chapter 2 has been published in Journal of Pathology, 2012.

in several different cancer types (Goetz et al., 2008b; Williams and Lisanti, 2005). Consistent with a dual role for Cav1 in tumor progression, we have previously shown that Cav1 negatively regulates EGFR signaling, but upon its Src-dependent tyrosine phosphorylation, promotes focal adhesion turnover and tumor cell migration (Goetz et al., 2008a; Joshi et al., 2008b; Lajoie et al., 2007b). Phospho-Cav1 (pY14Cav1) promotes cell polarization through activation of Rho GTPase and is directly associated with Rho/ROCK-dependent promotion of focal adhesion turnover and associated tumor cell migration and invasion (Grande-Garcia et al., 2007; Joshi et al., 2008b). More recently, Cav1 interaction with and activation of both RhoA and RhoC was shown to promote metastasis of B16 melanoma cell lines (Arpaia et al., 2011).

Gal3 is a galactose-specific lectin that binds and crosslinks glycoproteins forming a lattice that promotes receptor tyrosine kinase (RTK) signaling (Dennis et al., 2009a; Partridge et al., 2004a). Receptor recruitment to the lattice is a function of both N-glycan number (NXS/T sites) and branching of N-glycans through the action of the Golgi Mgat5 glycosyltransferase (Lau et al., 2007). Expression of the galectin lattice overrides Cav1 inhibition of EGFR signaling and functions in concert with tyrosine phosphorylated Cav1 to promote focal adhesion turnover and cell migration (Goetz et al., 2008a; Lajoie et al., 2007b). This led us to suggest that Cav1 is a conditional tumor suppressor whose suppressor function is overridden upon expression of Gal3 and the galectin lattice (Lajoie et al., 2009a). However, whether Gal3 and Cav1 work coordinately to promote human cancer development and progression has yet to be demonstrated.

Thyroid cancer is the most commonly diagnosed human endocrine malignancy and in the United States the incidence of thyroid cancer has more than doubled over the past 3 decades (Davies and Welch, 2006). Papillary carcinoma (PTC), follicular carcinoma (FTC), and Hurthle cell carcinoma are thyroid malignancies that are generally grouped together as

differentiated thyroid cancer (DTC) due to their common origin from follicular thyroid cells (LiVolsi and Asa, 1994). Current treatment includes surgery, radioactive iodine therapy, and TSH suppression and the outlook for the majority of individuals diagnosed with DTC is excellent (Cooper et al., 2009). Few DTC patients will go on to develop local recurrence, or regional or distant cancer metastasis and only approximately 1-2% eventually die from their disease (Nilubol and Kebebew, 2011). Paradoxically, anaplastic thyroid cancer (ATC), like DTC also of thyroid follicular cell origin, is considered one of the most fatal human solid tumors (O'Neill et al., 2010). ATC arises or 'transforms' from pre-existing DTC and thus represents the dysdifferentiated endpoint of thyroid cancer progression (Wiseman et al., 2003). Recently, several novel anticancer agents have been studied in both the laboratory and clinic for treatment of ATC and aggressive forms of DTC (Kojic et al., 2011).

Gal3 has been implicated in thyroid cancer tumor progression (Takenaka et al., 2003; Xu et al., 1995; Yoshii et al., 2001) and Gal3 has been reported to be the single most accurate marker for DTC diagnosis (Bartolazzi et al., 2001; Bartolazzi et al., 2008a; Chiu et al., 2010; Inohara et al., 1999; Wiseman et al., 2008). Here, we show that coordinate Cav1 and Gal3 expression represents a highly precise marker for DTC diagnosis, but also that they regulate tumor cell migration, Rho activation and FAK stabilization in focal adhesions of DTC cell lines, but not ATC cell lines. These studies therefore provide important validation of a role for concerted Cav1 and Gal3 regulation of adhesion dynamics and cell motility in thyroid cancer progression.

2.3 Materials and methods

2.3.1 Antibodies and reagents

Rabbit anti-Cav1, mouse anti-FAK, mouse anti-Gal3 and mouse anti-RhoA antibodies were purchased from Santa Cruz Biotechnology, phalloidin, Alexa 488, 568, or 647-conjugated

secondary antibodies and Lipofectamine-2000 from Invitrogen and HRP-conjugated mouse and rabbit secondary antibodies from Jackson ImmunoResearch Laboratories.

2.3.2 Cell lines

Two papillary (TCP1 and KTC1), one follicular (FTC133) and five anaplastic (ACT1, T235, T238, C648, HTH) human thyroid cell lines were a kind gift from Dr. Rebecca Schweppe, (University of Colorado, Denver, CO) and genetically validated. To minimize phenotypic drift, all cell lines were passaged two to three times after recovery from frozen stocks before initiating experiments and maintained in culture for a maximum of 10-12 passages.

2.3.3 Plasmids and small interfering RNAs

FAK-GFP plasmid and transfection was as described previously (Joshi et al., 2008b). Validated ON-TARGET plus SMARTpool small interfering RNAs (siRNA) for human Cav1 and Gal3 were from Dharmacon. Thyroid cancer cells were cultured in complete medium for 24 h before transfection with 2 mg of specific human Cav1 siRNA, a pool of four specific human Gal3 siRNA oligonucleotides (custom synthesized) or control siRNA (si*CONTROL* nontargeting siRNA #1, Negative control siRNA with at least 4 mismatches to any human, mouse, or rat gene. Microarray tested.; Dharmacon) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol and cells grown for 48 h prior to experimentation.

2.3.4 Immunofluorescence labeling

Immunofluorescence was as previously described (Shankar et al.). Briefly, cells were fixed with 3% paraformaldehyde for 15 min at room temperature, rinsed with PBS, permeabilized with 0.1% Triton X-100 in PBS/CM (1x PBS substituted with 0.1mM $CaCl_2$ and 1mM $MgCl_2$) containing 1% bovine serum albumin (BSA), and then incubated with primary and fluorescent secondary antibodies in PBS/CM containing 1% BSA. After labeling, the

coverslips were mounted in Airvol (Air Products, Inc.), and images collected with 60× or 100× planapochromat objectives (NA 1.35) of an FV1000 Olympus confocal microscope.

2.3.5 Western blotting

Cell pellets from 80% confluent cultures were washed with cold PBS and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA containing freshly added 2 mM DTT, 0.5 mM PMSF, 1 mM sodium vanadate, 2.5 mM sodium fluoride, and 1 μM leupeptin) for 30 min at 4°C, pelleted at 13,000 rpm at 4°C, and the supernatant collected and stored at −80°C. Equal amounts of proteins were separated on 12% SDS-PAGE, electroblotted onto nitrocellulose, probed with the indicated antibodies and HRP-conjugated secondary antibodies, and revealed by ECL. Band intensity was quantified by densitometry relative to b-actin.

2.3.6 Migration assay

Cell migration assays were as previously described (Shankar et al.). Briefly, forty-eight hours after siRNA transfection, cells were trypsinized, counted, and 3×10^4 cells transferred to uncoated (migration) 8-μm cell culture inserts (BD Falcon) in medium containing 2% serum and placed into 24-well plates containing complete medium. After 16 hours, non-migrating cells were removed from the top of the filter with a cotton swab and migrating cells on the bottom of the filter fixed with methanol/acetone, stained with 0.5% crystal violet and counted.

2.3.7 Rho activation assay

Cell lysates collected from control as well siRNA transfected cells were incubated with 15-20 μl (20-30 μg) glutathione-agarose beads bound to a GST-tagged fusion protein, (Rho Binding Domain of the Rho effector protein, Rhotekin, Millipore) for 1 h at 4°C and

centrifuged. Activated RhoA eluted from the beads and total RhoA in cell lysates were determined by western blotting with anti-RhoA antibodies.

2.3.8 FRAP measurements

FRAP was performed on a confocal microscope (FV1000; Olympus) equipped with a 60x planApochromat objective (NA 1.35; oil) and SIM scanner as described previously (Joshi et al., 2008b). Cells plated at low density for 24 h in an 8-well IDIBI chamber were transfected with FAK-GFP for 24 h or transfected with FAK-GFP and either Cav1 or Gal3 siRNA for 48h. Fluorescence recovery was followed at 4-s time intervals until the intensity reached a plateau and normalized to the prebleach intensity. Intensity ratios in the bleached area were compared before bleaching and after recovery to calculate mobile and immobile fractions using Prism 4 (GraphPad). Graphs are representative of a minimum of three independent experiments in which between 6 and 15 FAs were bleached.

2.4 Results

2.4.1 Coordinated expression of Gal3 and Cav1 promote DTC tumor cell migration

To evaluate the functional role of Cav1 and Gal3 in thyroid cancer progression, we studied two papillary (TCP1 and KTC1), one follicular (FTC133) and five anaplastic (ACT1, T235, T238, C648, HTH) thyroid cancer cell lines. Gal3 and Cav1 expression was significantly elevated in the TCP1, KTC1 and FTC133 DTC lines, as well as anaplastic ACT1 cells, relative to the other anaplastic cell lines (Figure 2-1A). Previously, elevated expression of Cav1 was shown to be associated with Rho activation in mouse embryo fibroblasts and cancer cell lines (Grande-Garcia et al., 2007; Joshi et al., 2008b). Significantly increased GTP-RhoA levels were detected in TPC1, KTC1 and FTC133 relative to ACT1 and T238 cell lines (Figure 2-1B). Silencing of Gal3 and Cav1 significantly down-regulated Rho activation

in TPC1 and KTC1 cells linking expression of both Gal3 and Cav1 to Rho activation in these DTC cells (Figure 2-1C). Gal3 and Cav1 co-expression therefore contribute towards RhoA activation in DTC cell lines but not the anaplastic ACT1 cell line.

Immunofluorescent labeling of the cell lines for Gal3 and Cav1 confirmed that DTC cell lines (KTC1, TPC1 and FTC133) and ACT1 expressed elevated levels of endogenous Gal3 and Cav1 relative to other anaplastic cell lines (representative images from T238 are shown) (Figure 2-2). Gal3 showed a nuclear and cytoplasmic labeling but was also found, particularly in FTC133 and TPC1 cells, in peripheral actin-rich lamellipodial regions. Cav1 showed a typical surface distribution to the rear of the cell and a minor fraction could be observed to extend to lamellipodial regions, as previously reported (Joshi et al., 2008a). Gal3 and Cav1 expressing ATC1 cells showed reduced spreading compared to the other cell lines and presented a more epithelioid morphology (Figure 2-2).

In a Boyden chamber assay, increased migration was observed for Gal3/Cav1 expressing TCP1, KTC1, FTC133 and ACT1 cell lines (Figure 2-3A). Gal3 and Cav1 specific siRNA treatment of TCP1, KTC1, FTC133 and ACT1 cells resulted in a greater than 90% knockdown of Gal3 and Cav1 protein levels, respectively (Figure 2-3B). Knockdown of either Gal3 or Cav1 significantly reduced the migration of TCP1, KTC1 and FTC133 DTC cell lines but not anaplastic ACT1 cells (Figure 2-3C). Elevated expression of Gal3 and Cav1 are therefore required for migration of DTC cells but their expression apparently does not functionally impact on the migratory behavior of anaplastic ACT1 cells.

2.4.2 Coordinate expression of Gal3 and Cav1 promote FAK stabilization in focal adhesions

Gal3 and Cav1 stimulation of cell migration has been shown previously to coordinately stabilize FAK-GFP in focal adhesions and promote focal adhesion turnover (Goetz et al.,

2008a). Immunofluorescent labeling for F-actin and focal adhesions showed that TCP1, KTC1 and FTC133 DTC cell lines and anaplastic T235 and T238 cell exhibited a similar cell morphology with individual cells presenting actin stress fibres, actin-rich lamellipodia and FAK-labeled focal adhesions (Figure 2-4A). In contrast, ACT1 cells formed epithelioid clusters and presented fewer focal adhesions. Indeed, quantification of the number of focal adhesions per cell showed no significant difference between the cell lines except for ACT1 that presented a significantly reduced number of focal adhesions per cell (Figure 2-4B).

Stabilization of FAK in focal adhesions is associated with FAK phosphorylation and consequent signaling events that lead to focal adhesion disassembly and cell migration (Goetz et al., 2008a; Hamadi et al., 2005; Joshi et al., 2008b). Cells were transiently transfected with FAK-GFP and fluorescence recovery after photobleaching (FRAP) used to selectively measure FAK-GFP dynamics in focal adhesions. Increased FAK exchange between focal adhesion and cytosolic pools, reflected in a higher mobile fraction, was consistently detected in Gal3 and Cav1 negative anaplastic T238 and T235 cells compared with Gal3 and Cav1-positive TCP1, KTC1 and FTC133 DTC cell lines. The ACT1 anaplastic cell line, although expressing Gal3 and Cav1, had a similar elevated FAK-GFP mobile fraction as the other anaplastic cell lines. Knockdown of Gal3 and Cav1 significantly increased FAK-GFP mobile fractions in focal adhesions of KTC-1 and TPC-1 cells compared to cells transfected with control siRNA (Figure 2-4B).

Next we wanted to test whether reintroduction of Cav1 or Gal3 alone or in combination in the anaplastic cell lines showing reduced Gal3 and Cav1 expression impacted FAK-GFP dynamics in focal adhesions. For this we transiently transfected the T238 anaplastic cell line with FAK-GFP, Cav1-RFP and Gal3-YFP and used FRAP to selectively measure FAK-GFP dynamics in focal adhesions (Figure 2-5). Gal3-YFP could be detected in the 488 nm channel used to detect FAK-GFP but its intensity was significantly reduced in the region of

peripheral focal adhesions (Figure 2-5A). Expression of Cav1 or Gal3 alone did not impact FAK-GFP exchange between focal adhesion and cytosolic pools (Figure 2-5B). The fact that Gal3-YFP alone does not affect FAK-GFP recovery provides an internal control that bleed-through of Gal3-YFP is not affecting FRAP measurements of FAK-GFP dynamics (Figure 2-5B). However, in cells co-transfected with both Cav1 and Gal3, FAK-GFP showed a significantly reduced mobile fraction within focal adhesions (Figure 2-5B). Gal3 or Cav1 alone are therefore insufficient to induce cell migration but are able to stabilize FAK-GFP when expressed together. Coordinate Cav1 and Gal3 expression in DTC, but not ATC, cell lines is therefore associated with increased cell migration, RhoA activation and FAK stabilization in focal adhesions.

2.5 Discussion

We show here that Gal3 and Cav1 are coordinately upregulated in DTC relative to benign tumors and that their expression is required for migration, Rho activation and FAK stabilization in focal adhesions of DTC but not ATC cells. These data confirm our previous reports of Gal3/Cav1 regulation of focal adhesion turnover and further demonstrate that Gal3 is also required for Cav1 regulation of Rho signaling in DTC (Goetz et al., 2008a; Joshi et al., 2008b). These data therefore define a critical role for the synergistic activity of these two membrane domain effectors in thyroid cancer differentiation.

Cav1 over-expression and its Src-dependent phosphorylation stimulate Rho and FAK activation leading to Rho/ROCK and Src-dependent focal adhesion dynamics, tumor cell migration and invasion and metastasis (Arpaia et al., 2011; Grande-Garcia et al., 2007; Joshi et al., 2008b). Prior work from our lab suggested that Gal3 and pY14Cav1 synergize to promote FAK stabilization in focal adhesions (Joshi et al., 2008b). However, previously we had yet to link Gal3 to pY14Cav1 activation of Rho and simulation of tumor cell migration.

The demonstration here that both Gal3 and Cav1 are required for the increased Rho activation and cell migration of Gal3/Cav1 expressing DTC cell lines confirms a role for Gal3 in the regulation of pY14Cav1 activity. Furthermore, we show that restoration of both Cav1 and Gal3, but not either alone, in the T238 anaplastic cell line is able to stabilize FAK-GFP in focal adhesions confirming the synergistic action of these membrane domain regulators.

Elevated Gal3 and Cav1 expression in anaplastic ACT1 cells does not promote RhoA activation or FAK stabilization in focal adhesions, and Gal3 and Cav1 knockdown does not affect ACT1 cell migration. While selective for one ATC cell line, distinct from the other thyroid cancer cell lines studied in that it exhibits an epithelioid morphology and reduced number of focal adhesions (Figure 2-4A), these data indicate that coordinate elevated expression of Gal3 and Cav1 are not necessarily associated with tumor cell migration. Other factors therefore impact on the ability of Gal3 and Cav1 to regulate focal adhesion signaling and tumor cell migration. Gal3 interacts with Mgat5-modified integrins and stimulates integrin activation (Lagana et al., 2006) and stimulates Cav1 phosphorylation (Goetz et al., 2008a). Cav1 phosphorylation is associated with Rho activation and FAK stabilization in focal adhesions and the promotion of Rho kinase and Src-dependent tumor cell migration and invasion (Joshi et al., 2008b). Consistently, overexpression of the caveolin scaffold domain (CSD) peptide and disruption of Cav1-Rho GTPase interaction was recently associated with reduced a5b1-integrin expression, and reduced Src-mediated metastasis of B16 melanoma cells (Arpaia et al., 2011). Cav1 and FAK are both Src substrates and a recent study of thyroid cancer cells showed that Src-dependent phosphorylation of FAK was a critical regulator of growth and invasion of both PTC and ATC cells (Schweppe et al., 2009). The demonstration here that both Gal3 and Cav1 are required for DTC Rho activation, FAK stabilization in focal adhesions and cell migration, indicates that Gal3 and the galectin lattice, through stimulation of integrin, FAK and Rho signaling, represents a critical

determinant of the ability of Cav1 to contribute to DTC progression, migration and aggressivity.

The data presented here support a functional role for coordinate expression of Gal3 and Cav1 in DTC, which appears to be lost once the tumors have transformed into ATC. Unfortunately, there is a subset of individuals diagnosed with DTC who go on to develop locoregional or distant thyroid cancer recurrence not amenable to current therapy. Recently, several novel anti-cancer agents for treatment of ATC and recurrent DTC have been investigated (Kojic et al., 2011). Targeting Gal3 in thyroid tumors expressing elevated levels of both Cav1 and Gal3 could conceivably switch Cav1 from a promoter to suppressor of tumor progression. Gal3 targeting agents have been previously described, including oral ingestion of pectin (Nangia-Makker et al., 2002) and a small molecule Gal3 inhibitor, Td131_1, that induces apoptosis and enhance chemosensitivity and radiosensitivity in PTC (Lin et al., 2009b). Our results not only confirm the diagnostic utility of Gal3 for DTC, but also suggest that Gal3 targeted anti-cancer agents may be of particular value for treatment of Cav1/Gal3 expressing DTC, and their associated metastases, and warrants further study.

Figure 2-1. DTC cell lines exhibit coordinate Gal3 and Cav1 expression and elevated RhoA activation.

A. Cell lysates of two papillary (TCP1 and KTC1), one follicular (FTC133) and five anaplastic (ACT1, T235, T238, C648, HTH) human thyroid cell lines were probed by Western blot with antibodies to Gal3, Cav1 and β-actin. TCP1, KTC1, FTC133, and ACT1 showed elevated level of both Gal3 and Cav1. Bands in Western blots were quantified by densitometry and normalized relative to β-actin (n = 3) and compared with the average intensity of T238 (mean±SEM; **, $P < 0.01$). B. RhoA-GTP pull-down assay was performed on TCP1, KTC1, FTC133, and ACT1 cells. RhoA-GTP pull-down and total RhoA fractions were probed by Western blot with anti-RhoA antibody. Bands in Western blots were quantified by densitometry and normalized relative to total RhoA (n = 3) compared with the average intensity of T238 (mean±SEM; **, $P < 0.01$). C. Cell lysates from TPC1 and KTC1 cells transfected with FAK-GFP and either Gal3, Cav1 or control siRNA were subjected to western blot for RhoA-GTP and total RhoA fractions with anti-RhoA antibody. Bands in western blots were quantified by densitometry and normalized relative to total RhoA (n = 3; mean±SEM; ***, $P < 0.001$).

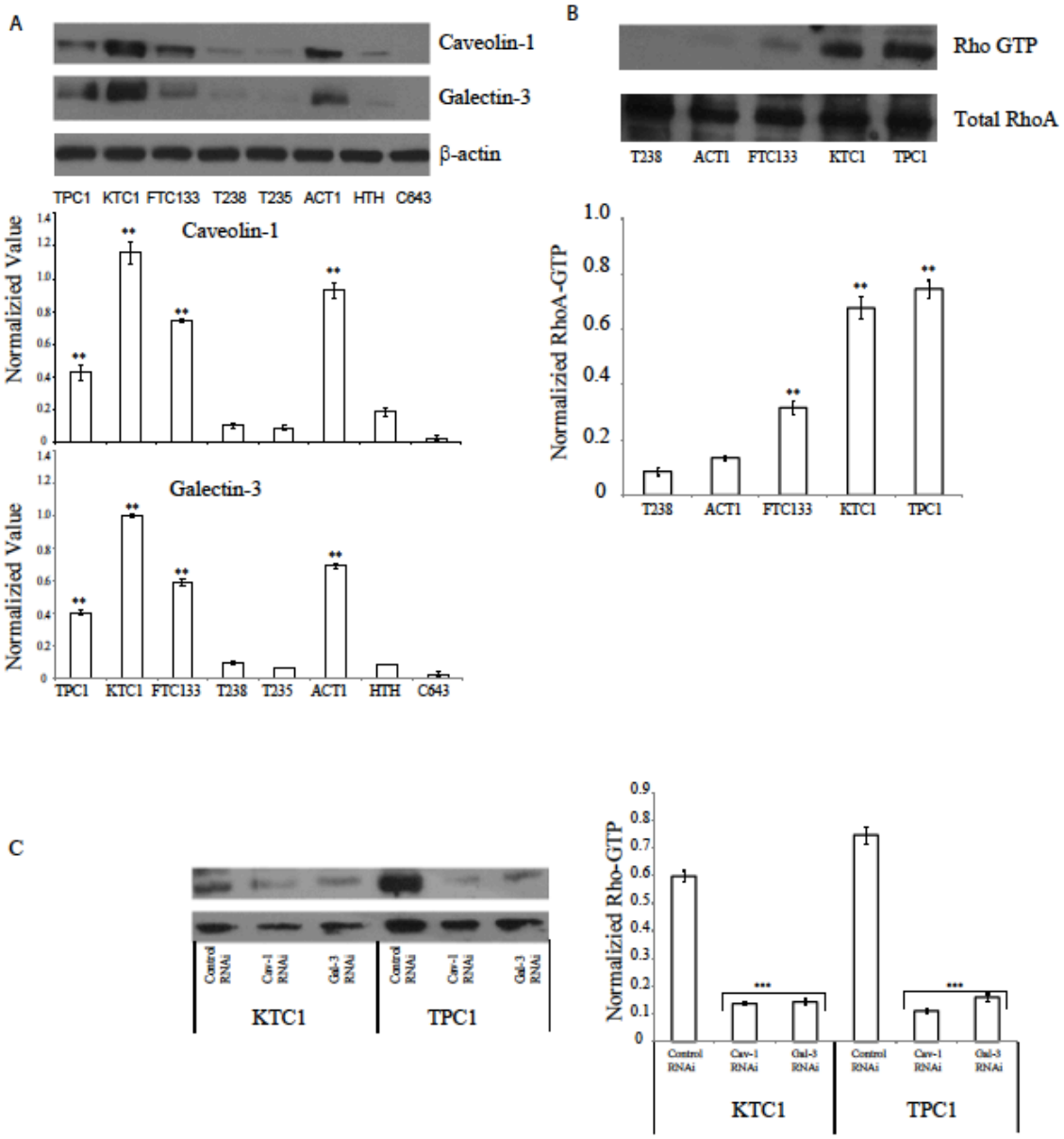

A
Caveolin-1
Galectin-3
β-actin
TPC1 KTC1 FTC133 T238 T235 ACT1 HTH C643
Normalizied Value
Caveolin-1
Galectin-3
B
Rho GTP
Total RhoA
T238 ACT1 FTC133 KTC1 TPC1
Normalizied RhoA-GTP
C
Control RNAi
Cav-1 RNAi
Gal-3 RNAi
KTC1
TPC1
Normalizied Rho-GTP

Figure 2-2. Gal3 and Cav1 localization in thyroid cancer cell lines.

