REGULATION OF TUMOR CELL METASTASIS BY CD151

By

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DEDICATION

To my family thank you for instilling in me the value of hard work and going after your dreams. To my mom, thank you for always supporting me and sacrificing to make sure that growing up I never went without, even if it meant you had to.

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Matise LA., Palmer TD., Ashby, WJ., Nashabi A., Chytil A., Aakre M., Pickup MW., Zijlstra A., Moses, HL., (2012). Lack of TGF-β signaling promotes collective cell invasion in tumor-stromal crosstalk. *Breast Cancer Research:* 14(4):R98.

Palmer, TD., Ashby, WJ., Lewis, JD., and Zijlstra, A. , (2011). Targeting tumor cell motility to prevent metastasis. *Advanced Drug Delivery Reviews:* 63(8):568–581 **Chapter 2 of this thesis**

Palmer, TD., Zijlstra, A. (2011) CD151. *USCD-Nature Molecule Pages.* (doi:10.1038/mp.aa004123.01) **Chapter 3 of this thesis**

Palmer, TD,, Lewis, JD., and Zijlstra A. (2011) Quantitative analysis of cancer metastasis using an avian embryo model. *Journal of visualized experiments*: (51), e2815.

LIST OF ABBREVIATIONS

ALCAM	Activated Leukocyte Cell Adhesion Molecule
Arp2/3	Actin Regulatory Protein 2/3
CAM	Chorioallantoic Membrane
CXCL-4	CXC Chemokine Ligand 4
DARC	Duffy Antigen Related Chemokine
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
FAK	Focal Adhesion Kinase
GAP	GTPase Activating Protein
GEF	Guanine Nucleotide Exchange Factor
HEp3	Human Epidermoid Carcinoma Cell Line 3
HGF	Hepatocyte Growth Factor
HNSCC	Head and Neck Small Squamous Cell Carcinoma
Ig-SF	Immunoglobulin Superfamily
ITGA3	Integrin Alpha 3
LEL	Large Extracellular Loop
MAb	Monoclonal Antibody
miRNA	microRNA
MMP	Matrix Metalloproteinase
PCa	Prostate Cancer
PETA-3	Platelet Endothelial Antigen-3
PKC	Protein Kinase C
SDF-1	Stromal Derived Factor-1

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEL	Small Extracellular Loop
SPARC	Secreted Protein Acidic and Rich in Cysteine
TERM	Tetraspanin Enriched Microdomain
TGFβ	Transforming Growth Factor Beta
WAVE	Wiskott Aldrich Syndrome Protein (WASP) Family Protein

REGULATION OF TUMOR CELL METASTASIS BY CD151

TRENIS PALMER

Dissertation under the direction of Professor Andries Zijlstra

Our lab has demonstrated that clustering of the tetraspanin CD151 on the surface of tumor cells inhibits cell migration and cancer metastasis. As a member of the tetraspanin superfamily CD151 associates with a number of proteins on the cell surface and functions as a molecular scaffolding protein when clustered into protein rich complexes known as Tetraspanin Enriched Microdomains (TERM). Treatment of tumor cells with the anti-CD151 monoclonal antibody (MAb) 1A5 promotes the localization of CD151 to the areas of cell-cell contact to promote adhesion and inhibit migration and metastasis through TERM formation. Due to the published associations with the laminin binding integrins (primarily α3β1), combined with the ability of CD151 to regulate α3β1-mediated adhesion, we hypothesized that it would be involved in the inhibition of migration and metastasis mediated by the clustering of CD151. We present data demonstrating that 1A5 recognizes the integrin-association epitope of CD151 and binds to integrin free CD151 ($CD151^{free}$). Additionally, we demonstrate that the expression of integrin α3 is not required for the ability of CD151 to control motility. We found the expression of $CD151^{free}$ increasingly present in the tumor tissue of patients with aggressive prostate cancer furthermore, $CD151^{free}$ staining functions an independent prognostic indicator of survival. The demonstration that α3β1 is not functionally involved in the regulation of migration by CD151 suggests that there are functions of CD151 related to motility that are independent of integrin associations. By proteomic analysis we elucidated the components of the CD151TERM complex and pulled down ALCAM/CD166. We demonstrate that ALCAM is required for the inhibition of migration as a component of the CD151-TERM complex. Mechanistically, the CD151/ALCAM immobility complex signals through Protein Kinase

Cα to activate the small GTPase Rap1A to control motility. Ultimately, sustained activation of Rap1 is sufficient to inhibit metastasis. This thesis defines the role of CD151 as a molecular integrator of migration and regulator of metastasis through its ability to control both cell-cell and cell-matrix adhesion.