TCP1, KTC1, FTC133, ACT1 and T238 thyroid cancer cells were immunofluorescently labeled with Gal3 (blue), Cav1 (green) antibodies and actin was labeled using Alexa647-phalloidin (red). TCP1, KTC1, FTC133, ACT1, cell lines showed increased expression of both Gal3 and Cav1 compared to the T238 anaplastic cell line (Similar results were obtained with the T235 ATC line). Scale bar: 10 μm.

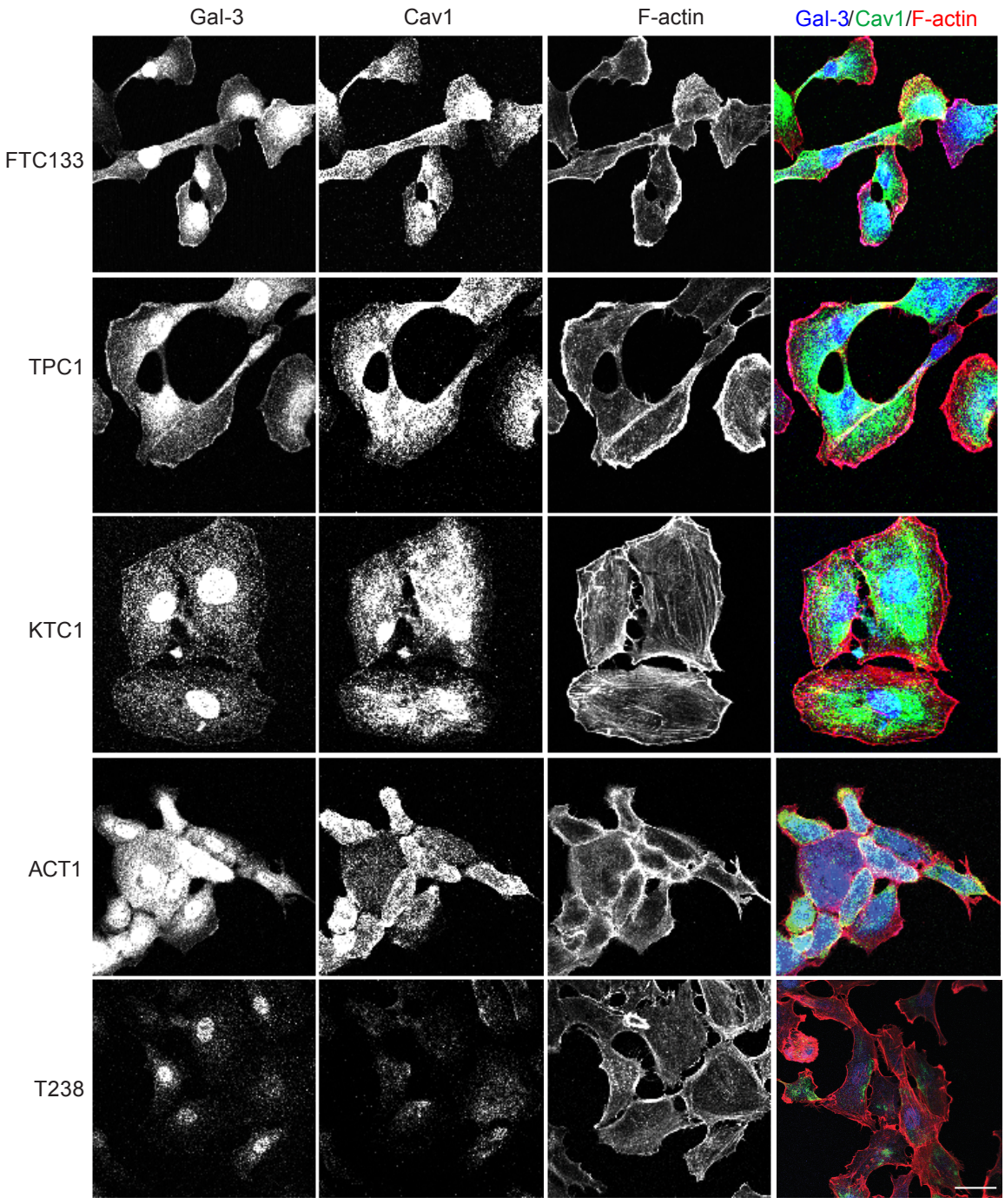

Gal-3
Cav1
F-actin
Gal-3/Cav1/F-actin
FTC133
TPC1
KTC1
ACT1
T238

Figure 2-3. DTC cell migration is Gal3 and Cav1-dependent.

DTC cell migration is Gal3 and Cav1-dependent. A. Boyden chamber Transwell migration assay of TCP1, KTC1, FTC133, ACT1, T235, T238, C648 and HTH cells was performed as indicated (n = 3) and significance determined relative to T238 (mean±SEM; **, P < 0.01; ***, P < 0.001). B. Cell lysates from TCP1, KTC1, FTC133, ACT cells transfected with control or specific siRNA against Gal3 and Cav1 were collected after 48 hours and Western blotted for Gal3, Cav1 and β-actin. C. Boyden chamber migration assay TCP1, KTC1, FTC133, ACT1 and T235 cells was performed 48 hours after transfection with control non-targeting siRNA (NT) or Cav1 and Gal3 siRNA as indicated (n = 3; mean±SEM; **, P < 0.01).

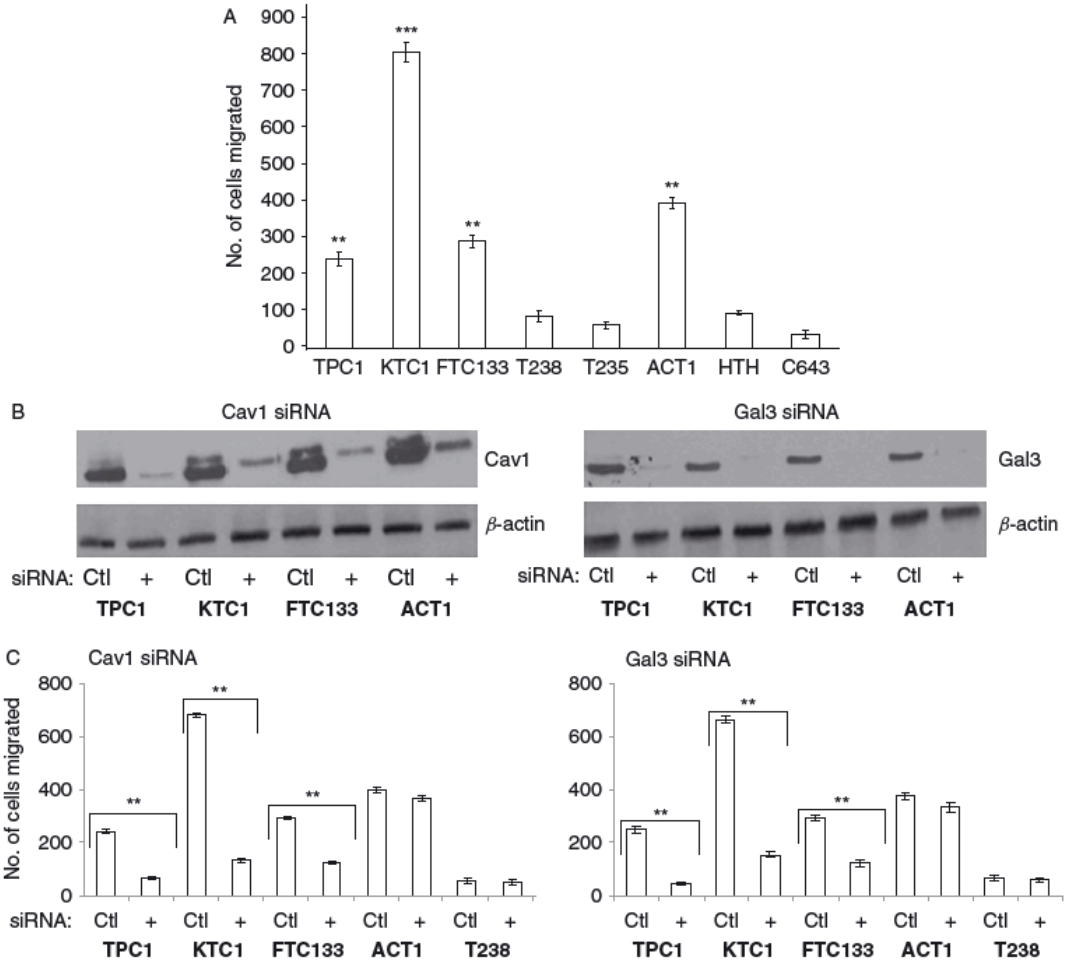


Figure 2-4. DTC Gal3 and Cav1 expression promotes FAK stabilization in focal adhesions.

A. TCP1, KTC1, FTC133, ACT1, T235 and T238 thyroid cancer cells were immunofluorescently labeled with Alexa568-phalloidin (red), and anti-FAK (green) antibodies and the number of focal adhesions (FAs) per cells was counted using ImagePro software. Bar, 20 μm. B. TCP1, KTC1, FTC133, ACT1, T235 and T238 cells were transiently transfected with FAK-GFP and subjected to FRAP of FAK-GFP in peripheral focal adhesions. Compiled recovery curves (left) and a graph showing average mobile fractions (right) are shown. C. TPC1 and KTC1 cells were transiently transfected with FAK-GFP and either Gal3, Cav1 or control siRNA before FRAP analysis of FAK-GFP in peripheral FAs. Compiled recovery curves for KTC1 and TPC1 cells (top) and a graph showing average mobile fractions (right) are shown. (n = 3; mean±SEM; ***, $P < 0.001$.)

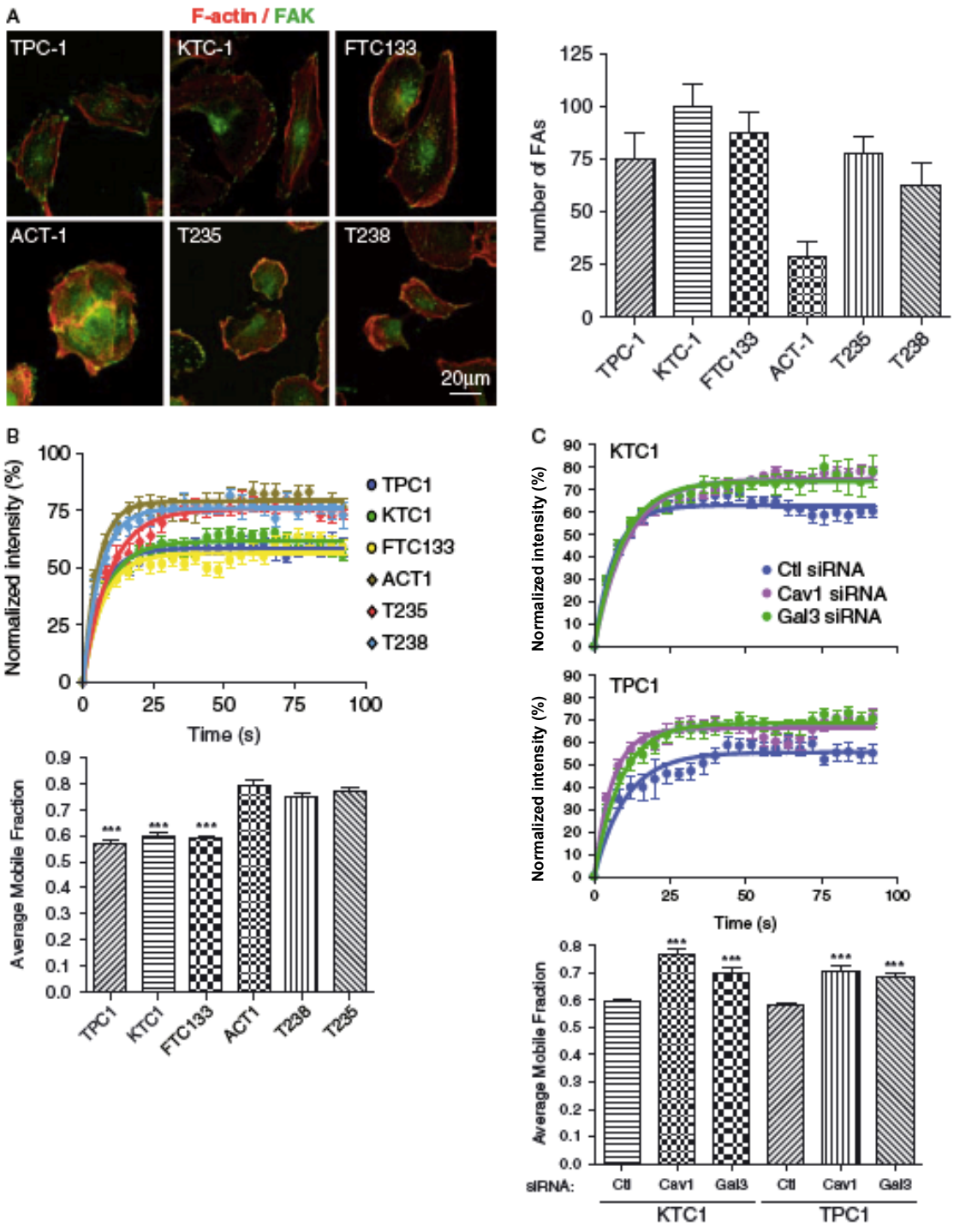
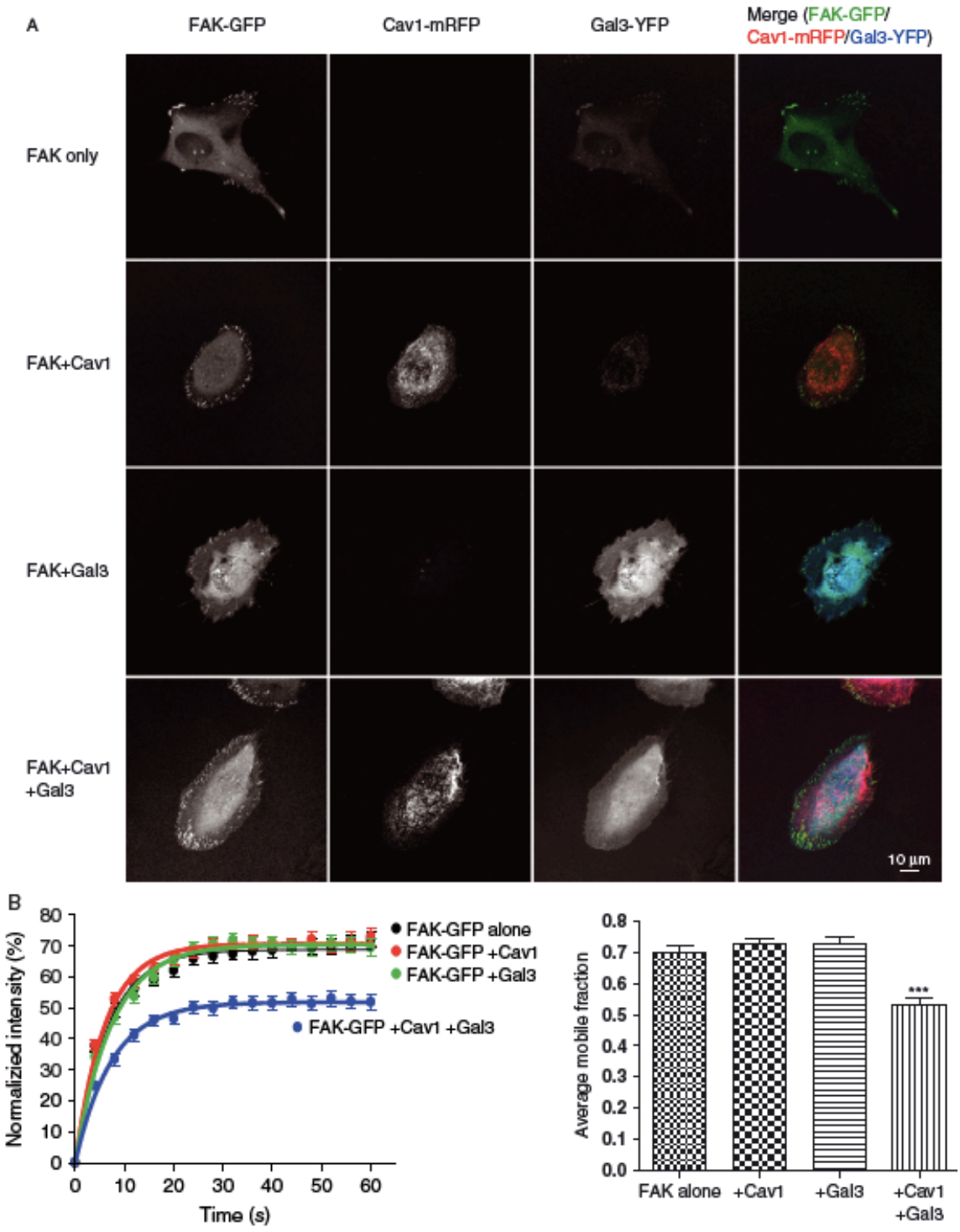

A
F-actin / FAK
TPC-1
KTC-1
FTC133
ACT-1
T235
T238
20μm
number of FAs
125
100
75
50
25
0
TPC-1
KTC-1
FTC133
ACT-1
T235
T238
B
Normalized intensity (%)
100
75
50
25
0
0
25
50
75
100
Time (s)
TPC1
KTC1
FTC133
ACT1
T235
T238
Average Mobile Fraction
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
TPC1
KTC1
FTC133
ACT1
T238
T235
C
KTC1
Normalized intensity (%)
90
80
70
60
50
40
30
20
10
0
Ctl siRNA
Cav1 siRNA
Gal3 siRNA
TPC1
Normalized intensity (%)
0
25
50
75
100
Time (s)
Average Mobile Fraction
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
siRNA:
Ctl
Cav1
Gal3
Ctl
Cav1
Gal3
KTC1
TPC1

Figure 2-5. Coordinate expression of Gal3 and Cav1 stabilize FAK-GFP in focal adhesions of anaplastic T238 cells.

A. T238 cells were transiently transfected with FAK-GFP alone or FAK-GFP with Cav1-mRFP or Gal3-YFP separately or with Cav1-mRFP and Gal3-YFP. Representative confocal images of T238 cells that were used for FRAP analysis, transfected with FAK-GFP alone, FAK-GFP with Cav1-mRFP, FAK-GFP with Gal3-YFP, and FAK-GFP with Cav1-mRFP and Gal3-YFP are shown. Gal3-YFP is detected in the FAK-GFP channel but peripheral labeling is significantly reduced relative to FAK-GFP labeling of focal adhesions. Scale bar: 10 μm. B. FAK-GFP in peripheral FAs was photobleached and recovery followed over time. Compiled recovery curves and a bar graph of average mobile fraction are shown. (n = 3; mean±SEM; ***, $P < 0.001$.)


A
FAK-GFP
Cav1-mRFP
Gal3-YFP
Merge (FAK-GFP/
Cav1-mRFP/Gal3-YFP)
FAK only
FAK+Cav1
FAK+Gal3
FAK+Cav1
+Gal3
10 μm
B
Normalizied intensity (%)
Time (s)
FAK-GFP alone
FAK-GFP +Cav1
FAK-GFP +Gal3
FAK-GFP +Cav1 +Gal3
Average mobile fraction
***
FAK alone
+Cav1
+Gal3
+Cav1
+Gal3

CHAPTER 3 Galectin-3 Overrides PTRF/Cavin-1 Reduction of PC3 Prostate Cancer Cell Migration *

3.1 Summary

Expression of Caveolin-1 (Cav1), a key component of cell surface caveolae, is elevated in prostate cancer (PCa) and associated with PCa metastasis and a poor prognosis for PCa patients. Polymerase I and Transcript Release Factor (PTRF)/cavin-1 is a cytoplasmic protein required for Cav1-dependent formation of caveolae. Expression of PTRF reduces the motility of PC3 cells, a metastatic prostate cancer cell line that endogenously expresses abundant Cav1 but no PTRF and no caveolae, suggesting a role for non-caveolar Cav1 domains, or Cav1 scaffolds, in PCa cell migration. Tyrosine phosphorylated Cav1 (pY14Cav1) functions in concert with Galectin-3 (Gal3) and the galectin lattice to stabilize focal adhesion kinase (FAK) within focal adhesions (FAs) and promote cancer cell motility. However, whether PTRF regulation of Cav1 function in PCa cell migration is related to Gal3 expression and functionality has yet to be determined. Here we show that PTRF expression in PC3 cells reduces FAK stabilization in FAs and reduces cell motility without affecting pY14Cav1 levels. Exogenous Gal3 stabilized FAK in focal adhesions of PTRF-expressing cells and restored cell motility of PTRF-expressing PC3 cells to levels of PC3 cells in a dose-dependent manner with a optimal concentration of 2 μg/ml. Exogenous Gal3 rescued the reduced FAK stabilization in FAs of PC3 cells due to Gal3 knockdown but not that due to Cav1 knockdown while Cav1 knockdown also prevented Gal3 rescue of FA-associated FAK stabilization in PTRF-expressing PC3 cells. Our data supports a role for PTRF/cavin-1, through caveolae formation, as an attenuator of the non-caveolar functionality of Cav1 in Gal3-pY14Cav1 signalling and regulation of FA dynamics and cancer cell migration.

* A version of Chapter 3 has been published in PLoS One, 2015.

3.2 Introduction

Cav1, a member of the caveolin protein family, is a key component of caveolae, the flask-shaped invaginations on the cell surface involved in many cellular processes such as vesicular transport, intracellular signaling and mechanical transduction (Parton and Del Pozo, 2013). Cav1 is involved in regulation of lipid rafts and of multiple cancer-associated processes including cell death and survival, cell migration and invasion, and tumor growth and metastasis (Goetz et al., 2008a; Navarro et al., 2004; Parton and Del Pozo, 2013; Williams and Lisanti, 2005). Cav1 expression is elevated in metastatic prostate cancer (PCa) cells and Cav1 has been evaluated as a prognostic marker of aggressive PCa (Gould et al., 2010; Yang et al., 1998; Yang et al., 1999). Cav1 has also been found associated with PCa metastasis in mouse and human PCa cell lines (Li et al., 2001a; Yang et al., 1998). PC3 is a metastatic prostate cancer cell line that expresses abundant levels of tyrosine phosphorylated Cav1 (pY14Cav1) but does not have caveolae on the cell surface for the lack of polymerase 1 and transcript release factor (PTRF)/cavin-1, required together with Cav1 for caveolae formation (Gould et al., 2010; Hayer et al., 2010; Hill et al., 2008; Joshi et al., 2008a). Overexpression of PTRF in PC3 cells decreases the cell motility via reduced matrix metalloprotease 9 (MMP9) production (Aung et al., 2011). Further studies have shown that PTRF/cavin-1 expression alters the PC3 cell secretome by affecting cholesterol dynamics and the actin cytoskeleton, and attenuates promotion of PCa progression by non-caveolar Cav1 microdomain (Inder et al., 2012; Moon et al., 2014).

Cell migration, a critical element of metastatic disease, is a dynamic and multistep process regulated through spatiotemporal feedback among actomyosin contraction, actin polymerization, and continuous disassembly and formation of adhesions (Gupton and Waterman-Storer, 2006; Lauffenburger and Horwitz, 1996; Ridley et al., 2003). Focal adhesions (FAs) are macromolecular assemblies that link the extracellular matrix and the

cytoskeleton and transmit mechanical force and regulatory signals (Burridge and Fath, 1989; Chen et al., 2003). Focal adhesion kinase (FAK) is the major kinase involved in FA signaling and regulates focal adhesion dynamics through its kinase domain (FRNK) and tyrosine 397 (Y397) autophosphorylation. Reduced FAK Y397 phosphorylation is associated with increased FAK exchange between FAs and cytosol, slower FA disassembly and reduced cell migration (Giannone et al., 2004; Hamadi et al., 2005). pY14Cav1 increases membrane lipid order in FAs and promotes FAK stabilization within FAs in multiple cancer cell lines including the PC3 PCa cell line, a cell line that expresses no endogenous PTRF and thus no caveolae, suggestive of a role for non-caveolar Cav1 scaffolds (Gaus et al., 2006; Goetz et al., 2008a; Joshi et al., 2008a). Cav1 is a major substrate of Src kinase and is phosphorylated on tyrosine 14 (Y14) (Glenney, 1989; Glenney and Zokas, 1989; Grande-Garcia et al., 2007; Li et al., 1996b). In human breast, colon and prostate cancer cell lines, Src-dependent phosphorylation of Cav1 promotes FAK stabilization in focal adhesions, focal adhesion turnover, RhoA signaling and cell migration and invasion (Joshi et al., 2008a). Galectin-3 (Gal3), a galactose-specific lectin family that preferentially binds cell surface GlcNAc-transferase V (Mgat5)-modified N-glycans, stimulates FAK and PI3K activation, enhances integrin activation and recruitment to fibrillar adhesions, and increases F-actin turnover (Dumic et al., 2006; Granovsky et al., 2000; Lagana et al., 2006; Saravanan et al., 2009). We have shown that pY14Cav1 and Gal3 act together to stabilize FAK within FAs and promote FA dynamics and cell migration and that coordinate expression of Cav1 and Gal3 distinguish differentiated thyroid cancer from benign (Goetz et al., 2008a; Shankar et al., 2012).

However, it has yet to be determined whether and how expression of PTRF and, thereby, caveolae, impacts the concerted Cav1-Gal3 regulation of FA dynamics and cell migration. We therefore used PC3 PCa cells that lack PTRF and caveolae but express Cav1 and show

Cav1 phosphorylation, to specifically determine the role of PTRF on Cav1-Gal3 regulation of FA dynamics and cancer cell migration.

3.3 Materials and methods

3.3.1 Antibodies, plasmids, siRNA and recombinant human Gal3-His

Bovine serum albumin (BSA), and mouse anti-β-actin antibodies were purchased from Sigma-Aldrich. Rabbit anti-FAK were purchased from Santa Cruz Biotechnology, Inc.; rabbit anti-pY14Cav1 was purchased from Cell Signaling, Inc.; mouse anti-PTRF and rabbit anti-Cav antibodies were purchased from BD Transduction Laboratories. HRP-conjugated mouse and rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Phalloidin and secondary antibodies conjugated to Alexa 488, 568, or 647 were purchased from Life Technologies, Thermo Fisher Scientific. PTRF-mCherry and mCherry plasmids were generous gifts from Dr. Michelle Hill (The University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia). FAK–GFP plasmid was as described previously (Joshi et al., 2008a). Validated ON-TARGET plus SMARTpool small interfering RNAs (siRNA) for Cav1 (mouse siCav1: L-0058415-00) and control siRNA (non-targeting siRNA no. 1, siCONTROLS: D-001210-01) were purchased from Dharmacon. Custom synthesized mouse Gal3 siRNA oligonucleotides duplexes (Henderson et al., 2006) were purchased from Dharmacon.

Recombinant human Gal3 tagged with 6XHis at C-terminal was generated using the plasmid, pHIS-Parallel2, described previously (Wallner et al., 2004). This plasmid and protocols were kind gift from Ludger Johannes (Institute Curie). In order to generate Gal3-His, we used BL21-DE3 bacteria (New England Biolabs). Briefly, overnight culture was re-inoculated (1:5) in fresh LB supplemented with ampicillin and cultured at 37°C on 225 rpm shaker untill the

OD600 reached 1.0 and then induced using 0.4 mM IPTG for 3 h at 30°C on 225 rpm shaker. Recombinant Gal3-His was purified on talon affinity matrix (Clontech) using the supplied protocol, transferred to 1x PBS prior to quick freezing in liquid N_2.

3.3.2 Cell culture and transfection

The human PC3 cell line was from American Type Culture Collection (ATCC) and maintained in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The PC3 cell lines stably expressing GFP or PTRF-GFP (PC3-GFP and PC3-GFP-PTRF) were generous gifts from Dr. Michelle Hill (The University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia) (Inder et al., 2012). All cell lines were passaged at least twice after recovery from frozen stocks before initiating experiments and maintained in culture for a maximum of 8 to 10 passages to minimize phenotypic drift.

Transfection was carried out following the standard protocols. Briefly, cells were plated in complete medium and incubated for 24 h at 37°C in a CO_2 incubator. On the next day, cells were washed with PBS and transfected with appropriate amounts of plasmid DNA or siRNAs using Lipofectamine 2000 (Life Technologies, Thermo Fisher Scientific). Cells were allowed to grow for 24 h (for plasmid) or 48 h (for siRNAs) prior to subsequent experiments.

3.3.3 Western blotting

Cell pellets from 80% confluent cultures were washed with cold PBS and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA containing freshly added 2 mM DTT, 0.5 mM PMSF, 1 mM sodium vanadate, 2.5 mM sodium fluoride, and 1 μM leupeptin) for 30 min at 4 °C, pelleted at 13,000 rpm at 4 °C, and the supernatant collected and stored at -80 °C. Equal amounts of proteins were separated on 12% SDS-PAGE, electroblotted onto nitrocellulose (GE Healthcare Life Science), probed

with indicated antibodies and HRP-conjugated secondary antibodies, and revealed by ECL (Merck Millipore).

3.3.4 Immunofluorescence labeling

Cells were fixed with 3% paraformaldehyde (PFA) for 15 min at room temperature, rinsed with PBS, permeabilized with 0.1% Triton X-100 in PBS/CM, blocked with PBS/CM containing 0.2% bovine serum albumin (BSA), and then incubated with primary and fluorescent secondary antibodies in PBS/CM containing 0.2% BSA. After labeling, the coverslips were mounted in CelVol (Celanese, Ltd.), and images acquired with the 100× planapochromat objectives (NA 1.35) of an FV1000 Olympus confocal microscope.

3.3.5 FRAP measurements

FRAP was performed on a confocal microscope (FV1000, Olympus) equipped with a 60x planapochromat objective (NA 1.35; oil) and SIM scanner. Cells were plated at low density on FN (10 µg/ml) for 24 h in an 8-well µ-slide chamber (ibidi), transfected with FAK-GFP or FAK-GFP plus mCherry or FAK-GFP plus PTRF-mCherry, and experiments performed 24 h later at 37°C. siRNA were transfected 48 h before FRAP experiments. When indicated, before FRAP experiments, the cells were treated with Gal3-His by changing the medium to serum-free medium containing the indicated concentration of Gal3-His for 10 min before changing back to bicarbonate-free RPMI medium. For each FRAP analysis, a prebleach frame was acquired followed by a single bleach event using the simultaneous and independent stimulation of the 405 line of the SIM scanner. Fluorescence recovery was followed at 4-s time intervals until the intensity reached a plateau. Fluorescence during recovery was normalized to the prebleach intensity. Intensity ratios in the bleached area were compared before bleaching and after recovery to calculate mobile fractions using

Prism 4 (GraphPad). Graphs are representative of a minimum of three independent experiments in which between 10 and 25 focal adhesions were bleached.

3.3.6 Migration assay

For migration assay, cells were trypsinized and counted, and 200,000 cells/well were transferred to uncoated 8-μm cell culture inserts (BD Falcon) in medium containing 2% serum and the assembly placed into 24-well plates containing complete medium. After 16 h, non-migrated cells were removed from the top of the filter with a cotton swab, and migrated cells on the bottom of the filter were fixed with 3% PFA, stained with 5% crystal violet and labeled cells counted. Cell counts were normalized to the PC3 group. Alternatively, 200,000 cells/well were transferred to the same inserts in serum-free medium with or without 2 μg/ml Gal3-His and were kept for 14 h before fixation and staining. Cell counts were normalized to the PC3 non-treated group.

3.4 Results

3.4.1 Expression of PTRF/cavin-1 decreases PC3 cell migration and disrupts FAK stabilization in focal adhesions

PC3 prostate cancer cells stably expressing PTRF-GFP (PC3-GFP-PTRF) show decreased cell motility compared with PC3 cells stable expressing GFP (PC3-GFP) or non-transfected PC3 cells (Figure 3-1A), as previously reported (Aung et al., 2011). The reduced migration of PC3-PTRF-GFP cells was not associated with an altered number of focal adhesions labeled for FAK (Figure 3-1B, C). To determine FAK stability in FAs, PC3 cells were transfected with FAK-GFP alone, FAK-GFP plus mCherry or FAK-GFP plus PTRF-mCherry and subjected to fluorescence recovery after photobleaching (FRAP) assay. As shown in Figure 1D, the expression of PTRF-mCherry increased the mobile fraction of FAK-GFP in FAs relative to PC3 cells transfected with FAK-GFP alone or FAK-GFP with mCherry. PTRF

expression therefore diminished FAK stabilization in FAs, indicative of increased exchange with cytoplasmic FAK and reduced FA disassembly (Giannone et al., 2004; Hamadi et al., 2005).

3.4.2 PTRF expression does not affect pY14Cav1 levels but additional Gal3 treatment restores FAK stabilization in the FA and cell motility of PTRF-expressing PC3 cells

Tyrosine-phosphorylated Cav1 (pY14Cav1) regulates the migration of multiple cancer cell lines including PC3 (Goetz et al., 2008a; Joshi et al., 2008a). Stable PTRF expression in PC3 cells, however, did not alter Cav1 expression levels or phosphorylation (Figure 3-2). As Gal3 has been shown to function together with pY14Cav1 to regulate FAK stability in FAs (Goetz et al., 2008a; Shankar et al., 2012), we tested whether exogenous Gal3 could restore FAK stabilization in FAs in PC3-PTRF-GFP cells. Addition of His-tagged Gal3 (Gal3-His) at concentrations from 1-4 μg/ml did not affect FAK stabilization within FAs in PC3 cells. However, in PTRF expressing PC3 - cells, addition of 1.5 or 2 μg/ml Gal3-His significantly restored the stabilization of FAK in FAs, although 2 μg/ml of Gal3-His contributed as the optimal concentration since it stabilized FAK in FAs to the largest extent and most significantly ($p<0.001$) compared with other concentrations of Gal3-His; addition of higher (3 or 4 μg/ml) or lower (1 μg/ml) concentrations of Gal3-His failed to restore the FA associated FAK stabilization (Figure 3-3A). The dose-dependence of the Gal-His stabilization of FAK in FAs is consistent with the concept of the galectin lattice, in which a critical ratio of Gal3 to glycan substrates enables optimal glycoprotein crosslinking that leads to receptor dynamics and signaling (Dennis et al., 2009b). A Transwell cell migration assay showed that the optimal 2 μg/ml Gal3-His enhanced the migration of all three cell lines (PC3, PC3-GFP and PC3-GFP-PTRF) to the largest extent and to the same level (Figure 3-3B). In contrast, 1, 1.5 and 3 μg/ml of Gal3-His had no effect on the cell migration of control PC3 and PC3-GFP

cells, but elevated PC3-GFP-PTRF cell migration to a smaller extent (Figure 3-3B). This was possibly due to Gal3 activation of small GTPases through integrins and ECM remodeling (Lagana et al., 2006; Saravanan et al., 2009). Higher concentration of Gal3-His, i.e. 4 µg/ml, failed to promote any of the cell migration significantly (Figure 3-3B). Addition of exogenous Gal3 protein therefore restores the deficient FAK stabilization in FAs and the reduced migration of PTRF-expressing PC3 cells in a dose-dependent manner.

3.4.3 Gal3 rescue of FA-associated FAK stabilization is Cav1-dependent

To determine whether endogenous Gal3 stabilizes FAK in FAs of PC3 cells, we depleted Gal3 with siRNA obtaining a consistent 90% reduction in Gal3 levels after 48 hours (Figure 3-4A). After 24 hours we transfected the cells with FAK-GFP alone or FAK-GFP together with PTRF-mCherry, and treated them or not with 2 µg/ml Gal3-His prior to FRAP analysis of FAK-GFP stability in FAs. As shown in Figure 4B, knockdown of Gal3 (Gal3 KD) diminished FAK stabilization in FAs, same as observed for PTRF-expressing PC3 cells, while siCTL had no effect on FAK stabilization compared to untransfected cells. Importantly, treatment with Gal3-His rescued FAK stabilization in FAs in both PTRF-expressing and Gal3 knockdown cells (Figure 3-4B).

We then knocked down Cav1 in PC3 cells (Cav1 KD) with a siRNA targeting human Cav1 (siCav1) (Figure 3-5A) before transfection with either FAK-GFP alone or FAK-GFP plus PTRF-mCherry and treatment with exogenous Gal3-His (2 µg/ml). siCav1 increased the mobile fraction of FAK-GFP in FAs in PC3 but not in PTRF-expressing PC3 cells, demonstrating that Cav1 selectively regulates FA dynamics in PC3 cells (Figure 3-5B). Further, Cav1 siRNA prevented the rescue of reduced FAK stabilization in FAs even in the presence of Gal3-His in PC3 cells and the rescue of FAK stabilization in FAs in Gal3-His treated PTRF-expressing PC3 cells (Figure 3-5B). Cav1 is therefore necessary for Gal3-

dependent FAK stabilization in FAs of PC3 cells. Concerted regulation of FA dynamics by Cav1 and Gal3 is therefore impacted by expression of PTRF and Cav1 recruitment to caveolae. This suggests that recruitment of Cav1 to caveolae alters the stoichiometric relationship between non-caveolar Cav1 and Gal3 that is critical to their coordinated regulation of FA dynamics and cancer cell migration.

3.5 Discussion

The cavin family includes PTRF/cavin-1, SDPR/cavin-2, PRKCDBP/cavin-3 and MURC/cavin-4, which share a conserved N-terminal domain comprised of heptad repeats of hydrophobic amino acids, possibly forming coiled coils, and a following region of several basic amino acids (Hansen and Nichols, 2010). Expression of the cavins is associated with the expression and stabilization of Cav1 and the formation and function of caveolae (Hansen and Nichols, 2010). Of the cavins, PTRF/cavin-1 is essential for caveolae formation and loss of PTRF results in loss of caveolae and downregulation of Cav1 protein levels (Hill et al., 2008; Liu and Pilch, 2008). Cav1 has been ascribed non-caveolar functions (Lajoie et al., 2009a; Lajoie et al., 2009b; Zheng et al., 2011) and stoichiometry between caveolins and cavins must necessarily influence the expression of not only caveolae but also non-caveolar Cav1 domains or Cav1 scaffolds. PC3 cells are an excellent cellular model to study non-caveolar functions of Cav1 as they lack PTRF and caveolae but show elevated levels of Cav1 (Gould et al., 2010). Indeed, expression of PTRF in PC3 cells neutralizes the non-caveolar Cav1 microdomains and alters the cell motility, secretion pathways and angiogenesis and lymphangiogenesis abilities in PC3 cells (Aung et al., 2011; Hill et al., 2012; Inder et al., 2012; Moon et al., 2014; Nassar et al., 2013b). Consistently, we show here that PTRF expression in PC3 cells limits Cav1-Gal3 regulation of FAK stabilization in FAs and cell motility.

Gal3 and pY14Cav1 function in concert to promote FA dynamics and cell motility. pY14Cav1 promotes cell polarization and migration and regulates membrane lipid order at FAs (Gaus et al., 2006; Goetz et al., 2008a; Grande-Garcia et al., 2007). Gal3 binding to cell surface Mgat5-modified N-glycans forms a galectin lattice that stimulates FAK and PI3K activation, and promotes integrin activation and F-actin turnover (Dumic et al., 2006; Granovsky et al., 2000; Lagana et al., 2006; Saravanan et al., 2009). Furthermore, in cells lacking the galectin lattice due to absence of Mgat5, pY14Cav1 is not sufficient to promote FA-associated FAK stabilization while expression of Mgat5 to form the galectin lattice increases cell spreading and induces FAs but requires pY14Cav1 to stabilize FAK within FAs (Goetz et al., 2008a). Consistently, coordinated expression of Cav1 and Gal3 is observed in differentiated thyroid cancer-derived cell lines and siRNA knockdown assays in the same cell lines show that both Cav1 and Gal3 are required to promote FAK stabilization in FAs and cell migration compared to benign thyroid lesion-derived cells (Shankar et al., 2012). More recent data showing that Gal3 is required for epidermal growth factor (EGF) signaling that induces Cav1 phosphorylation and promotes circular dorsal ruffle (CDR) formation, cell migration and fibronectin fibrillogenesis; these studies led to the suggestion of a Gal3-integrin-pY14Cav1 signalling module that mediates EGF signaling to Rho A (Boscher and Nabi, 2013).

Our data shows that PTRF expression in PC3 cells does not alter Cav1 or pY14Cav1 levels, which suggests that PTRF regulates pY14Cav1 function in FA dynamics through Cav1 recruitment to caveolae but not by altering Cav1/pY14Cav1 levels. Formation of caveolae is therefore proposed to regulate the expression and function of non-caveolar Cav1 domains. Indeed, it has been shown that preferentially expression of Cav1 in non-caveolar Cav1 domains due to an absence of PTRF is associated with advanced PCa and that expression of PTRF/caveolae neutralizes the non-caveolar Cav1 domains to slow down PCa progression (Moon et al., 2013). The fact that excess Gal3 can restore pY14Cav1-

dependent FA signalling indicates that stoichiometry between Gal3 and pY14Cav1 is critical to their ability to interact to regulate FA signaling and dynamics. Of a range of concentrations from 1-4 μg/ml, 2 μg/ml was optimal to rescue PTRF-expressing cells, highlighting the concentration dependence of Gal3 lattice function. Indeed, Gal3 stimulation of integrin-dependent fibronectin fibrillogenesis is concentration-dependent (Lagana et al., 2006). Integrins are modified by Mgat5 and Gal3 activates α5β1 integrin and recruits it to fibrillar adhesions (Demetriou et al., 1995; Lagana et al., 2006). Similarly, pY14Cav1 has been shown to interact with integrins and modulate the lipid order in FAs (del Pozo et al., 2005; Gaus et al., 2006; Radel and Rizzo, 2005; Wei et al., 1999). Our data suggests that stoichiometry of Gal3-pY14Cav1 is a critical regulator of the Gal3-pY14Cav1 signaling and that PTRF is a novel regulator of this signaling module. PTRF has been shown to alter exosomal secretion of PC3 cells (Inder et al., 2012) and whether PTRF disrupts the Gal3-pY14Cav1 module by limiting Gal3 secretion or by sequestering pY14Cav1 into caveolae and away from Cav1 scaffolds remains to be determined (Figure 3-6).

The stoichiometric relationship between Gal3 and Cav1 also relates to PCa progression. It has been demonstrated that higher Gal3 level correlates with increased metastasis potential of PCa cells (Wang et al., 2013a). In vitro cell migration is a surrogate assay for tumor cell invasion and metastasis. Our finding that addition of Gal3 promotes the cell migration of both PC3 prostate cancer cells and PTRF-expressing PC3 cells suggests that promotion of cancer cell migration may contribute to Gal3 function in tumor metastasis. Meanwhile it has also been shown that Gal3 is highly expressed in normal prostate cells but silenced in malignant prostate epithelial cells (LNCaP) through methylation of its promoter (Ahmed et al., 2007). Cav1 expression levels also vary with increased expression levels in PCa with LNCaP cells as an exception (Gould et al., 2010). Taken together, it is suggestive of a stoichiometric regulation of PCa progression by Gal3 and Cav1 levels where low levels of

Gal3 and Cav1 correlate with less aggressive prostate cancer whereas high levels of Gal3 are associated with either highly aggressive prostate cancer, if the cells express higher levels of Cav1, or normal prostate epithelium, if they express lower levels of Cav1. Both Cav1 and Gal3 are playing important roles in PCa progression. Cav1 has been evaluated as a prognostic marker of aggressive PCa since the 1990s (Yang et al., 1998; Yang et al., 1999). Gal3 has been recognized as an important regulator of PCa metastasis and several attempts have been performed to target Gal3 in PCa, using either the Gal3-binding cancer-associated Thomsen-Friedenreich glycoantigen (TF-Ag)-mimic lactose-L-leucine or a high-affinity Gal3-bingding glycopeptide purified from cod (Glinskii et al., 2012; Guha et al., 2013). Our study shows that Cav1 and Gal3 act in a concerted manner to restore FAK stabilization in focal adhesions and cell motility affected by PTRF, which implicates Cav1 and Gal3 as important regulators in PCa.

Figure 3-1. Expression of PTRF in PC3 cells reduces the cell motility by affecting FAK stabilization in FAs and not the number of FAs per cell.

(A) Transwell migration assay shows that PC3-GFP-PTRF cells migrate slower than wildtype PC3 and PC3-GFP cells. (B, C) Representative confocal images of PC3, PC3-GFP and PC3-GFP-PTRF cells and quantification of FAs per cell in each of the cell lines show that the number of FAs per cell is not significantly affected by PTRF expression in the cell. (D) Fluorescence Recovery After Photobleaching (FRAP) assay shows that FAK-GFP intensity recovery level is increased with PTRF-mCherry co-transfection compared with FAK-GFP alone or FAK-GFP and mCherry co-transfection. The FAK-GFP intensity recovery curve graph of one representative experiment and a bar graph of the FAK-GFP mobile fraction (calculated based on the fluorescence recovery plateau) are shown. ($n \geq 3$; ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$.)