CHAPTER I

INTRODUCTION

Tumor Cell Metastasis

Metastasis is the primary cause of cancer-related mortality. While we have made significant advances in the treatment of cancer, there are few if any treatments designed to target metastatic disease. Derived from the Greek word meaning displacement or movement, metastasis is traditionally defined as the spread of cancer cells from one organ to another non-adjacent organ or tissue. This is an overly simplistic definition but it offers a foundation for exactly what metastatic disease: a disease of mis-regulated cell motility. There are two principles reasons why treating metastasis has proved difficult: 1) the complexity of the metastatic process summarized in Figure 1,(1,2) and 2) the overall inefficiency of metastasis, for which it has been suggested that less than <0.001% of all circulating tumor cells give rise to secondary metastases(3). The process of metastasis is a cyclic process and each sequential step of the cascade must be completed in order for a tumor cell to form a metastatic lesion in a distant site. It begins with departure of cells from the primary tumor, a process that can be motivated by a number of factors including but not limited to the availability of oxygen and nutrient in the tumor microenvironment, space constraints mediated by rapid growth of the tumor and soluble factors that are able to serve as chemoattractants at the secondary site. Following successful departure from the primary tumor, disseminating tumors cells must be able to enter the vasculature (intravasate) and survive transport through the vascular system. Those cells that are able to successfully persist in the vasculature, be able to arrest in a capillary bed,and subsequently extravasate at the secondary site. Finally, the secondary site must be conducive to tumor growth before the disseminating tumor cells can establish a-