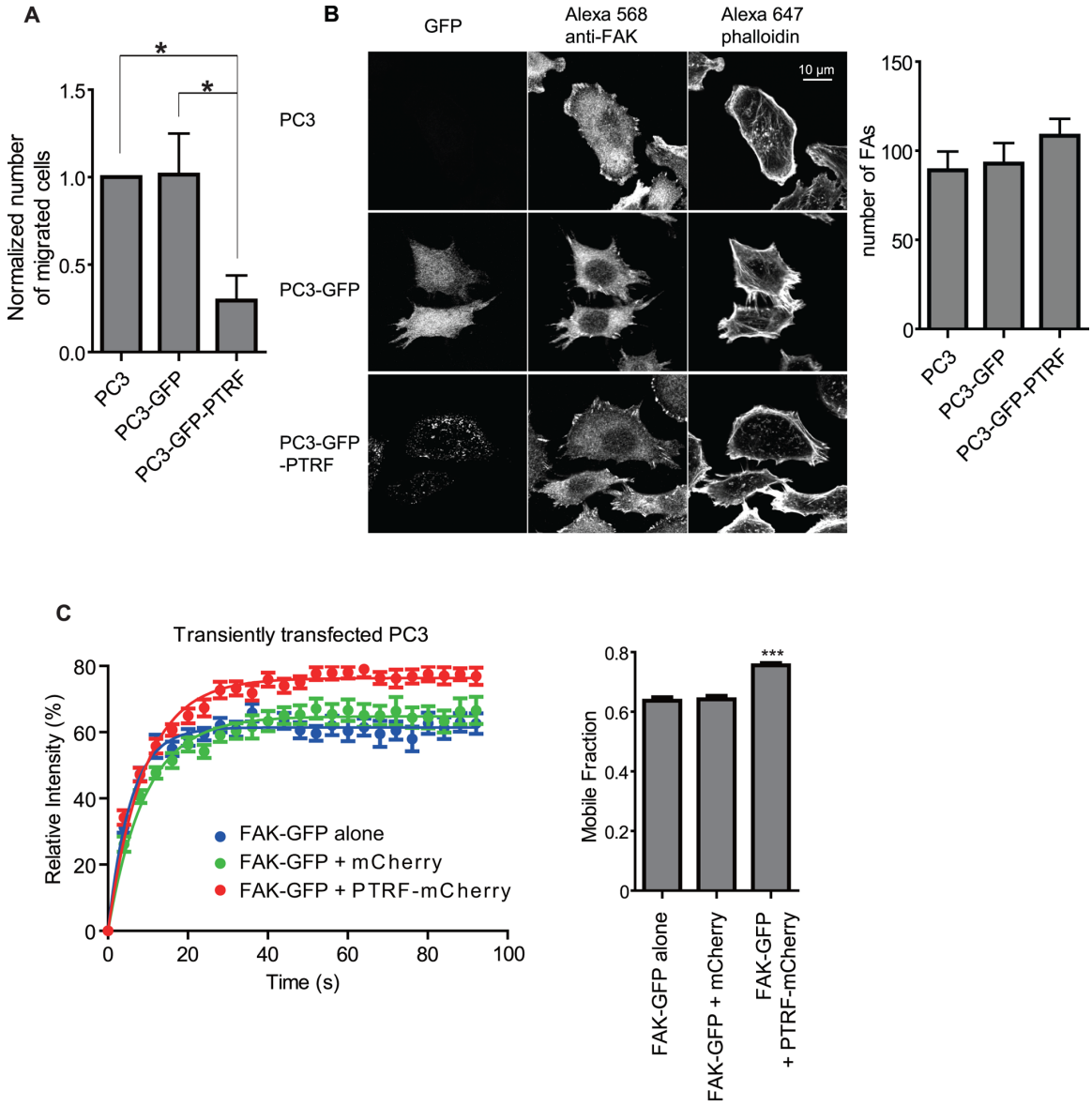

A
B
C
Normalized number of migrated cells
PC3
PC3-GFP
PC3-GFP-PTRF
GFP
Alexa 568 anti-FAK
Alexa 647 phalloidin
10 µm
number of FAs
Transiently transfected PC3
Relative Intensity (%)
Time (s)
FAK-GFP alone
FAK-GFP + mCherry
FAK-GFP + PTRF-mCherry
Mobile Fraction
***

Figure 3-2. Expression of PTRF in PC3 cells does not affect pY14Cav1 levels in the cell.

Western blot shows levels of GFP, GFP-PTRF, Cav1 and pY14Cav1 in PC3 wild-type cells (PC3), PC3 cells stably transfected with GFP (PC3-GFP) and PC3 cells stably transfected with GFP-PTRF (PC3-GFP-PTRF). Western blot band intensity of Cav1 and pY14Cav1 is quantified and normalized to that of β-actin and shows no significant difference of Cav1 expression or phosphorylation between PC3, PC3-GFP and PC3-GFP-PTRF cells. (n≥3; ***: p<0.001.)

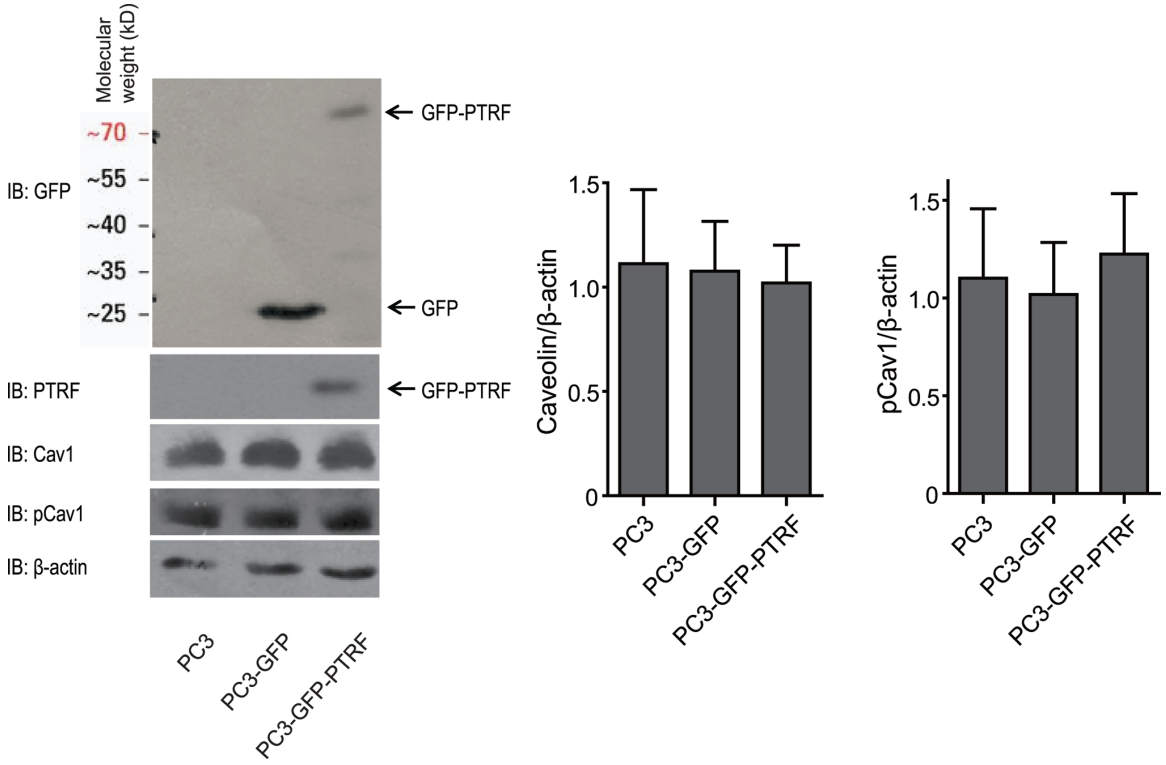


Figure 3-3. Exogenous Gal3 functions to stabilize FAK within FAs and to promote the cell migration in a dose-response manner.

(A) Mobile fraction of FAK-GFP in FAs calculated from the FRAP assay shows the FA-associated FAK stability in PC3 cells transfected with FAK-GFP alone or FAK-GFP plus PTRF-mCherry and subjected to Gal3-His treatment at 0, 1, 1.5, 2, 3 or 4 μg/ml concentrations. 2 μg/ml remains the optimal concentration of Gal3 to restore FAK stabilization in FAs in PTRF expressing PC3 cells; 1.5 and 3 μg/ml have less or none significant effects; while higher or lower concentrations fail to restore FAK stability in FAs in PTRF expressing PC3 cells. (B) Qantification of migrated PC3, PC3-GFP and PC3-GFP-PTRF cells in the transwell assay with Gal3-His treatment at 0, 1, 1.5, 2, 3 or 4 μg/ml concentrations shows that 2 μg/ml Gal3-His increases the cell migration of all cell lines while other concentrations of Gal3 increase the cell migration of PC3-GFP-PTRF cells to similar levels of control PC3 and PC3-GFP cells, with various significance, though. (NT: non-treated; $n \geqslant 3$; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.)

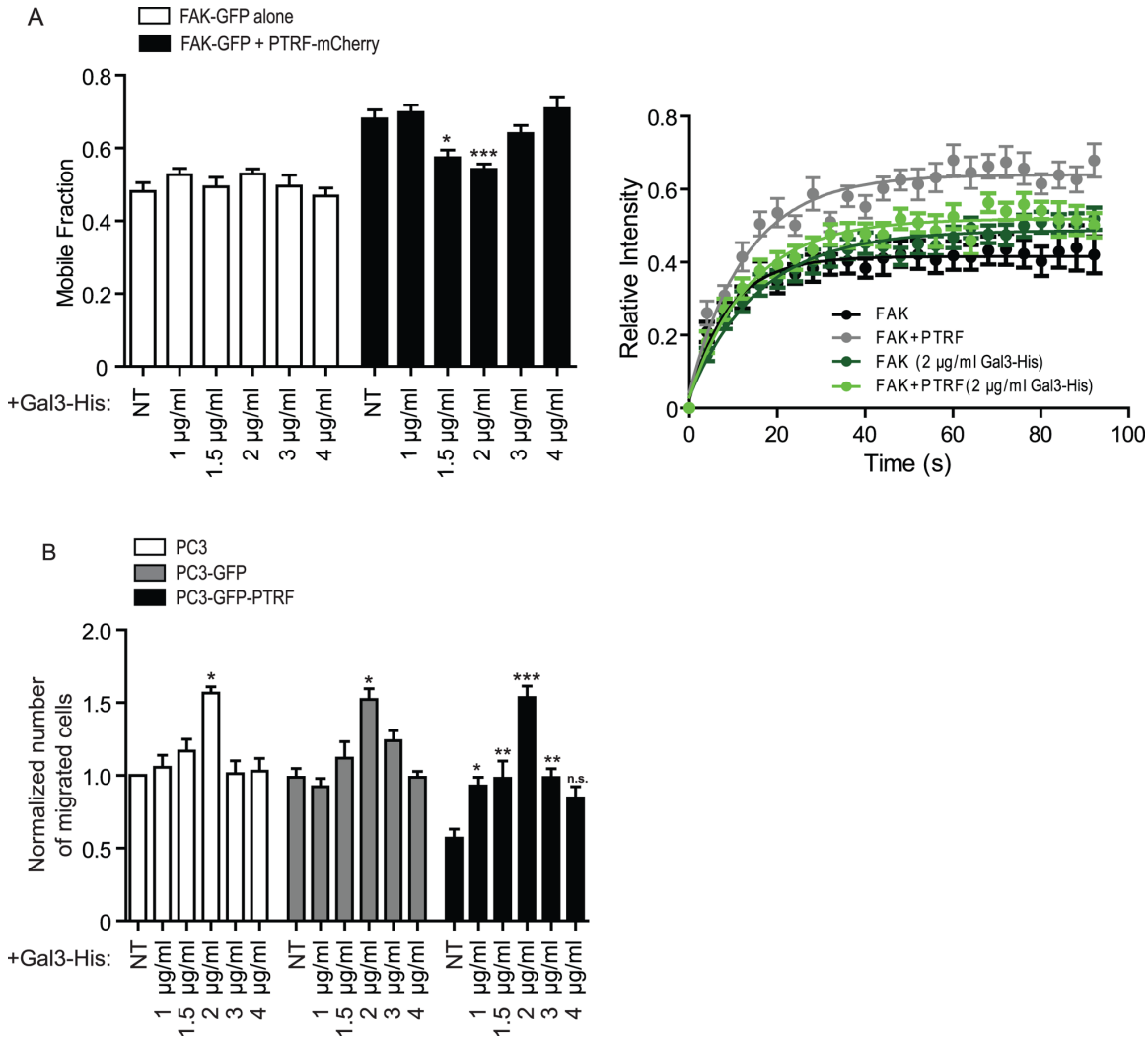

A
FAK-GFP alone
FAK-GFP + PTRF-mCherry
Mobile Fraction
0.8
0.6
0.4
0.2
0
*
***
+Gal3-His:
NT
1 µg/ml
1.5 µg/ml
2 µg/ml
3 µg/ml
4 µg/ml
Relative Intensity
FAK
FAK+PTRF
FAK (2 µg/ml Gal3-His)
FAK+PTRF(2 µg/ml Gal3-His)
Time (s)
0
20
40
60
80
100
B
PC3
PC3-GFP
PC3-GFP-PTRF
Normalized number of migrated cells
2.0
1.5
1.0
0.5
0
n.s.

Figure 3-4. Gal3 siRNA knockdown disrupts FAK stabilization in FAs, which is restored by 2 μg/ml Gal3-His.

(A) Western blot shows the efficiency of Gal3 siRNA knockdown. (B) FRAP assay of FAK-GFP shows FA-associated FAK stability of PC3 cells transfected with no siRNA, scramble control siRNA (siCTL) or the siRNA against human Gal3 (siGal3), and subjected to 2 μg/ml Gal3-His treatment. (n=3; ***: p<0.001.)

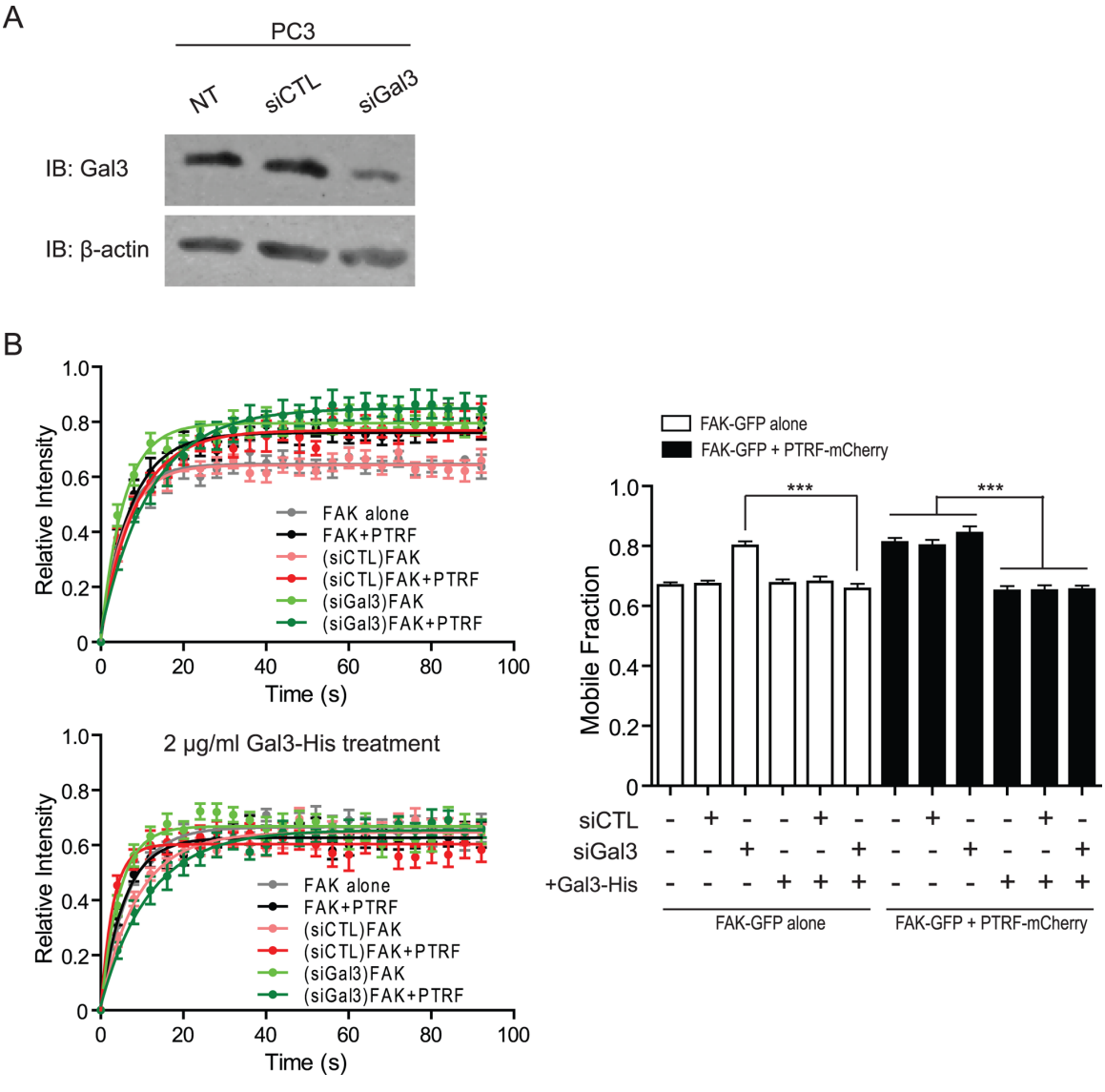


Figure 3-5. Cav1 siRNA knockdown disrupts FAK stabilization in FAs, which is not restored by 2 μg/ml Gal3-His treatment.

(A) Western blot shows the efficiency of Cav1 siRNA knockdown. (B) FRAP assay of FAK-GFP shows FA-associated FAK stability of PC3 cells transfected with no siRNA, scramble control siRNA (siCTL) or the siRNA against human Cav1 (siCav1), and subjected to 2 μg/ml Gal3-His treatment (n=3; ***: p<0.001).

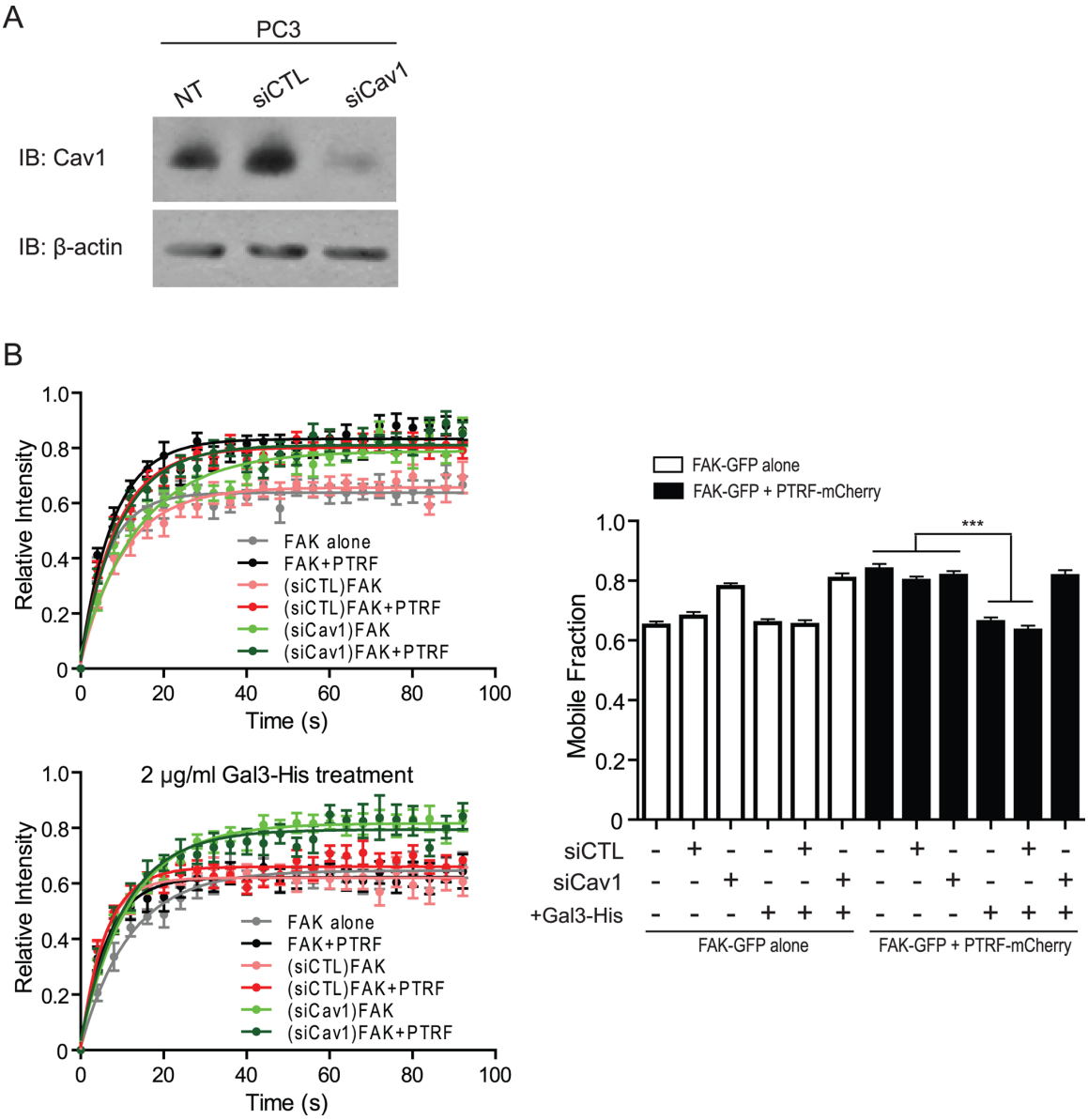
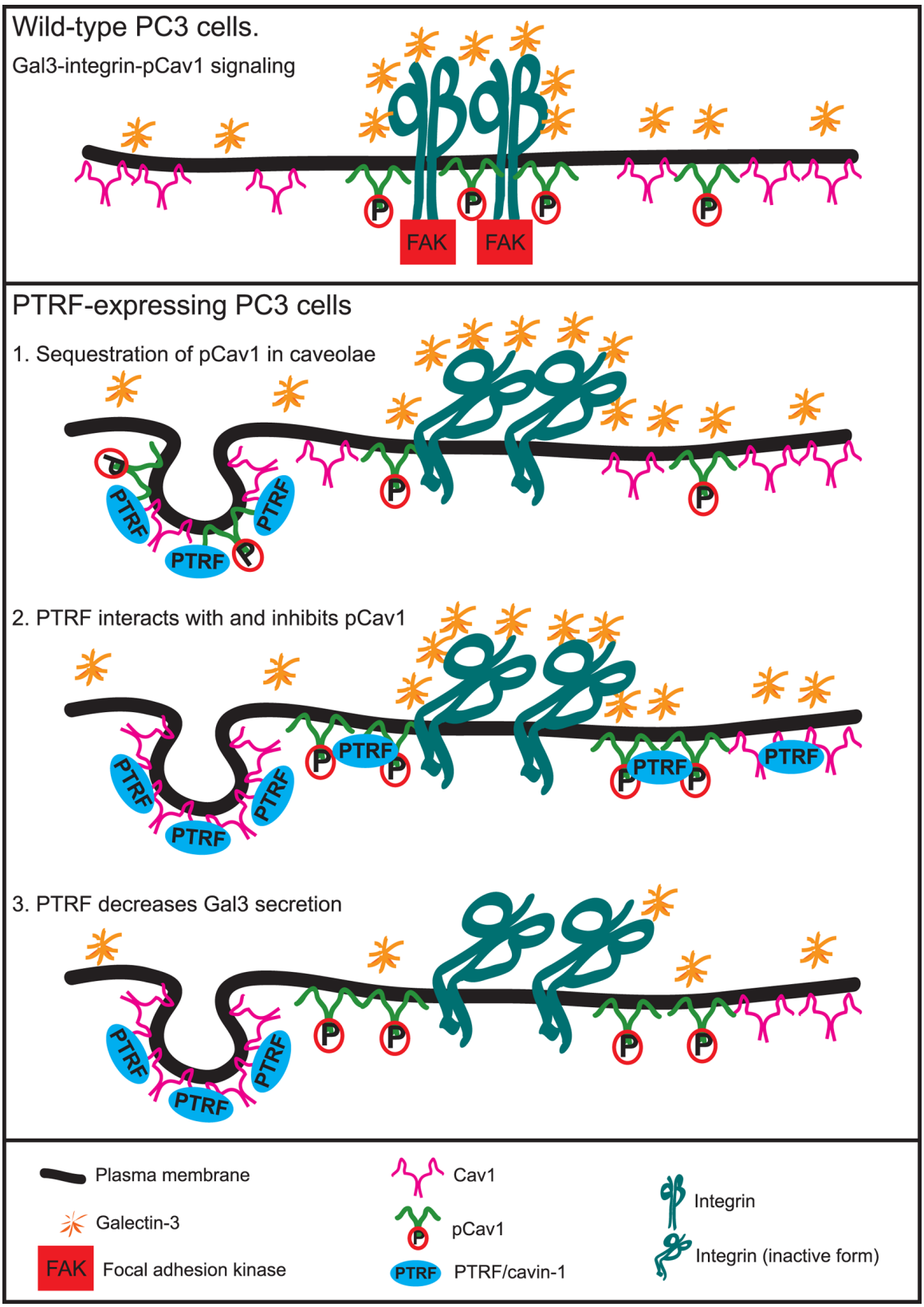


Figure 3-6. Summarized working models of affecting Gal3-pY14Cav1 function.