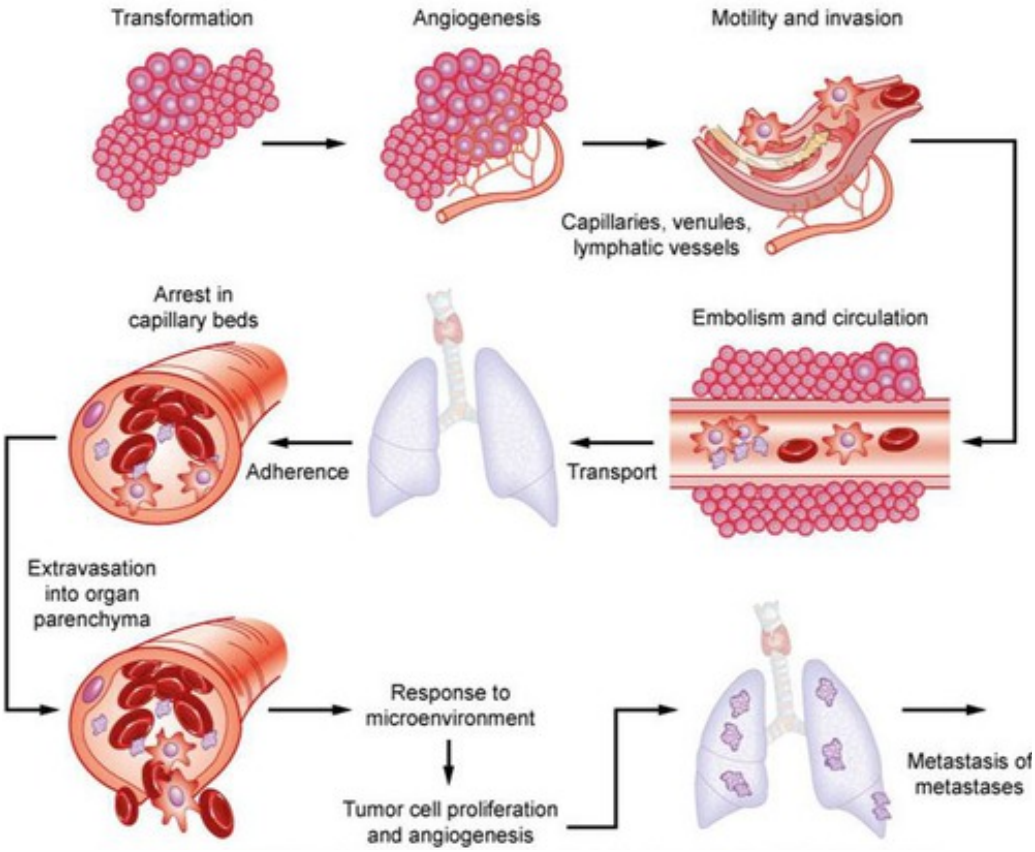

Fig.1. Cancer metastasis requires a complex series of events. Cancer cells must gain access to blood and lymphatic vessels, survive the trip to distant locations, move back into the tissues and initiate a new tumor. Because each of these steps is required the successful metastatic cell is rare compared with the huge numbers of cancer cells at the primary site. Consequently, metastasis usually only occurs late in cancer evolution. This figure modified and adapted from (2).

metastatic colony of tumor cells. Each step in the metastatic cascade requires unique properties not present in every tumor cell. The absence of specific traits or the inability to adapt leads to the elimination of tumor cells at each sequential step in this cascade. Although the disease is clearly pathological, the regulation of cell migration is inherent in the biology of normal cells. during non-pathological processes such as development and wound healing. This is demonstrated experimentally as many of the same processes that regulate the motility of normal cells are also involved in the acquisition of the motile metastatic phenotype of tumor cells. Therefore, we cannot discuss metastasis without an understanding of the process of cell migration in normal cells.

The Process of Cell Migration

Cell migration is required for development, wound healing, proper immune system function, and tumor cell metastasis . The steps of the migration cycle depicted in figure 2 can be summarized in five steps: 1). protrusion at the front of the cell 2). adherence to the substrate at the front of the cell which generates tractional force across the cell body 3). cell surface proteolysis of cell to matrix proteins 4). contraction of the cell body and 5). the release of cell-substrate adhesion at the rear of the cell(4). Although this is a very simplistic view of the process of cell motility like the metastatic cascade it is a highly coordinated process and in order for a cell to become motile the process must be completed successfully. Normal cells such as those that maintain the structural integrity of organs and those involved in maintaining epithelial cell barriers would prefer to stay immobile. Similarly, tumor cells would also prefer to remain non-motile. In normal and tumor cells the non-motile phenotype is primarily controlled by the expression of cell to cell adhesion molecules like E-cadherin. E is an important protein