Extracellular Gal3 and non-caveolar pY14Cav1 each stabilizes FAK within FAs dependent on each other. Expression of PTRF disrupts this function through three possible ways: 1) by recruiting pY14Cav1 away from non-caveolar Cav1 scaffolds and into caveolae; 2) by direct interaction with non-caveolar pY14Cav1; 3) by affecting Gal3 secretion and thus extracellular Gal3 concentration. Through which pathway PTRF affects Gal3-pY14Cav1 function on FA dynamics remains to be studied.


Wild-type PC3 cells.
Gal3-integrin-pCav1 signaling
FAK
FAK
PTRF-expressing PC3 cells
1. Sequestration of pCav1 in caveolae
PTRF
2. PTRF interacts with and inhibits pCav1
3. PTRF decreases Gal3 secretion
Plasma membrane
Galectin-3
FAK Focal adhesion kinase
Cav1
pCav1
PTRF/cavin-1
Integrin
Integrin (inactive form)

CHAPTER 4 Tyrosine Phosphorylated Caveolin-1 Regulates Vinculin Tension in Focal Adhesions through Its Scaffolding Domain

4.1 Summary

Caveolin-1 (Cav1), a key component of cell surface caveolae, is also a major Src kinase substrate. Tyrosine-14 (Y14) phosphorylated Cav1 (pY14Cav1) stabilizes focal adhesion kinase (FAK) within focal adhesions promoting focal adhesion disassembly/assembly and cancer cell motility. The caveolin scaffolding domain (CSD; D82-R101 in Cav1) interacts with multiple proteins however its role in pY14Cav1 function has yet to be determined. We report here that pY14Cav1-dependent FAK stabilization in focal adhesions and stimulation of prostate cancer cell migration is prevented by CSD mutations (F92A/V94A) and by membrane-permeable CSD competing peptides. Quantitative proteomic analysis of GST pull-downs with a N-terminal Cav1 polypeptide encompassing both Y14 and the CSD showed a 6-fold enrichment in binding of vinculin and, to a lesser extent, α-actinin, talin and filamin, to phosphomimetic Cav1Y14D(1-101) relative to non-phosphorylatable Cav1Y14F(1-101). Moreover, pY14Cav1 enhanced CSD-dependent vinculin tension in focal adhesions. The requirement for an intact CSD in pY14Cav1 focal adhesion stabilization and tension defines a functional interaction between Y14 phosphorylation and the CSD in pro-migratory Cav1 activity.

4.2 Introduction

Focal adhesions are a macromolecular complex in which transmembrane integrins and cytoplasmic proteins, including vinculin, talin, α-actinin and FAK, link the extracellular matrix (ECM) to the actin cytoskeleton (Burridge and Fath, 1989; Burridge et al., 1988; Gardel et al., 2010). Focal adhesions are key transmitters of the cellular response to mechanical forces mediated by actomyosin contractility and ECM rigidity (Burridge and Guilluy, 2015; Hoffman,

2014). Vinculin, a cytoplasmic component of focal adhesions, interacts with both the talin-integrin complex and the actin cytoskeleton and is closely involved in focal adhesion tension-induced signalling (Carisey et al., 2013; Cohen et al., 2006; del Rio et al., 2009; Kanchanawong et al., 2010; Ziegler et al., 2006). Upon activation at sites of cell adhesion, vinculin switches from a closed globular conformation to an extended conformation allowing binding of specific partners to the head (Vh) and tail (Vt) domains (Bakolitsa et al., 2004; Cohen et al., 2006; Ziegler et al., 2006). Active extended vinculin stabilizes various focal adhesion proteins, including FAK, within focal adhesions and activates integrins in an actin- and talin-dependent manner (Carisey et al., 2013). A fluorescence resonance energy transfer (FRET)-based vinculin tension sensor (VinTS) construct showed that force transmission across vinculin is selectively enhanced in leading edge focal adhesions and determines focal adhesion size and turnover (Grashoff et al., 2010; Hernandez-Varas et al., 2015).

Src kinase is a key regulator of focal adhesion signaling, phosphorylating multiple focal adhesion proteins including FAK and vinculin, and also tumor cell migration. Integrin-dependent autophosphorylation of focal adhesion kinase (FAK) tyrosine 397 (Y397) creates a high-affinity binding site for Src homology 2 (SH2)-containing proteins, such as Src kinase (Mitra and Schlaepfer, 2006). FAK-Src signaling is a key regulator of the dynamic recruitment of focal adhesion components and leading edge activity (Webb et al., 2004). Src phosphorylation of tyrosine 1065 (Y1065) of vinculin induces its conformation-based activation and tension (Huang et al., 2014; Zhang et al., 2004). Curiously, the major Src tyrosine phosphorylated substrate identified in an early study was caveolin-1 (Cav1), the caveolae coat protein (Glenney, 1989). Y14 phosphorylated Cav1 (pY14Cav1) stabilizes focal adhesion components promoting focal adhesion turnover and directional cell migration (Boscher and Nabi, 2013; Goetz et al., 2008a; Grande-Garcia et al., 2007; Joshi et al.,

2008a). Here we reveal that pY14Cav1 induces vinculin tension in focal adhesions of prostate cancer cells.

Cav1 contains a highly conserved caveolin scaffolding domain (CSD; amino acids 82-101), including the essential F92TVT95 segment, which mediates the interaction of Cav1 with multiple signaling molecules, such as Src family tyrosine kinases, growth factor receptors, endothelial nitric oxide synthase (eNOS) and G proteins (Couet et al., 1997a; Garcia-Cardena et al., 1997; Hoop et al., 2012; Li et al., 1996a; Li et al., 1995; Nystrom et al., 1999). The CSD is also required for Cav1 regulation of integrin-dependent endocytosis, indicating a potential role for the CSD in integrin-dependent cell adhesion and migration (Hoffmann et al., 2010). A cell-permeable peptide sequence from antennapedia (AP) fused to the CSD sequence (amino acids 82-101 of Cav1), termed AP-Cav, mimics CSD function inhibiting eNOS and blocking NO release from endothelial cells *in vitro* (Bernatchez et al., 2005; Bucci et al., 2000; Gratton et al., 2003). AP-Cav inhibition of eNOS decreases vasodilation, inflammation and hyperpermeability of tumor microvasculature thereby blocking tumor angiogenesis and delaying tumor progression *in vivo* (Bernatchez et al., 2005; Bucci et al., 2000; Gratton et al., 2003). This suggests that the CSD mimicking peptides may represent an effective anti-cancer therapy (Williams and Lisanti, 2005). However, whether AP-Cav peptide also affects pro-migratory functions of Cav1 in tumor cells is not known.

Cav1 Y14 phosphorylation induces conformational changes that spatially separates Cav1 molecules within the Cav1 oligomer and has been predicted to alter the conformation and/or accessibility of the CSD, facilitating CSD interaction with other proteins (Shajahan et al., 2012; Zimnicka et al., 2016). Indeed, Y14 phosphorylation of Cav1 increases eNOS binding, an interaction that has been mapped to a 10-amino acid sequence within the CSD (Chen et al., 2012; Trane et al., 2014). Our data shows that the phosphomimetic Y14D Cav1 mutation enhances Cav1 binding to focal adhesion proteins. Further, pY14Cav1 stabilization of focal

adhesion proteins, as well as promotion of vinculin tension and cell migration, requires an intact CSD and is inhibited by AP-Cav peptides. These studies define a novel role for the CSD in pro-migratory pY14Cav1 function in tumor cells.

4.3 Materials and methods

4.3.1 Antibodies and reagents

Bovine serum albumin solution (BSA, 30%), and mouse anti-β-actin antibodies were purchased from Sigma-Aldrich. Rabbit anti-Cav1 and rabbit anti-FAK were purchased from Santa Cruz Biotechnology, Inc.; rabbit anti-pY14Cav1 and rabbit anti-Cav antibodies from Transduction Laboratories. HRP-conjugated mouse and rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Phalloidin and secondary antibodies conjugated to Alexa 488, 568, or 647 were purchased from Life Technologies, Invitrogen. PP2 and Y27632 were purchased from EMD Millipore. Latrunculin A and Jasplakinolid were purchased from Sigma-Aldrich.

4.3.2 Plasmids

C-terminal–tagged myc/mRFP-tagged Cav1wt, Cav1Y14F, Cav1Y14D and Cav1Y14R under control of the cytomegalovirus promoter in pcDNA3 plasmid has been described previously (Goetz et al., 2008a; Joshi et al., 2008a). The Cav1 scaffolding domain mutant (Cav1F92A/V94A) and the combined mutants (Cav1Y14F/F92A/V94A and Cav1Y14D/F92A/V94A) were generated using following sets of primers (F92A/V94A-F: 5’cac cac cgc cac tgc gac gaa ata ctg g3', F92A/V94A-R: 5'cca gta ttt cgt cgc agt ggc ggt ggt g3' and Cav1-BamHI-R: 5'ggg gat ccc tat ttc ttt ctg ca agt tga tgc gga c3', Cav1-HindIII-F:5'gga agc tta gca tgt ctg ggg gca aat ac3') where either Cav1wt or its Y14 mutants were used as templates for post-overlapping extension. The final PCR amplified products, Cav1F92A/V94A, Cav1Y14F/F92A/V94A, or Cav1Y14D/F92A/V94A, were TA-cloned

(Invitrogen), restriction digested (by HindIII) and sequence verified before sub-cloning back into pRFP-N1 at EcoR1-BamH1 restriction sites. Restriction enzymes (EcoR1 and BamH1) were purchased from New England Biolabs. T4 ligase was purchased from Invitrogen.

4.3.3 Cell culture, transfection and drug treatment

The human DU145 cell line was from American Type Culture Collection (ATCC) and maintained in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Stable DU145 cell lines expressing dsR or the Cav1 constructs were generated by transfecting dsR or the Cav1 constructs expression vectors using Effectene (Qiagen). Neomycin-resistant cells were selected for 15 d with 400 μg/mL of geneticin (Life Technologies, Invitrogen), and resistant colonies were trypsinized and sorted for mRFP positives by fluorescence-activated cell sorting. Pooled mRFP-positive cells were allowed to recover and expanded in complete medium supplemented with geneticin. All cell lines were passaged at least twice after recovery from frozen stocks before initiating experiments and maintained in culture for a maximum of 8 to 10 passages to minimize phenotypic drift. Transient transfections with Lipofectamine 2000 (Life Technologies, Invitrogen) were performed using standard protocols and cells used after 24 h.

Transient plasmid transfection was done 24 h after plating of the cells or siRNA transfection, using Lipofectamine 2000 (Life Technologies, Thermo Fisher Scientific) following the manufacturer's protocol. Experiments were performed 24 h post plasmid transfection. In order to knockdown Cav1, cells were cultured in complete medium for 24 h before transfection with specific mouse Cav1 siRNA or control siRNA smartpools (mouse siCav1: L-0058415-00, siCONTROLS: D-001210-01; Dharmacon) using Lipofectamine 2000 transfection reagent (Life Technologies, Thermo Fisher Scientific) following the manufacturer's protocol.

Where indicated, cells were treated with 10 μM PP2 for 30 min, 20 μM Y27632 for 1 h, 150 nM Jasplakinolide for 2 h or 150 nM latrunculin A for 30 min.

4.3.4 Western blotting

Cell pellets from 80% confluent cultures were washed with cold PBS and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA containing freshly added 2 mM DTT, 0.5 mM PMSF, 1 mM sodium vanadate, 2.5 mM sodium fluoride, and 1 μM leupeptin) for 30 min at 4 °C, pelleted at 13,000 rpm at 4 °C, and the supernatant collected and stored at -80 °C. Equal amounts of proteins were separated on 12% SDS-PAGE, electroblotted onto nitrocellulose (GE Healthcare Life Science), probed with indicated antibodies and HRP-conjugated secondary antibodies, and revealed by ECL.

4.3.5 Immunofluorescence labeling

Cells were fixed with 3% paraformaldehyde (PFA) for 15 min at room temperature, rinsed with PBS, permeabilized with 0.1% Triton X-100 in PBS/CM, blocked with PBS/CM containing 2% bovine serum albumin (BSA), and then incubated with primary and fluorescent secondary antibodies in PBS/CM containing 2% BSA. After labeling, the coverslips were mounted in CelVol (Celanese, Ltd.) or Prolong Gold (Life Technologies, Thermo Fisher Scientific), and images acquired with a 60× or 100× planapochromat objectives (NA 1.35) of an Olympus FV1000 confocal microscope or with a 100x HC PL APO objective (NA 1.40; oil) of a Leica TCS SP8 confocal microscope.

4.3.6 FRAP and FRET measurements

FRAP was performed on an Olympus FV1000 confocal microscope equipped with a 60x planapochromat objective (NA 1.35; oil) and SIM scanner. Cells were plated at low density on FN (10 μg/ml) for 24 h in an 8-well μ-slide chamber (ibidi), transfected with denoted

plasmid constructs, and experiments performed 24 h later at 37°C. PP2 was applied to cells at 10 μM for 30 min before imaging. Alternatively, 18 h after transfection, the culture medium was replaced with serum-free medium containing 10 μM AP or AP-CAV for 6 h. For each FRAP analysis, a prebleach frame was acquired followed by a single bleach event using the simultaneous and independent stimulation of the 405 nm laser line of the SIM scanner. Fluorescence recovery was followed at 4-s time intervals until the intensity reached a plateau. Fluorescence intensity during recovery was normalized to the prebleach intensity. Normalized intensity value of the recovery plateau in each bleached area was calculated as the mobile fraction using Prism 4 (GraphPad). Graphs are representative of a minimum of three independent experiments in which between 10 and 25 focal adhesions were bleached.

FRET was performed on a Leica TCS SP8 confocal microscope with a 100x HC PL APO objective (NA 1.40; oil) using FRET AB (acceptor bleaching) mode. Cells were plated at low density on FN (10 μg/ml) in an 8-well μ-slide chamber (ibidi), transfected with denoted plasmid constructs 24 h post plating, and fixed with 3% PFA 24 h post transfection. Cells were then permeablized with 0.1% Triton X-100, blocked with 2% BSA, incubated with specific primary antibodies (mouse anti-myc tag or rabbit anti-Cav1) and Alexa Fluor 647 conjugated secondary antibodies. For FRET acceptor bleaching, ROIs were generated on visible focal adhesions for bleaching of the Venus channel. Both a pre-bleach image and a post-bleach image of both channels were taken and FRET efficiency was determined by the Leica software (LAS AF).

4.3.7 Migration assay and cytotoxicity assay

For migration assay, cells were trypsinized, counted, and transferred to uncoated 8-μm cell culture inserts (BD Falcon) in medium containing 2% serum and the assembly placed into 24-well plates containing complete medium with 10% serum. After 16 h, non-migrated cells

were removed from the top of the filter with a cotton swab, and migrated cells on the bottom of the filter were fixed with 3% PFA, stained with 5% crystal violet and labeled cells counted. Cell counts were normalized to the control DU145 group. Alternatively, after 2 h, medium in the inserts was replaced with serum-free medium containing 10 μM AP or AP-CAV and after 6 h the non-migrated cells removed, migrated cells fixed, stained, and counted. Cell counts were normalized to the DU145 plus AP treatment group. For the cytotoxicity assay, cells were trypsinized, counted and plated in complete medium for 24 h, which was replaced with serum-free medium (control) or serum-free medium containing 10 μM AP or AP-CAV for 6 h, after which the cells were fixed, stained, counted and normalized to the control group for each cell line.

4.3.8 GST pull-down and proteomics analysis

Cav1 (wild-type, Y14F, Y14D) fragments containing amino acids 1-101 were subcloned into pGEX-4T1 plasmid (GE Healthcare). GST, GST-Cav1Y14D and GST-Y14F plasmids were transformed into the BL21 E.coli strain and sequence verified clones were induced with 0.4mM IPTG 30ºC for 3h following the supplier's protocol. GST fusion proteins were bound to Glutathione-S-Sepharose (GE Healthcare) following the manufacturer's protocol and prepared beads (validated by SDS-PAGE and GST, Cav1 western blots) were stored at 4ºC for further use.

Pull-down assays with GST, GST-Cav1Y14D and GST-Cav1Y14F beads were performed following a protocol described previously (Garcia-Carden et al., 1997). Briefly, about 15 μg total protein lysate was incubated with the GST, GST-Cav1Y14D or GST-Cav1Y14F beads on a rotor at 4ºC for 3 h, washed and subjected to thrombin cleavage (0.1 U/rxn) in reaction buffer (20 mM Tris-HCl, pH-8.0, 100 mM NaCl, 0.3mM $CaCl_2$, 1 mM DTT and 0.1% Triton X-100). Reaction mixtures were incubated overnight at 4ºC. The supernatant was collected

and subjected to quantitative proteomics analysis using formaldehyde labeling. The eluate from GST-Cav1Y14D, GST-Cav1Y14F and GST alone pull-downs were labeled with CH_2O (light), CD_2O (medium) and $^{13}CD_2O$ (heavy) formaldehydes, respectively, which gave +28 Da, +32 Da and +36 Da mass shift to the peptides, respectively.

The peptide mixtures were analyzed on an Orbitrap Velos, as described (Imami et al., 2013), and the mass spectra were used to identify and quantify proteins using the MaxQuant package (Cox and Mann, 2008). Proteins having ≥2 peptides were considered for further bioinformatics analysis using the Quantitative Proteomics P-value Calculator (QPCC) (Chen et al., 2014). A distribution-free permutation method based on replicated log (ratio) was applied to the raw peptide ratios to identify significantly altered proteins. Of the significantly changed proteins, only those with a fold-change greater than 1.5 were considered further.

4.4 Results and discussion

4.4.1 The CSD mediates pY14Cav1-dependent FAK stabilization in focal adhesions and cell migration

Tyrosine phosphorylated Cav1 stabilizes FAK in focal adhesions and promotes Src- and ROCK-dependent migration of metastatic cancer cells (Goetz et al., 2008a; Joshi et al., 2008a). DU145 prostate cancer cells express Cav1 and caveolae but exhibit no detectable pY14Cav1 (Gould et al., 2010; Joshi et al., 2008a). Transient expression of Cav1wt and phosphomimetic Cav1Y14D promotes Src/ROCK-dependent FAK stabilization and cell migration of DU145 cells (Joshi et al., 2008a) making these cells an excellent model to selectively study the contribution of pY14Cav1. To determine the role of the CSD in pY14Cav1-dependent FAK stabilization in focal adhesions and cell migration, we introduced F92A/V94A mutations (Lajoie et al., 2007a; Li et al., 1996a; Nystrom et al., 1999) into wild-type, Y14F dominant negative mutant and Y14D phospho-mimetic mutant of C-terminal

Myc- and mRFP-tagged Cav1 (Figure 4-1A). Stable transfection of these Cav1 constructs in DU145 cells resulted in detection by immunoblotting of a higher molecular weight band corresponding to recombinant Cav1 in addition to endogenous Cav1 (Figure 4-1B). An antibody against pY14Cav1 selectively detected recombinant Cav1-myc-mRFP in Cav1wt expressing cells (Figure 4-1B). All constructs were expressed at levels lower than that of the endogenous Cav1. Expression of the constructs containing the F92A/V94A mutation was further reduced (Figure 4-1B). By confocal microscopy, all constructs were expressed and exhibited the typical cell surface distribution of wild-type Cav1 (Figure A-1).

To measure FAK stabilization in focal adhesions, we applied fluorescence recovery after photobleaching (FRAP) to peripheral FAK-GFP expressing focal adhesions of the DU145 Cav1 stables. In contrast to Cav1wt and the Cav1Y14D mutant and similar to Cav1Y14F, the F92A/V94A CSD mutants of Cav1 were unable to stabilize FAK within focal adhesions (Figure 4-1C and D). To ensure that differential stable expression levels of the Cav1 mutants (Figure 4-1B) did not impact the FRAP data, we transiently transfected DU145 cells with the Cav1-mRFP constructs together with FAK-GFP and selectively analyzed cells expressing similar levels of mRFP. As for the stable transfectants, the F92A/V94A CSD mutants of Cav1 prevented Cav1wt and Y14D stabilization of FAK in focal adhesions (Figure A-2). Transwell cell migration assays showed that stable expression of F92/V94A CSD mutants and Y14F Cav1 mutants reduced DU145 cell migration whereas Cav1Y14D increased DU145 cell migration (Figure 4-1E).

We then treated the DU145 Cav1 stable transfectants with 10 μM of control AP or AP-Cav peptides for 6 hours and monitored the mobile fraction of focal adhesion-associated FAK-GFP by FRAP. AP-Cav, but not AP, diminished FAK stabilization in focal adhesions induced by both Cav1wt and Cav1Y14D (Figure 4-2A). In contrast, only DU145-Cav1wt cells, but not DU145-Cav1Y14D cells, showed diminished FAK stabilization in focal adhesions in

response to PP2 inhibition of Src kinase (Figure 4-2B). Consistently, AP-Cav selectively reduced the migratory ability of all the DU145 transfectants to the level of the DU145-Cav1Y14F cells without affecting cell viability (Figure 4-2C and D). AP-Cav peptide therefore prevents focal adhesion FAK stabilization within focal adhesions and reduces cell motility that is dependent on pY14Cav1.

4.4.2 pY14Cav1 interaction with vinculin

To determine the impact of Y14 phosphorylation on Cav1 binding partners, and in particular focal adhesion proteins, we constructed GST-conjugates of a Cav1 construct containing amino acids 1-101, including both the Y14 phosphorylation site and the CSD. GST pull-downs from DU145 whole cell lysates were analyzed by quantitative mass spectrometry (Figure 4-3A). Consistent with reports of Cav1-integrin interaction (del Pozo et al., 2005; Salanueva et al., 2007; Wary et al., 1998), integrin β1 was detected but did not show preferred binding to GST-Cav1(1-101)Y14D or Y14F (Y14F/Y14D ratio 0.432, SD=0.281, P value 0.1718, n=3). The other focal adhesion proteins detected (vinculin, α-actinin -4, talin-1 and filamin-A/B) all showed significantly preferred binding to GST-Cav1(1-101)Y14D compared to Y14F, with vinculin showing the most robust binding preference for GST-Cav1(1-101)Y14D (Y14F/Y14D ratio 0.150, SD=0.023, P value 0.0090, n=2). Indeed, coimmunoprecipitation of filamin A with Cav1 is Src-dependent supporting its preferred interaction with Cav1Y14D in our proteomic analysis (Sverdlov et al., 2009). Western blotting of GST pull-downs confirmed the preferential binding of vinculin to GST-Cav1Y14D compared to GST and GST-Cav1Y14F (Figure 4-3B). As for FAK, vinculin-Venus showed increased stabilization in focal adhesions of Cav1wt and Cav1Y14D DU145 cells but not in cells expressing F92A/V94A or Y14F Cav1 mutants (Figure 4-3C). pY14Cav1 therefore interacts with and regulates vinculin stabilization in focal adhesions in a CSD-dependent manner.

4.4.3 CSD-dependent pY14Cav1 regulation of vinculin tension

Based on the enriched binding of vinculin to GST-Cav1Y14D and increased vinculin tension at leading edge focal adhesions (Grashoff et al., 2010), we expressed vinculin tension sensors together with myc-tagged Cav1 mutants in DU145 cells to examine the pY14Cav1 regulation of vinculin tension in focal adhesions. FRET efficiency of tension sensor (VinTS) and control tail-less (VinTL) constructs of vinculin were tested by fluorescence resonance energy transfer (FRET) acceptor bleaching. As shown in Figure 4-4A and B, VinTL showed constant high FRET efficiency equivalent to that of VinTS in control or Cav1Y14F transfected DU145 cells. VinTS, but not VinTL, displayed lower FRET efficiency in Cav1wt-myc and Cav1Y14D-myc transfected DU145 cells suggesting that pY14Cav1 increases vinculin tension in focal adhesions.

We then used prostate cancer cell lines that have different Cav1 and pY14Cav1 levels to test the role of endogenous Cav1 in vinculin tension. LNCaP does not express Cav1, DU145 expresses Cav1 but has no detectable pY14Cav1, and only PC3 has endogenous pY14Cav1 (Gould et al., 2010; Joshi et al., 2008a). PC3 showed significantly more vinculin tension (less FRET efficiency) than DU145 and LNCaP cells (Figure 4-4C and D). Blocking F-actin remodelling (ROCK inhibitor Y27632) and actin polymerization (Latrunculin A, LatA) relieved vinculin tension (high FRET efficiency) in PC3 cells, while stabilization of actin filaments with jasplakinolide (Jasp) induced high vinculin tension (low FRET efficiency) in all three cell lines (Figure 4-4C and D). In PC3 cells, vinculin tension was diminished by PP2 treatment and Cav1 knockdown and therefore is both Cav1- and Src-dependent (Figure 4-5A). Endogenous pY14Cav1 therefore regulates vinculin tension in focal adhesions.

Similarly, PP2 reduced vinculin tension in Cav1wt expressing DU145 cells (Figure 4-5B). However, PP2 was also able to reduce vinculin tension in Cav1Y14D cells (Figure 4-5B),

which contrasts the inability of Src inhibition to reverse Cav1Y14D-dependent FAK stabilization and enhanced tumor cell migration (Joshi et al., 2008a). Y14 phosphorylation of Cav1 therefore appears to be critical to the stabilization of focal adhesion components but additional Src activity is required for vinculin tension. Vinculin is activated by Src phosphorylation on Y1065 and this stabilizes various components of focal adhesions, including FAK, in a force-dependent manner (Carisey et al., 2013). Indeed, PP2 disrupts the Src-dependent activation of vinculin and tension development in airway smooth muscle cells (Huang et al., 2014; Zhang et al., 2004). Further, increased tension drives Src-dependent Cav1 Y14 phosphorylation (Joshi et al., 2012; Radel and Rizzo, 2005). At the same time Cav1 promotes Csk-dependent inactivation of Src and prevents Src/p190RhoGAP inhibition of RhoGTPase signaling, thereby driving cell polarization and motility (Grande-Garcia et al., 2007; Place et al., 2011). Together, this suggests that Src-dependent stabilization of focal adhesion proteins by pY14Cav1 is a central component of a feedback loop that enables temporal vinculin activation and unfolding, resulting in focal adhesion tension.

A scaffolding function for pY14Cav1 at focal adhesions is supported by the requirement for the CSD in the regulation of pY14Cav1 activity in focal adhesions and tumor cell migration, as shown here. Increased vinculin tension in PC3 cells is disrupted by treatment with the AP-Cav peptide but not the control AP peptide; neither LNCaP and DU145 cell vinculin tension levels were affected by either AP or AP-Cav treatment (Figure 5C). Further, both F92A/V94A mutation and the AP-Cav peptide treatment reversed the increased vinculin tension in Cav1wt and Cav1Y14D expressing DU145 cells (Figure 5D,E). These data support a role for the CSD in pY14Cav1-mediated protein interactions and in regulating pY14Cav1 function at focal adhesions.

Roles for the CSD and the caveolin-binding motif (CBM), with which it is proposed to interact (Couet et al., 1997a), in protein-protein interactions have been challenged based on

accessibility concerns; the CBM is predicted to be within hydrophobic core regions of the binding protein while the CSD is predicted to be closely associated with the membrane (Ariotti et al., 2015; Byrne et al., 2012; Collins et al., 2012). However, Y14 phosphorylation increases the spacing between Cav1 N-termini and may thereby expose an otherwise hidden CSD (Chen et al., 2012; Zimnicka et al., 2016), altering CSD binding partners. In addition to mediating interaction with signaling molecules, the CSD also mediates Cav1 oligomerization and caveolae formation (Kirkham et al., 2008). Y14 Cav1 phosphorylation induces caveolae swelling and internalization (Zimnicka et al., 2016). However, how pY14Cav1 functions at caveolae and how this regulates focal adhesion dynamics and tension, remain to be determined.

Disruption of pY14Cav1-dependent focal adhesion stabilization, vinculin tension and cell migration by the membrane-permeable CSD mimicking peptide, AP-Cav, defines a critical role for the CSD in focal adhesion dynamics and migration of tumor cells. The AP-Cav peptide inhibits angiogenesis and delays tumor progression *in vivo* (Bucci et al., 2000; Gratton et al., 2003) such that CSD mimicking peptides may represent an effective anti-cancer therapy (Williams and Lisanti, 2005). Our data suggests that therapeutic use of CSD mimicking peptides may also impact pY14Cav1-dependent tumor cell migration, enhancing the potential value of these CSD-targeted reagents as anti-cancer therapeutics.

Figure 4-1. CSD mutation prevents pY14Cav1 regulation of FAK focal adhesion stabilization and cell migration.

(A) Schematics showing the combined Cav1 Y14-CSD mutant constructs. (B) Western blot of Cav1 and pY14Cav1 in DU145 and stably transfected DU145 cells (rec: recombinant; end: endogenous.) (C) Representative FRAP images of DU145-Cav1wt (untreated and PP2-treated) and DU145-Cav1F92A/V94A cells transiently transfected with FAK-GFP. (D) Bar graphs of intensity recovery curves and mobile fraction of FAK-GFP in focal adhesions of stably transfected DU145 cell lines (Data represent mean±SEM from one of three independent experiments. n>8 for each cell line. One-way ANOVA with Tukey post-test; ***, $p<0.001$). (E) Bar graph of of the number of migrated DU145 and stably transfected DU145 cells (normalized to non-transfected DU145 cells) in Transwell migration assays (n=5; two-tailed unpaired t-test; *, $p<0.05$; **, $p<0.01$).

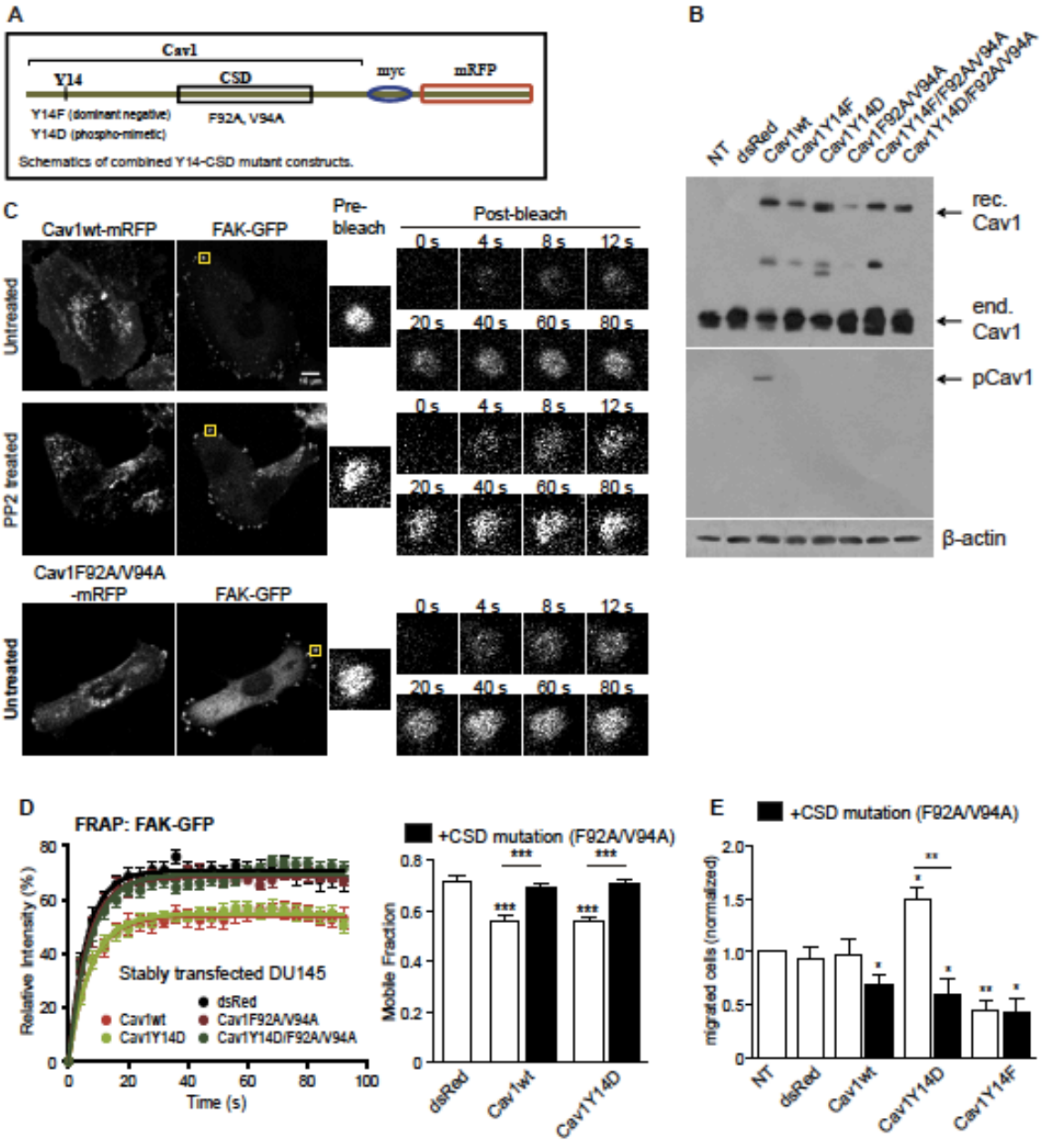

A
Cav1
Y14
CSD
myc
mRFP
Y14F (dominant negative)
Y14D (phospho-mimetic)
F92A, V94A
Schematics of combined Y14-CSD mutant constructs.
B
NT
dsRed
Cav1wt
Cav1Y14F
Cav1Y14D
Cav1F92A/V94A
Cav1Y14F/F92A/V94A
Cav1Y14D/F92A/V94A
rec. Cav1
end. Cav1
pCav1
β-actin
C
Cav1wt-mRFP
FAK-GFP
Pre-bleach
Post-bleach
0 s
4 s
8 s
12 s
20 s
40 s
60 s
80 s
Untreated
PP2 treated
Cav1F92A/V94A -mRFP
FAK-GFP
Untreated
D
FRAP: FAK-GFP
Relative Intensity (%)
Time (s)
Stably transfected DU145
dsRed
Cav1wt
Cav1F92A/V94A
Cav1Y14D
Cav1Y14D/F92A/V94A
+CSD mutation (F92A/V94A)
Mobile Fraction
dsRed
Cav1wt
Cav1Y14D
E
+CSD mutation (F92A/V94A)
migrated cells (normalized)
NT
dsRed
Cav1wt
Cav1Y14D
Cav1Y14F

Figure 4-2. The CSD mimicking peptide AP-Cav diminishes pY14Cav1-dependent FAK focal adhesion stabilization and cell migration.

(A) Intensity recovery curves and mobile fraction bar graphs for FAK-GFP in focal adhesions from FRAP assays of the stable DU145 Cav1 transfectants treated with AP or AP-Cav peptide for 6 h (Data represent mean±SEM from one of three independent experiments. n>8 for each cell line. Two-tailed unpaired t-test; ***, $p<0.001$). (B) Mobile fraction of FAK-GFP in focal adhesions of DU145 (NT) and stably transfected DU145 cell lines (Cav1 constructs as indicated) untreated or PP2-treated (Control: DU145 cells transfected with FAK-GFP only). Bar graph represents mean±SEM of three independent experiments (n>10 for each cell type/treatment for each experiment; One-way ANOVA with Tukey post-test; ***, $p<0.001$). (C) Quantification of migrated cell numbers in Transwell migration assays of DU145 (NT) and stably transfected DU145 cells (Cav1 constructs as indicated) treated with AP or AP-Cav for 6 h (n=5; two-tailed unpaired t-test; ***, $p<0.001$). (D) Quantification of adherent cells after treatment with AP or AP-Cav for 6 h as a measure of cell viability of non-transfected DU145 (NT) and stable DU145 Cav1 transfectants as indicated. The numbers of cells were normalized to that of untreated cells. No significant difference detected with One-way ANOVA with Tukey post-test (n=5).

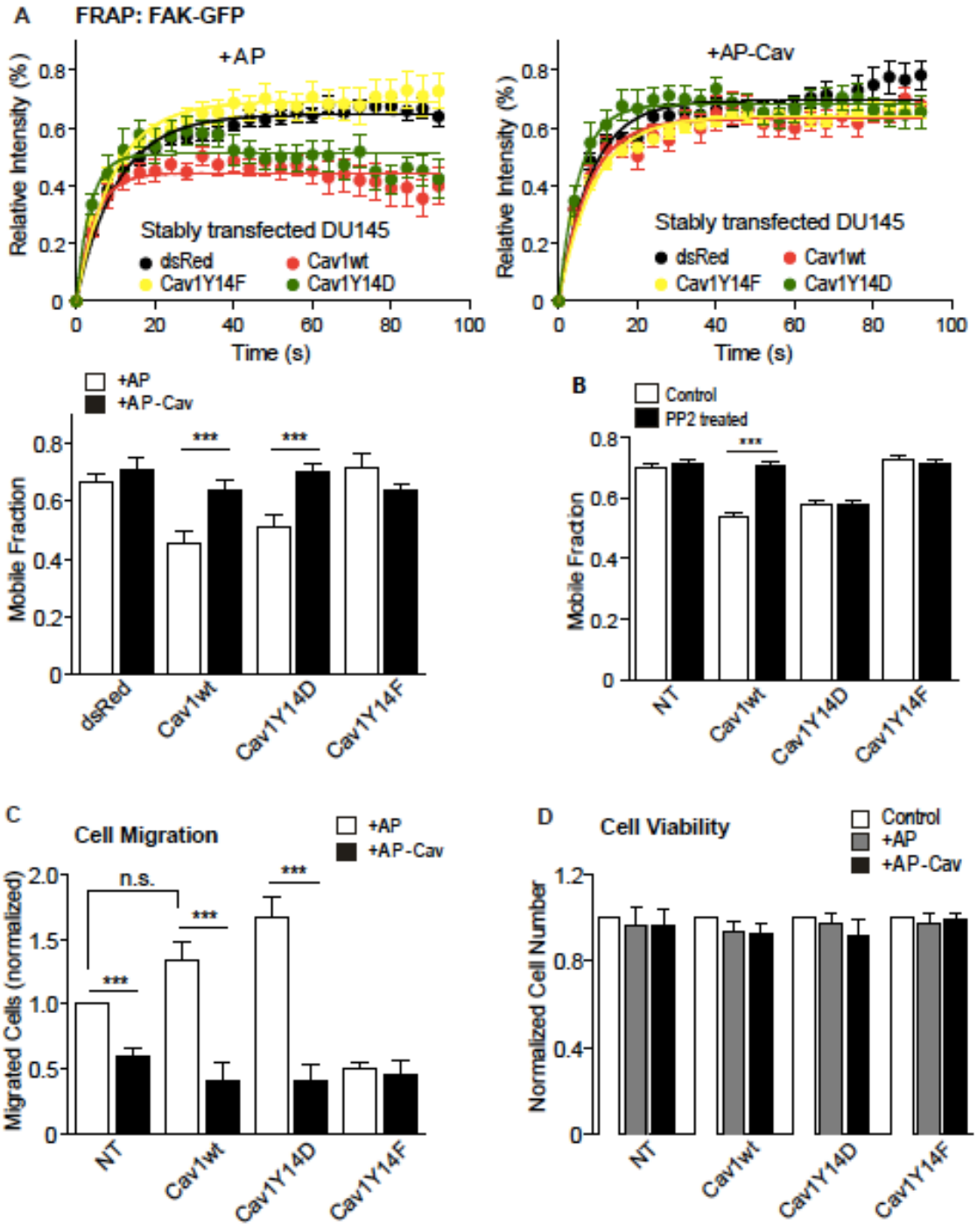

A
FRAP: FAK-GFP
+AP
+AP-Cav
Relative Intensity (%)
Time (s)
Stably transfected DU145
dsRed
Cav1wt
Cav1Y14F
Cav1Y14D
Mobile Fraction
B
Control
PP2 treated
NT
C
Cell Migration
Migrated Cells (normalized)
n.s.
D
Cell Viability
Normalized Cell Number

Figure 4-3. pY14Cav1 interacts with vinculin and regulates its stability within focal adhesions.

(A) A list of focal adhesion proteins detected by quantitative proteomics analysis of GST-Cav1Y14D and GST-Cav1Y14F pull-down elution. (Data represent 6 independent experiments. N, number of experiment repeats that detect the same protein; Average # peptides, average number of peptides detected that match the protein; binding ratio Cav1Y14F/Cav1Y14D, the ratio of the quantity of the binding partner detected from the elution from GST-Cav1Y14F versus that from GST-Cav1Y14D.) (B) Western-blot of elution from GST pull-down labeled with an anti-vinculin antibody showing the preferential binding of vinculin with GST-Cav1Y14D. Blots were quantified with the intensity of the vinculin blots and the bar graph represents mean±SEM of six independent experiments. (One-way ANOVA with Tukey post-test; *, $p<0.05$ compared with both others.) (C) Intensity recovery curve and mobile fraction bar graphs of FRAP assays on vinculin-Venus within focal adhesions of non-transfected DU145 (NT) and stable DU145 Cav1 transfectants as indicated. Intensity recovery curves represent one of three independent experiments; mobile fraction bar graph represents mean±SEM of three independent experiments. ($n>12$ for each cell line for each experiment; One-way ANOVA with Tukey post-test; ***, $p<0.001$.)

A

Gene ID	Protein name	N	average # peptides	Binding ratio Cav1Y14F/Cav1 Y14D		
				Mean	SD	P value
P18206-2	Isoform 1 of Vinculin	2	3	0.150	0.023	0.0090
Q5TCU6	Talin 1	3	13	0.257	0.038	0.0260
O43707	Alpha-actinin-4	3	29	0.271	0.015	0.0230
Q5HY54	Filamin-A	5	33	0.289	0.033	0.0090
O75369-2	Isoform 2 of Filamin-B	4	45	0.311	0.040	0.0280
P05556-2	Isoform Beta-1B of Integrin beta-1	3	5	0.432	0.281	0.1718

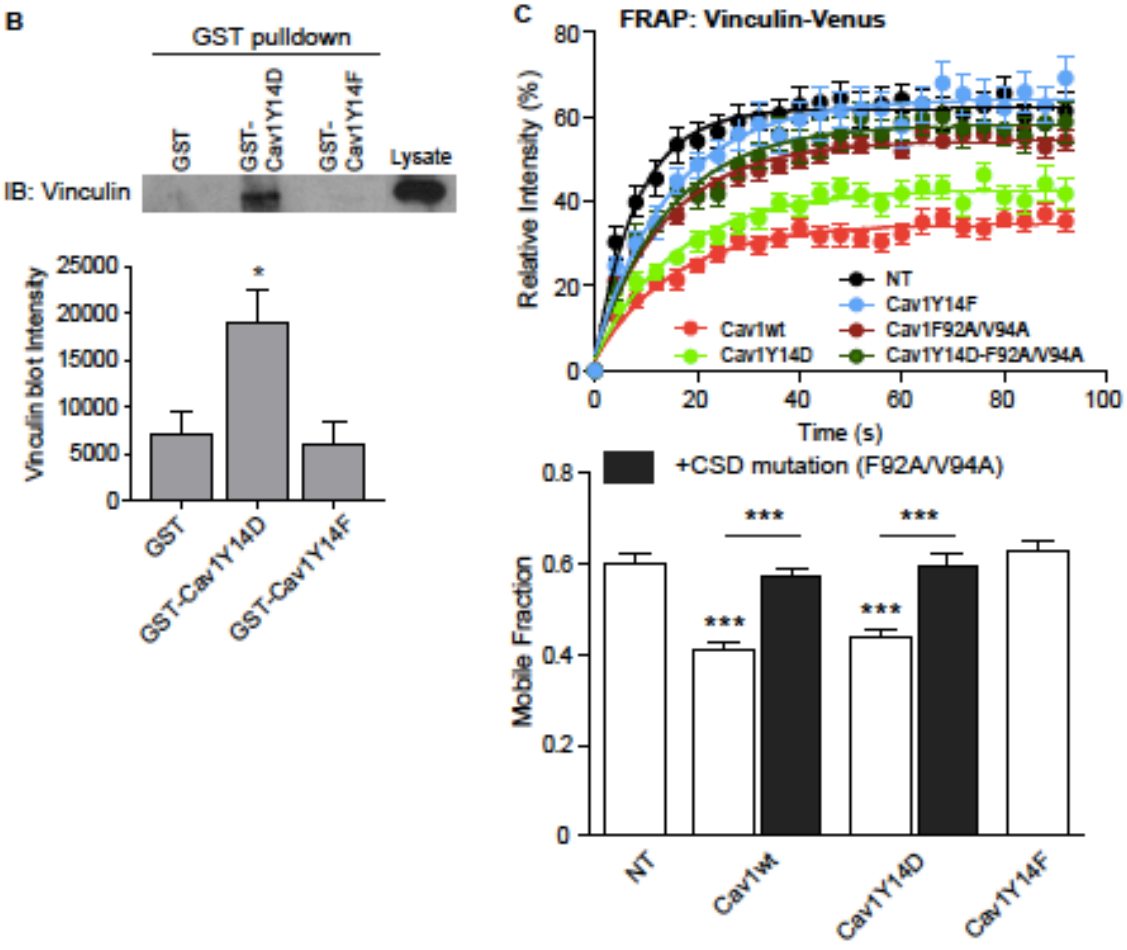


Figure 4-4. CSD-dependent pY14Cav1 regulation of vinculin tension in metastatic prostate cancer cells is actin- and ROCK-associated.