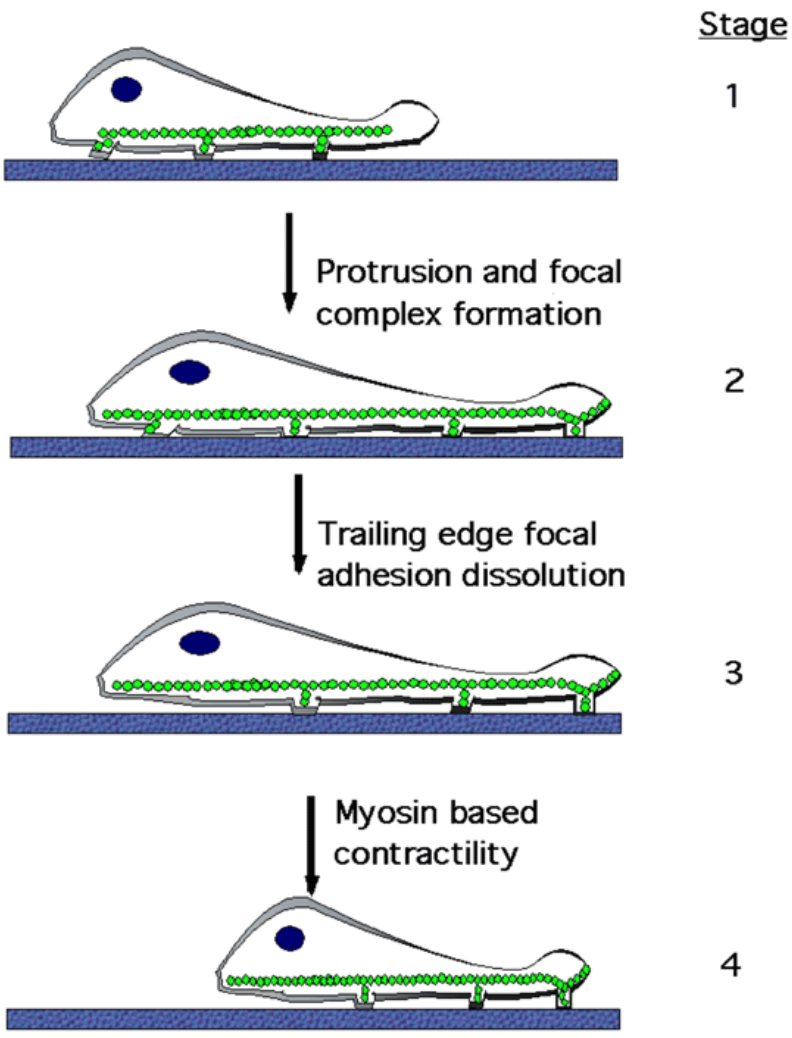

Fig. 2. Generalized model of Cell Motility. The steps in the cell motility cycle can be divided into steps which include 1). protrusion 2). adhesion to the substrate 3). cell surface proteolysis 4). contraction of the cell body and 5) de-adhesion at the rear cell which completes the migration cycle. This figure modified and adapted from (4).

the processes and development and metastasis. Its expression regulates cohesive nature of epithelial cells and their ability to respond to pro-migratory stimuli. While much of what we know about cell motility has been generated from analyses of single cell migration, this thesis will primarily focus on the migration of epithelial cells that are engaged in cell-cell interactions.

The Epithelial Mesenchymal Transition and Metastasis

For most tumors the progression toward metastatic behavior involves digression away from its original epithelial behavior and gain a certain level of phenotypic plasticity. This plasticity can occur through de- or trans-differentiation which can be accomplished by undergoing a cellar program of Epithelial-to-Mesenchymal cell Transition (EMT). EMT is primarily associated with the loss or mis-localization of the epithelial cadherin (E-cadherin). E-cadherin is highly expressed at epithelial cell-cell junctions and is involved in the maintenance of epithelial cell architecture. During embryonic development E-cadherin is expression is necessary for a number of processes including gastrulation and organ morphogenesis(5). It was initially characterized as a “invasion suppressor” because its loss is routinely associated with the promotion of tumor cell motility and invasion(6). The loss of E-cadherin is generally considered an early step in the progression to metastatic disease and in patient samples of invasive/metastatic tumors E-cadherin expression is either loss or mis-localized further supporting the role of E-cadherin as a regulator of metastasis (7,8). Although E-cadherin is a prominent example of a protein that regulates the motility of normal cells, there are many cell adhesion molecules involved in the process of tumor cell metastasis.

Involvement of Cell Motility Processes in Metastasis

Investigations into the phenomenon of EMT and an analysis of the cellular behaviors associated with the loss of E-cadherin provide clues to suggest that motility is involved in cancer cell metastasis. One of the major consequences associated with EMT

is an increase in cell motility. Additionally a wealth of experimental data generated both *in vitro* and *in vivo* has demonstrated that the expression of motility-related genes in cancer cells is sufficient to drive metastasis in many cell lines and tumor models(9). In the following sections this concept will be supported by expounding upon the steps involved in migration while also making connections to the overall metastatic process.

Cell protrusion and re-arrangement of the actin cytoskeleton

The initial steps involved in the regulation of motility are associated with re-arrangement of the actin cytoskeleton. Examples of proteins involved in this process include Arp 2/3, cortactin, and N-WASP. Re-arrangement of the actin cytoskeleton is primarily associated with the formation of two actin rich structures filopodia and lamellapodia, two structures with well defined functions. Filipodia formation is involved in the sensing of extracellular cues and surveillance of the extracellular microenvironment. Lamellipodia are thought to be the actual motors that pull the cell along the substratum. Arp2/3 is a seven subunit protein complex that serves as a site for actin nucleation and is believed to be important for regulating protrusive events at the front of the cell. It can be activated by a number of proteins including Wiskott Aldrich Syndrome Proteins (WASP) and cortactin. Arp2/3 activity and expression is involved in the regulation of fibroblast migration and-expression of Arp 2 and 3 is sufficient to promote the migration of gastric cancer cells(10). Arp2/3 expression is up-regulated in many tumors including; breast,colorectal, and lung. Additionally, the knockdown of Arp2/3 is sufficient to inhibit cancer cell invasiveness (11). Cortactin is an Arp2/3 binding protein routinely up-regulated in head and neck small squamous cell carcinoma (HNSCC), breast and lung cancer. It is involved in the formation of invadapodia, actin-rich structures involved in extracellular matrix degradation. Phosphorylation of cortactin is thought to be required for its ability to regulate migration. The WASP family of proteins also bind Arp2/3 binding protein and activate the complex in response to signals that induce migration. The family

consists of five proteins all of which are involved in some aspect of tumor cell motility at the front of the cell (12). One member of the family N-WASP, is decreased in metastatic breast cancer and is inversely correlated with poor patient outcome.

Cell-extracellular matrix interactions

Cell-ECM interactions stabilize newly synthesized protrusions at the leading edge of the migrating cell**.** These interactions with the matrix are largely controlled adhesion receptors such as members of the integrin family. Integrins are heterodimeric cell surface receptors consisting of an α and β subunit and, upon activation, are able to bind to their cognate matrix ligands and subsequently control adhesion. In addition to their abilities to control migration integrins by modulating cell-to-cell and cell-to-matrix adhesion(13,14), they are also able to control changes in morphology, survival and proliferation. They are abundantly expressed in numerous cells and tissues in all organisms while some cells express multiple integrins. Integrin activation is regulated by two principle mechanisms: 1). outside in activation and 2). inside out activation. Outside-in integrin activation is primarily associated with integrin binding to its extracellular matrix ligand. ECM binding promotes signal transduction mechanisms related to changes in cell morphology and migration. Epithelial cells express a number of different integrins on the surface including α2β1, α6β4, α3β1 and αvβ3 (14,15). The expression of these integrins have been analyzed in different human cancers including: breast, melanoma , and ovarian. While the phenotypes associated with their expression may vary, they are generally implicated in the regulation of invasive disease processes.

Cell-cell adhesion

As mentioned earlier, cell-cell adhesion is important for maintaining epithelial integrity and also for controlling tumor cell escape from the primary tumor. Cell-to-cell adhesion can be mediated by three classes of adhesion receptors: cadherins, integrins and member of the immunoglobulin super-family (IgG-Sf)(16). Members of the IgG-Sf have been implicated in the progression of a number of cancers including: melanoma, pros-

tate, ovarian, colon and breast. Members of this family include ALCAM, VCAM and L1-CAM(17). Structurally IgG proteins are characterized by the presence of at least one N-terminal extracellular IgG-like domain and a very short C-terminal tail(18). They can form two types of interactions on the cell surface homotypic and heterotypic. Homotypic interactions involve the association of the IgG-SF protein with itself on adjacent cells while, heterotypic interactions occur between IgG-SF and known ligands While E-cadherin is routinely loss IgSF members are routinely upregulated especially in migrating sheets of tumor cells. The up-regulation of IgSF proteins is especially important in the progression of metastasis and colonization at the secondary site.

Detachment and de-adhesion at the rear of the celll

The mechanisms associated with detachment at the rear of the cell and rear retraction remain poorly understood. It has been suggested that rear detachment is primarily due to to integrin inactivation and subsequent endocytosis. Furthermore, the loss of integrin interaction with the actin cytoskeleton is potentially involved as well. Although the process is not completely understood some of the proteins involved are known. In migrating fibroblasts the endocytosis of αvβ3 integrin appears to be involved. Additionally, in some cell types Rho/Rock activity is believed to be required. Myosin IIa has been demonstrated to act downstream of the Rho/ROCK activity to regulate actinomyosin contraction. The calpain family of cell surface proteases are responsible for the cleavage of a number of cell surface proteins that make up the integrin adhesive networks including Talin and FAK to regulate de-adhesion at the rear of the cell.