(A) Representative images of acceptor photobleaching FRET assay on vinculin tension sensor (VinTS) and control vinculin tailless (VinTL) in DU145 cells co-transfected with Cav1wt-myc. Cells were fixed and Cav1-myc constructs detected by immuno-labeling with anti-myc tag antibody. Insets to the right show enlarged focal adhesions (a, b, c, d) color-coded for FRET efficiency (Scale bar, 10 μm). (B) Quantification of FRET efficiency of VinTS or VinTL of indicated DU145 Cav1 transfectants. (C) Representative images of pre-bleach and FRET efficiency of PC3 cells, untreated (Control) or treated with Y27632, Latrunculin A (LatA) or Jasplakinolide (Jasp). (D) Quantification of VinTS FRET efficiency in focal adhesions of LNCaP, DU145 and PC3 cells, untreated (Control) or treated with Y27632, Latrunculin A (LatA) or Jasplakinolide (Jasp) as indicated. For B and D, data represent mean±SEM of three independent experiments (n>20 for each cell type/treatment for each experiment; One-way ANOVA with Tukey post-test for B and Two-way ANOVA with Dunnett post-test for D; ***, $p<0.001$.)

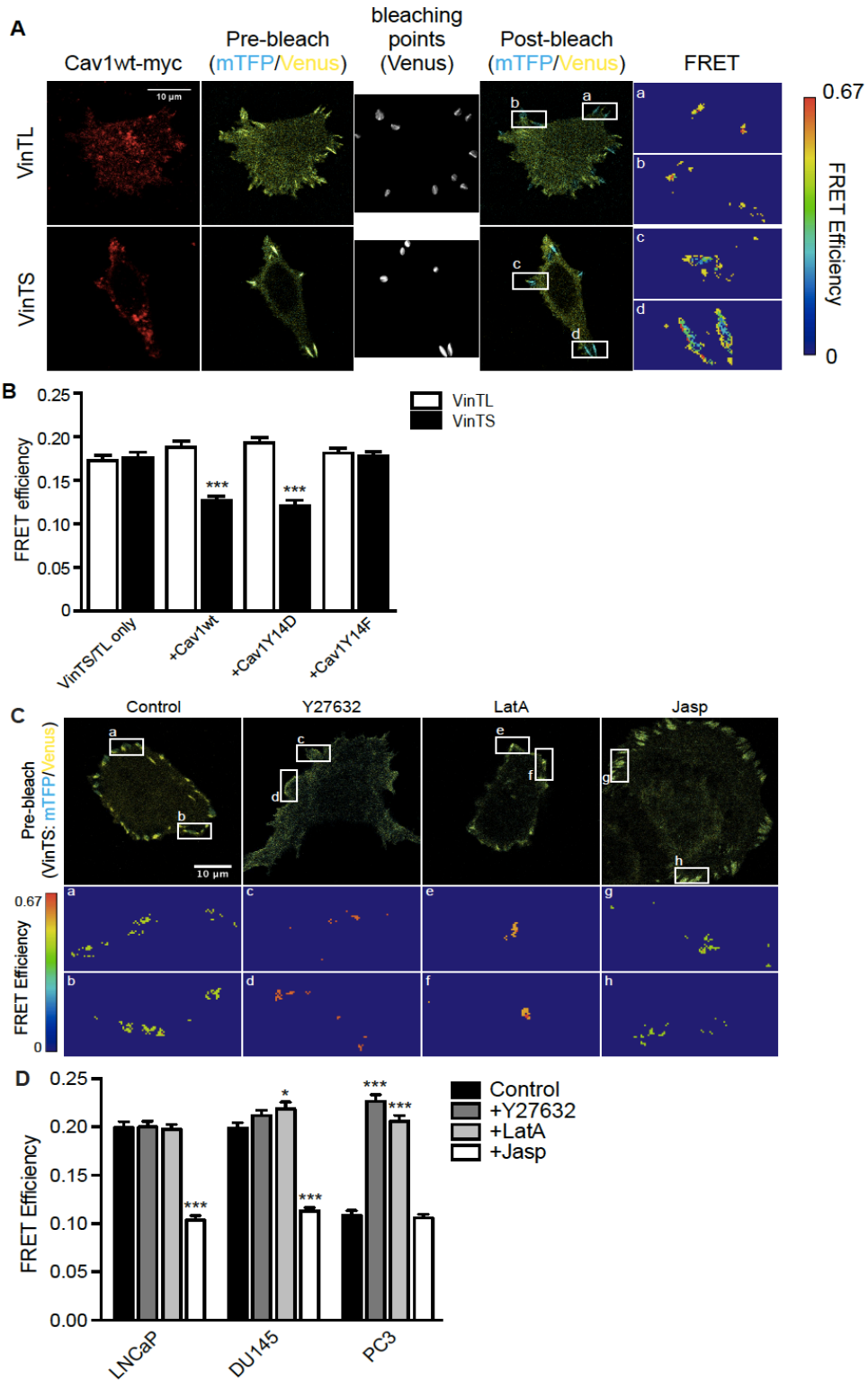
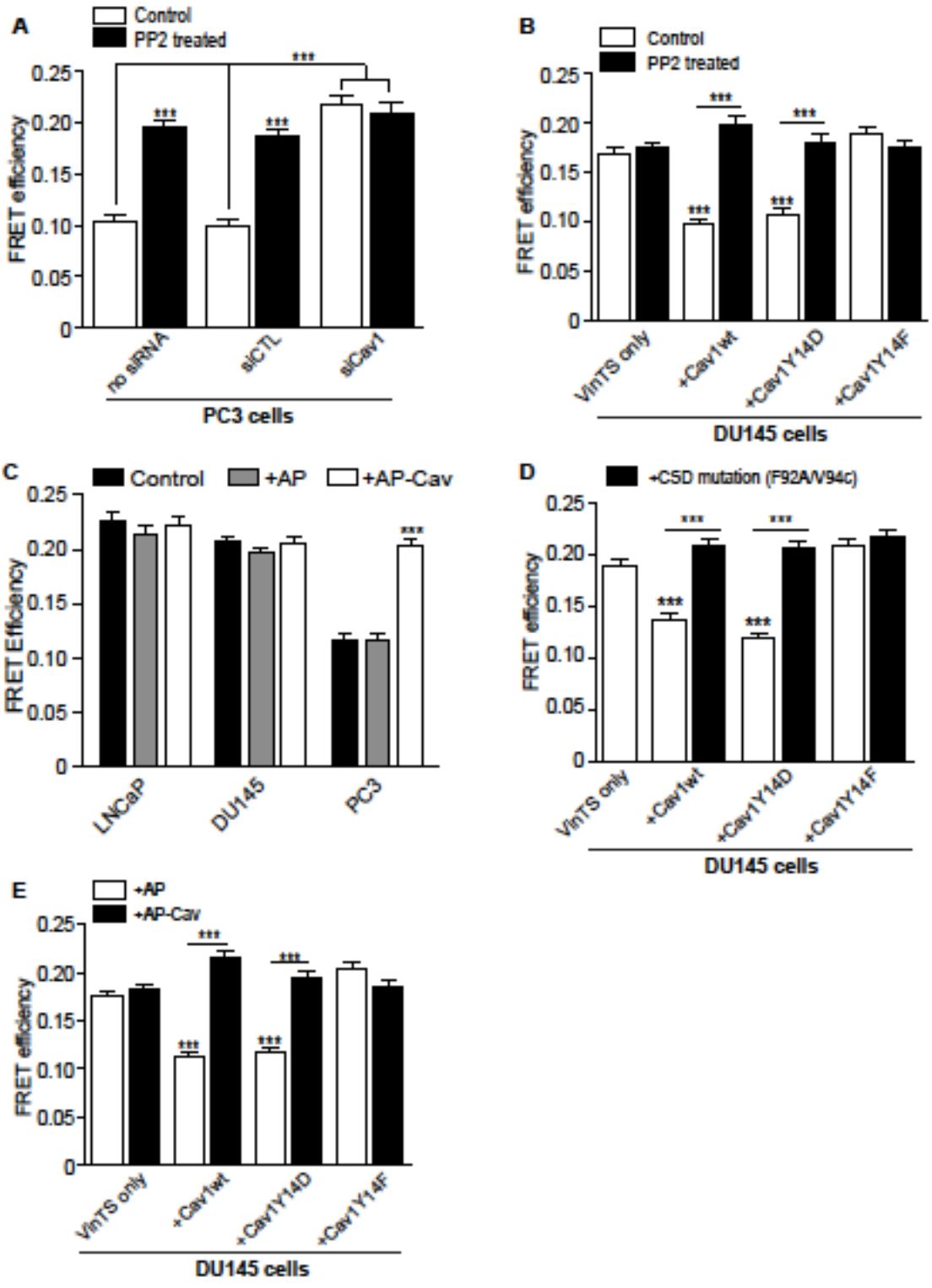

A
Cav1wt-myc
Pre-bleach (mTFP/Venus)
bleaching points (Venus)
Post-bleach (mTFP/Venus)
FRET
VinTL
VinTS
10 µm
0.67
FRET Efficiency
0
B
VinTL
VinTS
FRET efficiency
0.25
0.20
0.15
0.10
0.05
0
***
***
VinTS/TL only
+Cav1wt
+Cav1Y14D
+Cav1Y14F
C
Control
Y27632
LatA
Jasp
Pre-bleach (VinTS: mTFP/Venus)
10 µm
FRET Efficiency
0.67
0
D
FRET Efficiency
0.25
0.20
0.15
0.10
0.05
0.00
Control
+Y27632
+LatA
+Jasp
***
*
***
***
***
LNCaP
DU145
PC3

Figure 4-5. pY14Cav1 induces vinculin tension in a CSD-dependent manner.

(A) Quantification of FRET efficiency of VinTS in focal adhesions of siRNA transfected PC3 cells (no siRNA, siCTL or siCav1) and/or PP2 treatment. (B) Quantification of FRET efficiency of VinTS of DU145 Cav1 transfectants as indicated, untreated (control) or treated with PP2. (C) Quantification of VinTS FRET efficiency in focal adhesions of LNCaP, DU145 and PC3 cells, untreated (Control) or treated with AP/AP-Cav as indicated. (D, E) Quantification of FRET efficiency of VinTS of variously transfected DU145 cells as indicated, untreated (D) or treated with AP/AP-Cav for 6 h (E). Data represents mean±SEM of three independent experiments. (n>20 for each cell type/treatment for each experiment; One-way ANOVA with Tukey post-test; ***, $p<0.001$).


A
Control
PP2 treated
FRET efficiency
0.25
0.20
0.15
0.10
0.05
0
***
no siRNA
siCTL
siCav1
PC3 cells
B
Control
PP2 treated
FRET efficiency
VinTS only
+Cav1wt
+Cav1Y14D
+Cav1Y14F
DU145 cells
C
Control
+AP
+AP-Cav
FRET Efficiency
LNCaP
DU145
PC3
D
+C5D mutation (F92A/V94c)
FRET efficiency
VinTS only
+Cav1wt
+Cav1Y14D
+Cav1Y14F
DU145 cells
E
+AP
+AP-Cav
FRET efficiency
VinTS only
+Cav1wt
+Cav1Y14D
+Cav1Y14F
DU145 cells

CHAPTER 5 Discussion

The work in this thesis has demonstrated regulation of focal adhesions and tumor cell migration by Cav1 through multiple mechanisms including cooperating with the Gal3/galectin lattice, functioning through specific membrane domains, i.e. Cav1 scaffolds, and functioning through interaction between the pY14 and CSD domain of Cav1. Specifically, we found: (1) a synergistic expression and function of Cav1 and Gal3 in malignant thyroid cancer cells that regulates Rho activation, focal adhesion dynamics and cell migration; (2) a reduction of cell migration through affecting focal adhesion dynamics by PTRF/cavin-1 overexpression and caveolae formation in PC3 prostate cancer cells, which is overridden by reinforced Cav1-Gal3/galectin lattice function through additional Gal3; and (3) the CSD-dependent pY14Cav1 regulation of focal adhesion dynamics and tension and cell migration through interaction with focal adhesion proteins. My results have provided further understanding on the regulation mechanisms of Cav1 function – through co-effector Gal3/galectin lattice, through different membrane microdomains and through its molecular domain functions and interactions - and on Cav1 regulation of focal adhesion dynamics and tension in metastatic tumor cells. In this chapter, I will: 1) discuss the multiple mechanisms of Cav1 function regulation and their integration; 2) present an integrated model for Cav1 regulation of focal adhesion dynamics and tension; and finally, 3) discuss insights into cancer progression obtained through the presented works.

5.1 Multiple mechanisms of Cav1 function regulation

In this section I will discuss the multiple mechanisms demonstrated in the presented works for regulation of Cav1 functions in mediating focal adhesion dynamics and tumor cell migration, and how these mechanisms interact and integrate to regulate Cav1 function.

5.1.1 Regulation of Cav1 function through different Cav1 microdomains

Cav1 forms different types of membrane microdomains and the differential functions of these Cav1 microdomains have been implicated in studies of various cancer cells (Goetz et al., 2008b; Moon et al., 2014). PC3, an aggressively metastatic prostate cancer cell line that expresses Cav1 endogenously but not PTRF/cavin-1 nor caveolae, offers a great cell model for studying functions of the differential Cav1 microdomains on the cell surface. Previous studies have found that overexpression of PTRF/cavin-1 drives formation of caveolae and attenuated functions of non-caveolar Cav1 microdomains (Cav1 scaffolds), reducing cell polarity and motility and delaying cancer progression and metastasis (Aung et al., 2011; Hill et al., 2008; Hill et al., 2012; Moon et al., 2013). Further study of such alteration of Cav1 microdomains revealed that it inhibits angiogenesis and lymphangiogenesis of tumors formed by these cells (Nassar et al., 2013b); and that it affects the rearrangement of actin cytoskeleton and cholesterol distribution to alter the secretome and content of cell-derived extracellular vesicles (EVs), which further impairs reformation of the cancer metastases niche (Inder et al., 2014; Inder et al., 2012). These all indicate the pro-cancer properties of Cav1 scaffolds but not caveolae.

In Chapter 3, our findings using the same cell line further the understanding of how different Cav1 microdomains regulate cancer cell migration and cancer metastasis. Indeed, we found that attenuation of non-caveolar Cav1 scaffolds through formation of caveolae by PTRF/cavin-1 expression reduces stabilization of FAK within focal adhesions, suggesting Cav1 scaffolds regulation of cancer cell motility is mediated through changes to focal adhesion dynamics. We thus demonstrate a role for non-caveolar Cav1 scaffolds in increasing cancer cell motility through promoting focal adhesion dynamics. Our findings suggest that possible targeting of Cav1 as therapy for cancer should consider such roles of these Cav1 microdomains.

Studies have shown that caveolae and Cav1 scaffolds have different physical charateristics. Caveolae are well defined with their consistent invaginated morphology and size (Parton and Del Pozo, 2013; Yamada, 1955). Biogenesis and recycle of caveolae with key coat proteins caveolins and cavins and assisting proteins EHD2 and Pacsin2 have been well studied, and so have the functions of caveolae in multiple cellular activities (Goetz et al., 2008b; Parton and Del Pozo, 2013). Non-caveolar Cav1 scaffolds have been found as functional microdomains in cholesterol-rich rafts formed by oligomerized Cav1 that exhibit immobility and inhibitory functions on cell surface receptor signaling (Hill et al., 2008; Lajoie et al., 2009a; Lajoie et al., 2007a). Cav1 scaffolds are smaller structures compared to caveolae and are proposed to correlate to the SDS-stable Cav1 oligomer of ~15 Cav1 molecules, which are shown to have impact on the lipid raft proteome (Lajoie et al., 2009a; Lajoie et al., 2007a; Monier et al., 1995; Zheng et al., 2011). However, better ways to distinguish Cav1 scaffolds and caveolae need to be developed, given the limited resolution of confocal and conventional fluorescence microscopies, and the poor antigenicity and incompatibility of electron microscopy with living specimens. Stimulated emission depletion (STED) microscopy, a super-resolution microscopic technique, has clearly shown comparative characteristics of Cav1 scaffolds and caveolae in size and distribution (Zheng et al., 2011). Further studies applying super-resolution microscopies can promise good characterization of these Cav1 microdomains and better ways to distinguish them.

5.1.2 Regulation of Cav1 function through structural motif interaction

Within the Cav1 protein, both Y14 phosphorylation and the CSD domain are involved in Cav1 signaling interaction with specific partners. Cav1 is a major Src tyrosine phosphorylated substrate and its phosphorylated Y14 facilitates interaction with SH2-containing proteins (Glenney, 1989; Lee et al., 2000; Li et al., 1996b). The CSD domain of Cav1 has long been shown to be an important domain responsible for the signaling and

interaction of Cav1 with both upstream receptors and downstream effector molecules (Goetz et al., 2008b; Li et al., 1996a; Li et al., 1995). Recently, the functionality of the CSD domain has been reassessed for its high proximity with cell membrane and low accessibility to binding partners (Ariotti et al., 2015; Byrne et al., 2012; Collins et al., 2012). However, Cav1 Y14 phosphorylation is shown to induce conformational changes that increase the spacing between Cav1 molecules and may extend the CSD from the membrane, and therefore alter the accessibility of the CSD to facilitate its interaction with other molecules (Shajahan et al., 2012; Zimnicka et al., 2016). Indeed, our study in Chapter 4 shows a novel functional interaction between Cav1 Y14 phosphorylation and the CSD that regulates prostate cancer cell migration, whereby the CSD is required for pY14Cav1 regulation of focal adhesion dynamics and tension, while Y14 phosphorylation enhances Cav1 interaction with focal adhesion proteins. Taken together, Cav1 Y14 phosphorylation represents a key regulator of Cav1 signaling that controls not only SH2-based interactions but also the CSD-dependent interactions and signaling.

Other than inducing conformational changes, Cav1 Y14 phosphorylation may also regulate Cav1 signaling through compartmentalization. pY14Cav1 has been shown to be enriched at cellular protrusions and focal adhesions (Beardsley et al., 2005; Lee et al., 2000). Such focal adhesion localization of pY14Cav1 was challenged as the monoclonal anti-pY14Cav1 antibody was shown to cross-react with phospho-paxillin (Hill et al., 2007). Nevertheless, immunofluorescent imaging with a cross-absorbed polyclonal anti-pY14Cav1 antibody still showed the leading-edge localization of pY14Cav1 (Nomura and Fujimoto, 1999). Consistently, a phospho-mimetic Cav1Y14D construct exhibited a preferential localization at the leading edge compared to dominant negative Cav1Y14F mutant, by live cell time-lapse imaging (Joshi et al., 2008a). Indeed, our findings in Chapter 4 showed that the phospho-mimetic Y14D mutant of the GST-Cav1 construct had robustly higher binding affinity to

multiple focal adhesion components compared to non-phosphorylated Y14F mutant, which also suggests the close proximity of pY14Cav1 with cell leading edge and focal adhesions. Thus our study also supports the pY14Cav1 localization at focal adhesions. Such localization of pY14Cav1 indicates a Y14 phosphorylation (pY14) regulation of Cav1 signaling by compartmentalization to cell periphery and adhesions to facilitate pY14 and CSD interaction with specific partners, e.g. focal adhesion proteins, and trigger correlated signaling. A high-specificity and high-resolution detection of endogenous pY14Cav1 should be done in future to further demonstrate and characterize the regulation of Cav1 compartmentalization by pY14.

5.1.3 Gal3/galectin lattice regulation of Cav1 function

Cav1 plays complex roles in tumor progression and has been shown to be both a tumor suppressor and promoter in a cancer type- and progression phase-dependent manner. The CAV1 gene has been localized to the D7S522 locus, q31.1–q31.2 region of chromosome 7, a tumor suppressor region that is often deleted in many human cancer types; meanwhile, Cav1 is a poor prognostic marker in various types of cancer and promotes tumor growth and metastasis (Goetz et al., 2008b; Williams and Lisanti, 2005). Hence, Cav1 is considered to be a conditional tumor suppressor; one way to regulate its suppressor-promoter functions is by interaction with a co-effector, e.g. the Gal3/galectin lattice (Goetz et al., 2008b). Indeed, the galectin lattice overrides Cav1 negative regulation of growth factor signaling, i.e. EGFR signaling, allowing EGFR signaling to activate Cav1 Y14 phosphorylation and lead to consequent Rho activation and actin reorganization, as well as pY14Cav1-galectin lattice concerted regulation of focal adhesion dynamics and cell migration (Boscher and Nabi, 2013; Goetz et al., 2008a; Lajoie et al., 2007a). Our data in Chapters 2 and 3 both showed the Gal3/galectin lattice regulation of Cav1 functions in different cancer types. On one hand, as shown in Chapter 2, Cav1 and Gal3 are expressed synergistically in malignant differentiated

thyroid cancer cells and both - not either one alone - are required and sufficient to promote Rho activation, focal adhesion dynamics and cell migration, facilitating malignancy of the cancer. On the other hand, as shown in Chapter 3, Gal3 overrides PTRF/cavin-1 down-regulation of pY14Cav1 functions, promoting focal adhesion dynamics and cell migration, possibly through reinforced pY14Cav1-Gal3/galectin lattice concerted function. Consistently, previous studies have found that the Gal3/galectin lattice enables an outside-in integrin signaling that stimulates Cav1 phosphorylation and Rho activation, leading to pY14Cav1-Gal3/galectin lattice co-function in regulating focal adhesion dynamics, Rho activation and cell migration (Boscher and Nabi, 2013; Goetz et al., 2008a). Such a Gal3-integrin-Cav1 signaling provides a possible working model for Cav1-Gal3/galectin lattice interaction (Boscher and Nabi, 2013). These studies and our findings in Chapters 2 and 3 demonstrate an important role for Gal3/galectin lattice in regulating Cav1 function, which possibly serves as a functional switch for Cav1 to facilitate cancer progression and is required for Cav1 function in cancer progression.

5.1.4 An integrated multi-mechanism regulation of Cav1 functions

Our findings in Chapters 2, 3 and 4 introduced multiple mechanisms for the regulation of Cav1 functions – through the co-effector Gal3/galectin lattice, through formation of various membrane microdomains and through molecular domains of Cav1 and their modification. Cav1 plays complex roles during cancer progression and it is very possible that all these mechanisms are used to regulate Cav1 functions that promote tumor growth and further metastasis, i.e. an interactive integration of these mechanisms in Cav1 function. Indeed, interactions of these mechanisms have been demonstrated. Mgat5-deficient, and therefore galectin lattice-depleted, mouse mammary tumor cells show a deficiency in caveolae as well, which further alters proteome and signaling at lipid raft areas on the cell membrane (Lajoie et al., 2007a; Zheng et al., 2011). Gal3/galectin lattice overrides the functional changes

caused by the PTRF/cavin-1 expression and caveolae formation, as shown by our study in chapter 3. These studies suggest that Gal3/galectin lattice has an impact on the formation and signaling of Cav1 microdomains. Gal3/galectin lattice also has an influence on Cav1 molecule modification – the galectin lattice facilitates EGFR signaling to stimulate Cav1 Y14 phosphorylation, and co-functions with pY14Cav1 to induce Rho activation and promote actin reorganization, focal adhesion dynamics, ECM remodeling and cell migration (Boscher and Nabi, 2013; Goetz et al., 2008a). Cav1 Y14 phosphorylation, induced by EGF signaling and Src kinase, has been shown to induce the formation of membrane caveolae, and increase caveolae internalization by increasing the spacing between Cav1 molecules leading to caveolae swelling and binding to cytoskeleton proteins (Aoki et al., 1999; Orlichenko et al., 2006; Zimnicka et al., 2016). This suggests that pY14Cav1 is a regulator of caveolae formation and internalization. Consistently, pY14Cav1 promotes Cav1 expression and caveolae formation in response to tension in metastatic tumor cells (Joshi et al., 2012), demonstrating an impact of Cav1 domain modification on Cav1 membrane microdomain regulation of Cav1 functions. Cav1 microdomains, on the other hand, influence pY14Cav1-dependent functions. Induction of caveolae by PTRF/cavin-1 in caveolae-deficient PC3 prostate cancer cells reduced cell polarity and motility (Aung et al., 2011; Hill et al., 2012). In Chapter 3, we showed that such changes in Cav1 microdomains decreased cell motility by disrupting pY14Cav1-dependent focal adhesion dynamics, indicating that pY14Cav1 may need to function through Cav1 scaffolds while introduction of caveolae by overexpressing PTRF/cavin-1 diminished such pY14Cav1 function possibly by compartmentalizing pY14Cav1 to caveolae with less accessibility to focal adhesions. Future studies should investigate how Cav1 Y14 phosphorylation and differential Cav1 microdomains may influence the Gal3/galectin lattice, which will further our understanding of the integrated multi-mechanism regulation of Cav1 functions.