Adhesion controls migration and metastasis

The loss of cell-cell adhesion in epithelial derived cancers can ultimately determine whether or not a tumor cell becomes motile and departs the primary tumor. Independent of other pro-migratory factors in the tumor microenvironment the loss of cell-cell contact is required. An in-depth analysis of the metastatic cascade demonstrates that at various steps in the cascade the promotion or inhibition of cell-cell adhesion could be

important .This fact has not been demonstrated any more clearly than with experiments focusing on the loss or over-expression of E-cadherin in tumor cells. Equally important are the cell specific interactions with the extracellular matrix. The matrix can be both supportive and inhibitory to the migration and the metastasis of tumor cells. The role of adhesion to adjacent cells and to the matrix as a tumor cell progresses through the metastatic cascade is depicted in Figure 3.While some adhesion is important, too much adhesion to the matrix can inhibit cell motility.The balance of adhesion must be properly regulated such that it is optimal. This fact is depicted in the bell-shaped curve of cell motility (Fig 4) which demonstrates that in instances where neither minimal adhesion nor maximal adhesion facilitates motility(19). Indeed, migration is maximal when a dynamic optimal adhesion has been accomplished. Overall, adhesion is a key factor in the regulation of cell motility and tumor cell metastasis, although there maybe some debate as to how important individual adhesive mechanisms are to the overall process of metastasis. In this thesis I will introduce the idea that the cell surface protein CD151 can control numerous aspects of adhesion and regulate migration and metastasis by connecting two seemingly divergent molecules to function as a molecular integrator of cell migration.

The Tetraspanin CD151

CD151, a member of the tetraspanin family was initially isolated as a platelet cell surface antigen. It is expressed in a number of cells and tissues in addition to platelets including epithelial cells, endothelial cells, and megakaryocytes(20,21). CD151 is considered a metastasis promoter gene. It is routinely up-regulated at the invasive front of migratory cells and its expression is able to promote cell motility. In a mouse model of spontaneous prostate cancer development on a CD151 null background metastasis is diminished further demonstrating that the expression of CD151 promotes metastasis. As a member of the tetraspanin family CD151 interacts with a number of proteins on the cell surface forming macromolecular cell surface complexes known as Tetraspanin Enriched Microdomains (TERMS). The most well-known interactions involve the laminin binding