5.2 The relationship between tension and stabilization/dynamics of focal adhesions

In Chapter 4, we showed that focal adhesion tension, represented by vinculin tension, correlated with the stabilization of the focal adhesion components FAK and vinculin, in cells with various Cav1 expression profiles. The less mobile Y397 phosphorylated FAK forms a FAK-Src signaling complex at focal adhesion sites that further recruits focal adhesion adaptor proteins such as paxillin and Crk to promote focal adhesion dynamics and cell migration (Giannone et al., 2004; Mitra and Schlaepfer, 2006; Webb et al., 2004). Meanwhile, mechanical force has been shown to be an important regulator of focal adhesion maturation and dynamics. Directional force is proposed to conformationally change focal adhesion proteins and unmask their binding sites for newly recruited proteins, leading to focal adhesion growth and reinforced ECM-cytoskeleton linkages (Vogel and Sheetz, 2006). Indeed, mechanical force generated by both Rho/ROCK-mediated actomyosin contractility and ECM rigidity promotes the formation and growth of small adhesion-associated actomyosin bundles that recruit the adaptor proteins vinculin and zyxin (Gardel et al., 2010). Such force also induces integrin β_1 binding to ECM, which stimulates recruitment and activation of FAK leading to FAK-Src signaling and recruitment of further focal adhesion components as described above (Gardel et al., 2010). Consistent with this, our lab previously showed a diminished stabilization of FAK within focal adhesions upon disruption of intracellular tension by ROCK inhibitor Y27632 (Joshi et al., 2008a).

Cytoplasmic focal adhesion adaptor protein vinculin is proposed to be closely involved in focal adhesion tension-induced signaling since it locates between the talin-integrin complex and the actin cytoskeleton and interacts with both (Cohen et al., 2006; del Rio et al., 2009; Kanchanawong et al., 2010; Ziegler et al., 2006). Tension has been shown to play an interactive role in recruitment and signaling of vinculin itself. A constitutively active vinculin

mutant shows less turnover from focal adhesions and leads to enlarged focal adhesions, a phenomenon normally seen in a high-tension environment (Balaban et al., 2001; Cohen et al., 2006; Humphries et al., 2007). Tension, on the other hand, is required for maintaining vinculin in focal adhesions, since tension-disrupted cells lose vinculin from focal adhesions and stop further recruitment of vinculin to focal adhesions (Carisey et al., 2013). Nevertheless, the stabilized active vinculin activates integrins and stabilizes other focal adhesion proteins within focal adhesions (Carisey et al., 2013), again linking tension to dynamics of focal adhesions.

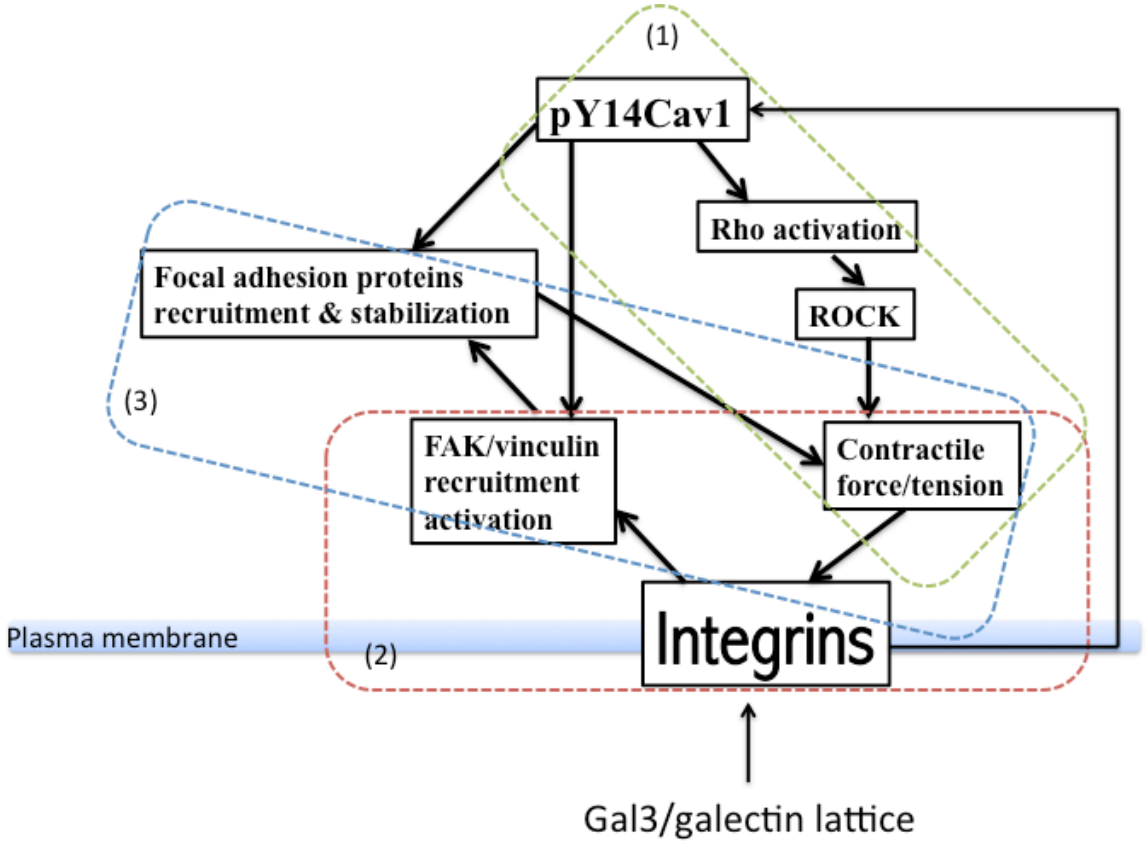
It is reasonable to consider that tension and dynamic growth of focal adhesions have an interactive relationship. A recent study on the relation between cell adhesion size and tension, using live cell imaging with FRET-base vinculin tension sensor (VinTS), found a plastic relationship between the size and tension of cell adhesions: positive and negative correlations appear alternatively and repeatedly within the life time of adhesion complexes (Hernandez-Varas et al., 2015). Also, initial changes in adhesion complexes often start with the size change and subsequently greater changes are led by tension alteration (Hernandez-Varas et al., 2015). These data suggest a complex interaction between tension and the dynamics of focal adhesions. A live-cell, time-lapse analysis of vinculin tension in focal adhesions, applying FRET assay of VinTS in live-cell time-lapse imaging and analysis of spatiotemporal distribution of the vinculin tension, should be able to resolve our findings in Chapter 4 with more details.

Cav1, especially pY14Cav1, stimulates Rho activation leading to cell polarization and contraction (Goetz et al., 2011; Grande-Garcia et al., 2007; Joshi et al., 2008a). Together with our data in Chapter 4 showing correlated regulation of focal adhesion protein stabilization and focal adhesion tension, a possible model for pY14Cav1 regulating focal adhesion dynamics and tension can be proposed: 1) pY14Cav1 induce cellular tension

through Rho activation; 2) tension promotes integrin activation, recruitment of other focal adhesion proteins, and stabilization and activation of vinculin and FAK, which is facilitated by pY14Cav1 as well; 3) stabilized active vinculin and FAK recruit and stabilize further focal adhesion components leading to reinforced focal adhesion architecture and tension transmission (Figure 5-1). Intriguingly, our lab has previously shown that pY14Cav1 is a tension sensor that transmits tension signaling that increases Cav1, and thus pY14Cav1, expression levels (Joshi et al., 2012). In combination with our findings in Chapter 4, it defines a positive feedback loop between tension and pY14Cav1 levels, adding another dimension to the signaling network of pY14Cav1 regulation of focal adhesion dynamics and tension.

Figure 5-1. Integrated regulation of focal adhesion dynamics and tension by pY14Cav1 and Gal3/galectin lattice.

Three-step pY14Cav1 regulation of focal adhesion dynamics and tension involves: (1) pY14Cav1 promotes Rho activation leading to actin contractile force through ROCK (green dashed box); (2) intracellular contraction-derived mechanical tension induces integrin activation leading to recruitment and activation of FAK and vinculin to cell adhesion sites, which is also facilitated by pY14Cav1 (red dashed box); (3) active FAK and vinculin recruit further focal adhesion proteins and promote focal adhesion growth, leading to reinforced ECM-cytoskeleton linkages and tension transmission (blue dashed box). Gal3/galectin lattice is also involved in such regulation network by facilitating integrin outside-in signaling that stimulate Cav1 phosphorylation.



5.3 Insights into cancer progression

Transformation and progression of cancer involve many cellular changes to achieve self-sufficient growth signaling, unlimited growth and proliferation, angiogenesis abilities, and tissue invasion and metastasis, namely the hallmarks of cancer (Hanahan and Weinberg, 2000). In the presented works, we found that cancer cells adapt various strategies, changing Cav1 and Gal3 expression levels, compartmentalization and molecular modification, to facilitate the transformation and progression of cancer.

Although commonly depleted in benign tumors, Cav1 and Gal3 are shown to be required for malignant tumors in various cancer types (Ellerhorst et al., 1999; Goetz et al., 2008b; Williams and Lisanti, 2005; Xu et al., 1995). In Chapter 2 we showed synergistic expression of Cav1 and Gal3 in malignant differentiated thyroid cancer cells but not in benign hyperplasia and that both Cav1 and Gal3 are required for the increased cell motility of the malignant thyroid cancer cells. Similar regulation is also present in prostate cancer, where both Cav1 and Gal3 expression is lost in benign adenoma and less aggressive prostate tumor cells but is present and increases with malignancy and metastasis (Ellerhorst et al., 1999; Gould et al., 2010). These results suggest both Cav1 and Gal3 as biomarkers showing diagnosis for malignancy and poor prognosis with possible metastases in various cancer types including thyroid cancer and prostate cancer.

Specific compartmentalization of Cav1 and Gal3 is implicated in cancer progression. One example comes from studies of metastatic prostate cancer cell line PC3, which is PTRF/cavin-1- and caveolae-deficient (Gould et al., 2010). Cav1 in this cell line function predominantly through non-caveolar Cav1 scaffolds while an introduction of caveolae by PTRF/cavin-1 overexpression attenuates the non-caveolar Cav1 function and consequently reduces cell motility, alters raft dynamics and secretome and inhibits tumor progression and

angiogenesis (Aung et al., 2011; Inder et al., 2012; Moon et al., 2013; Nassar et al., 2013b). This suggests that aggressive prostate cancer cells may preferentially compartmentalize Cav1 to Cav1 scaffolds in order to facilitate these various functions in cancer progression. Our findings in Chapter 3 demonstrated that the PTRF/cavin-1 induction of caveolae in PC3 cells decreased cell migration through affecting focal adhesion dynamics, identifying a role for non-caveolar Cav1 scaffolds in regulating focal adhesion dynamics. Studies have also found Cav1 in prostate cancer patient serum and PC3 cell-secreted prostasomes, small vesicles normally secreted by the prostate gland, facilitating prostate tumor progression (Llorente et al., 2004; Tahir et al., 2001). Similarly, specific compartmentalization of Gal3 is also reported in prostate cancer. Gal3 expression in aggressive prostate cancer cells gets excluded from nucleus, with cytoplasmic Gal3 regulating cell survival and proliferation (Califice et al., 2004; Fukumori et al., 2006; van den Brule et al., 2000). Meanwhile, cell surface Gal3/galectin lattice is required for aggressive prostate cancer metastasis (Glinskii et al., 2012; Wang et al., 2009a). Gal3 is also found in prostasomes and facilitates the bone metastases of prostate cancer (Kovak et al., 2013; Nakajima et al., 2016). These studies suggest specific compartmentalization of Cav1 and Gal3 (e.g. Cav1 scaffolds, nucleus-excluded cytoplasmic Gal3, cell surface galectin lattice and prostasome) to be an important process of prostate cancer progression, which can be potentially evaluated as biomarkers for aggressive prostate cancer.

Specific molecular modifications of Cav1 and Gal3 are also found to facilitate cancer progression. For example, Gal3 is modified in aggressive prostate cancer cells – cleaved Gal3 is found in prostasomes to slower the osteoclast differentiation and specifically reform the bone microenvironment facilitating the prostate tumor metastases (Nakajima et al., 2016). For Cav1, Y14 phosphorylation has been shown to actively promote cancer cell migration through Rho activation, focal adhesion dynamics, actin reorganization and ECM

remodeling, etc., which do not occur without Cav1 Y14 phosphorylation (Boscher and Nabi, 2013; Goetz et al., 2008b). With the assistance from Gal3/galectin lattice, growth factor signaling induces Cav1 phosphorylation, which further facilitates Cav1 functions promoting cell migration (Boscher and Nabi, 2013; Lee et al., 2000). Our data in Chapter 4 showed that pY14 of Cav1 interacts with the CSD domain functionally leading to promoted focal adhesion dynamics and tension and cell motility, demonstrating an important role for the CSD domain in mediating pY14Cav1-dependent functions. Both an F92A/V94A mutation and a CSD mimicking peptide AP-Cav can efficiently inhibit the pY14Cav1-dependent focal adhesion dynamics and tension and consequent cell migration. Indeed, application of such CSD mimicking peptide as anti-cancer therapy has been proposed (Williams and Lisanti, 2005), which has been shown to block angiogenesis and tumor progression *in vivo* (Bucci et al., 2000; Gratton et al., 2003). Our findings in Chapter 4, that pY14Cav1 induces focal adhesion tension, are consistent with previous studies showing pY14Cav1 activates Rho activation (Boscher and Nabi, 2013; Goetz et al., 2011; Joshi et al., 2008a). Together with previous findings that pY14Cav1 drives further expression and phosphorylation of Cav1 in response to tension (Joshi et al., 2012), it possibly defines a positive feedback loop between tension and pY14Cav1, adding another mechanism for cancer cells to regulate Cav1 phosphorylation. Given the role for caveolae as membrane buffers to release tension (Sinha et al., 2011), caveolae may also function as a mediator for the feedback loop between pY14Cav1 and tension to reach a balanced status and to stabilize pY14Cav1 levels. These studies demonstrate the important roles for modification of Cav1 and Gal3, especially Cav1 Y14 phosphorylation, in cancer progression. Potential treatments to inhibit Cav1 phosphorylation or pY14Cav1 functions (e.g. CSD mimicking peptides) should be evaluated for cancer therapeutics.

5.4 Final remarks

The works in this thesis demonstrate multiple mechanisms for the regulation of Cav1 function in tumor cells facilitating cancer progression. Cav1 is expressed synergistically with its co-effector Gal3 in malignant thyroid cancer cells and both are required to regulate focal adhesion dynamics and cell migration, emphasizing the Cav1-Gal3/galectin lattice co-function in cancer cells. The mechanism for this co-function between Cav1 and Gal3/galectin lattice remains to be further understood. Switch of Cav1 microdomains from Cav1 scaffolds to caveolae induced by PTRF/cavin-1 expression in PC3 prostate cancer cells reduces cell motility through affecting focal adhesion dynamics while the effects can be overridden by additional Gal3; this demonstrates that microdomain alteration of Cav1 function can be overridden by reinforced Cav1-Gal3/galectin lattice functions. Further work on how PTRF/cavin-1 overexpression and Cav1 microdomain switch lead to attenuated focal adhesion dynamics and cell migration is required. Finally, a newly demonstrated functional interaction between Y14 phosphorylation and the CSD domain of Cav1 was shown to regulate focal adhesion dynamics and tension in metastatic prostate cancer cells, showing a mechanism for regulation of Cav1 functions through its domain interaction. In summary, these works make a significant contribution to understanding the multiple Cav1 functions in cancer progression and provide valuable views for developing therapeutic targets for cancer.

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APPENDICES: Supplementary Data

Figure A-1. Characterization of stably Cav1 transfected DU145 cell lines.

Representative confocal images of DU145 and the stably transfected DU145 cells labeled with anti-FAK antibody and phalloidin to show the morphology, the distribution of the mRFP-tagged Cav1 constructs and the expression of focal adhesions.

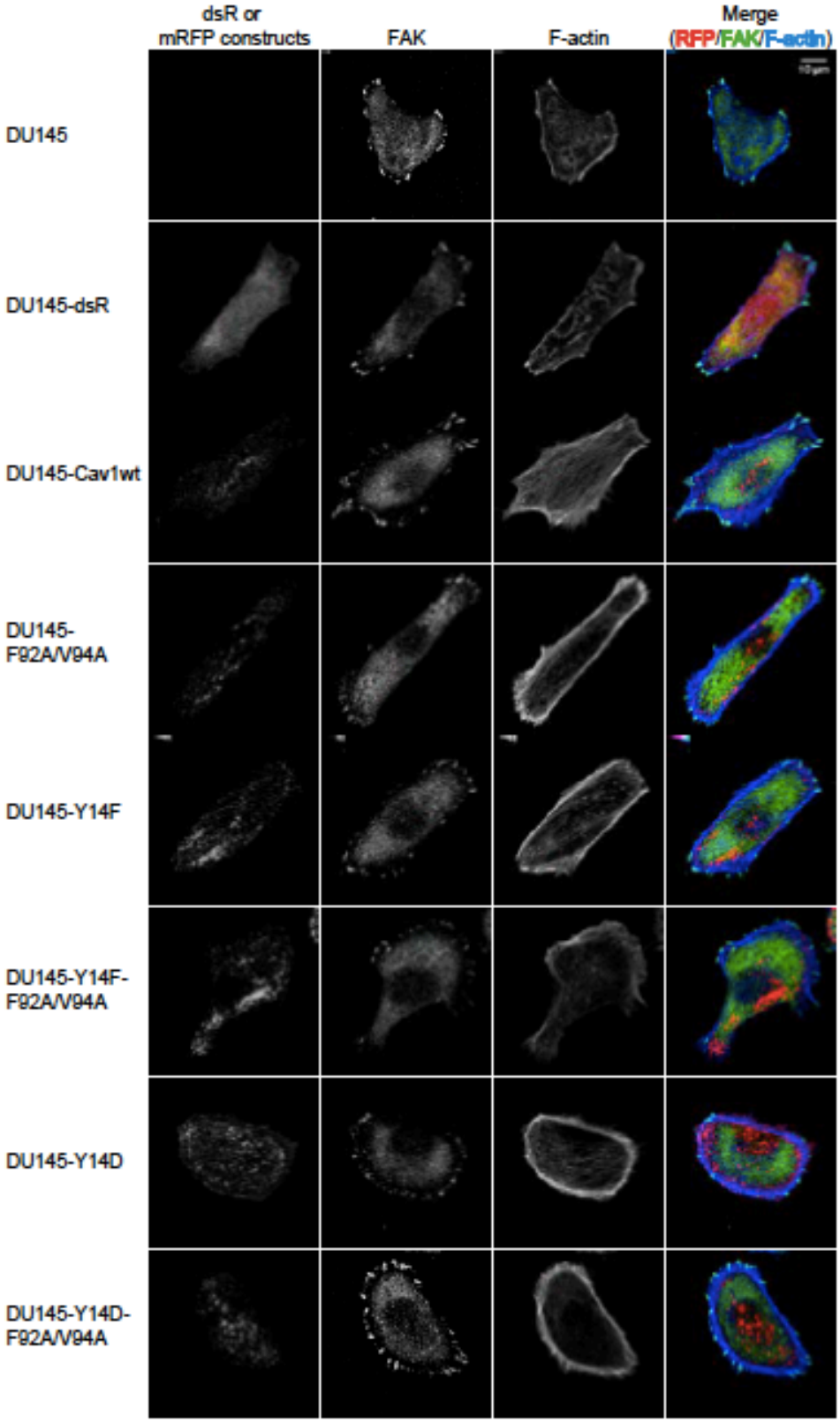
dsR or mRFP constructs
FAK
F-actin
Merge (RFP/FAK/F-actin)
DU145
DU145-dsR
DU145-Cav1wt
DU145-F92A/V94A
DU145-Y14F
DU145-Y14F-F92A/V94A
DU145-Y14D
DU145-Y14D-F92A/V94A

Figure A-2. Transiently transfected DU145 cells validating FAK-GFP FRAP data of stably transfected DU145 cells.

DU145 cells were transiently transfected with FAK-GFP and the indicated Cav1-mRFP mutant constructs. Cells expressing similar intensity levels of mRFP were analyzed by FRAP for FAK-GFP in focal adhesions. Graphs show FAK-GFP mobile fractions and average mRFP intensity of cells analyzed. (Data represent mean±SEM of three independent experiments. n>10 for each experiment for the FAK-GFP mobile fraction graph and n>4 for each experiment for the mRFP intensity graph. One-way ANOVA with Tukey post-test; ***, p<0.001.)

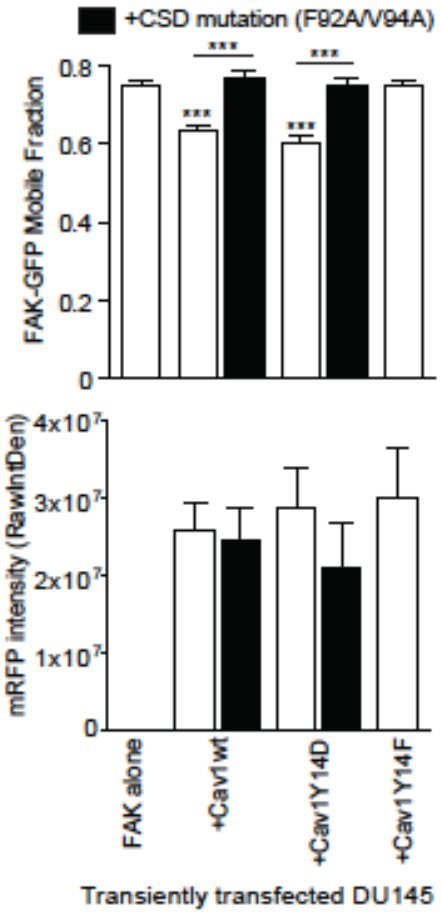

Figure A-3. Cav1 knockdown in PC3 cells.

Western blot of Cav1 and β-actin of PC3 cells with no siRNA transfection or transfected with control siRNA (siCTL) or siRNA targeting Cav1 (siCav1).