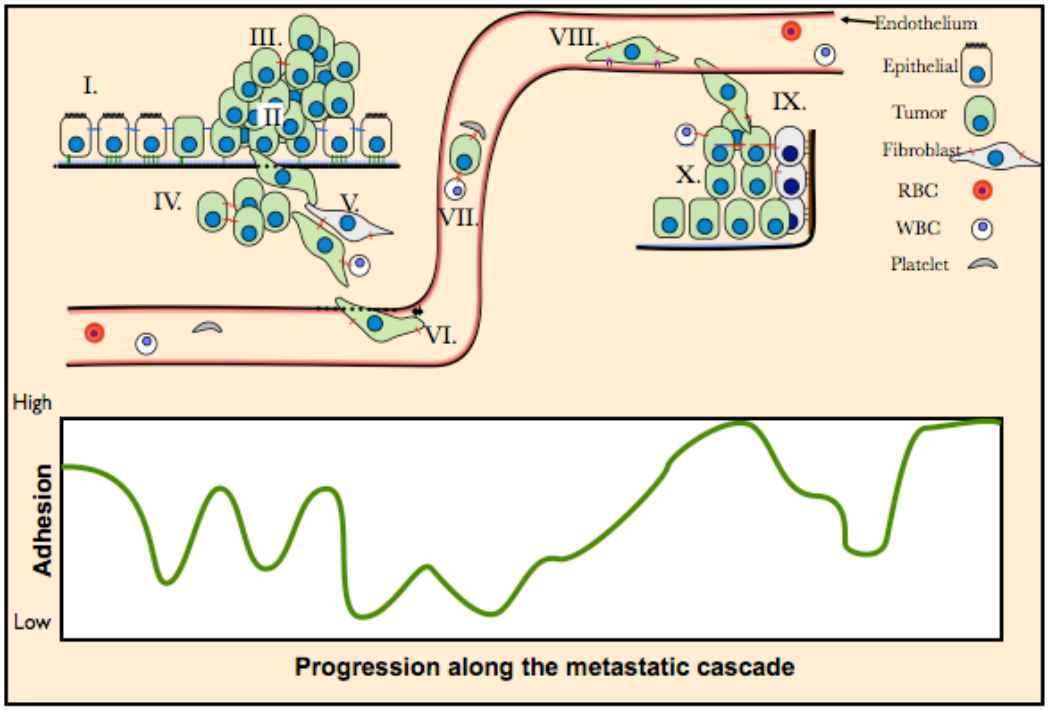

Fig.3.Cell-cell adhesion in the metastatic cascade: I). Adhesion molecules stabilize the interactions between normal cells, II). Tumors cells adhere to each other and normal cells in the microenvironment III). Cell to cell interactions in the tumor continue to be relatively high. IV). During the process of matrix invasion and degradation adhesion is decreased to a certain extent in order to allow migration some cells leave as single cells, adhesion is maintained between sheets of migrating cells. V). Migrating tumor cells interact with fibroblasts which can generate tracks for tumor cells to migrate on VI).During intravasation adhesion is minimal although the tumor cells can adhere to the endothelium VII). During transit in the vasculature tumor cells can interact with other blood cells including white blood cells, platelets, and fibroblasts. Cell to cell adhesion is high. VIII). At the secondary site prior to extravasation the tumors arrest by adhering to the blood vessel wall. IX). During the process of extravasation adhesion is decreased in order to allow for the tumor cells to transendothelial migrate. X). In the secondary site adhesion cell to cell adhesion is increased in order to allow colonization and growth This figure created by Andries Zijlstra 2013.

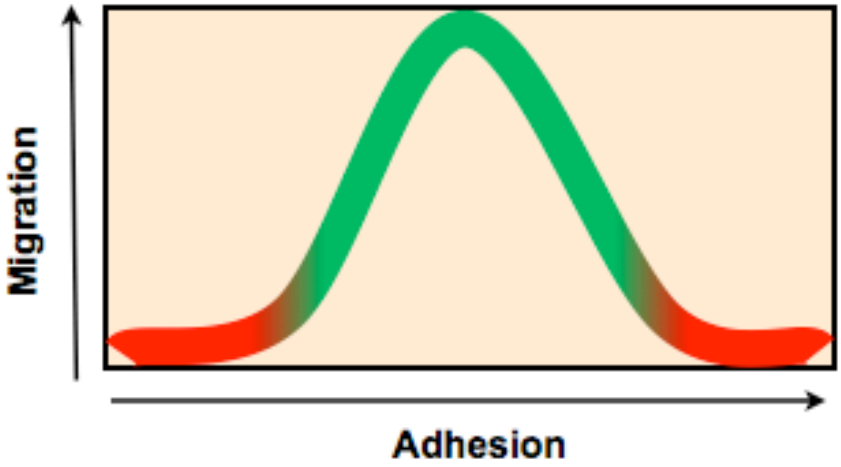

Fig. 4. Relationship between adhesion and migration potential. In order for tumor cells to migrate their must be a balance between adhesion and migration. Too little adhesion can inhibit migration but so can too much adhesion This figure modified and adapted from (19).

integrins α3β1 and α6β4. Through interactions with these proteins CD151 is involved in the regulation of cell-matrix adhesion and motility. The CD151 TERM complex contains a number of proteins in addition to the laminin binding integrins including: cadherins and immunoglobulin proteins(22). Recently, it was demonstrated that CD151 interacts with and regulates the function of the matrix metalloprotease MMP-14 ((23) demonstrating another role for CD151 in motility and migration by regulating matrix degradation.

Purpose of this study

The purpose of this study was two fold 1). to determine the molecular mechanisms associated with the regulation of motility and metastasis by clustering of CD151 and 2). determine the protein components of the CD151TERM complex on the cell surface in oder to elucidate the protein(s) involved in the regulation of motility and metastasis through association with CD151. Due to the published associations of CD151 with α3β1 I that it would be an attractive target, I further hypothesized that clustered CD151 associates with the integrin and through this association motility and metastasis are inhibited (Fig. 5). This thesis will also focus on the potential of targeting molecular mechanisms of motility in metastasis using CD151 as an example.

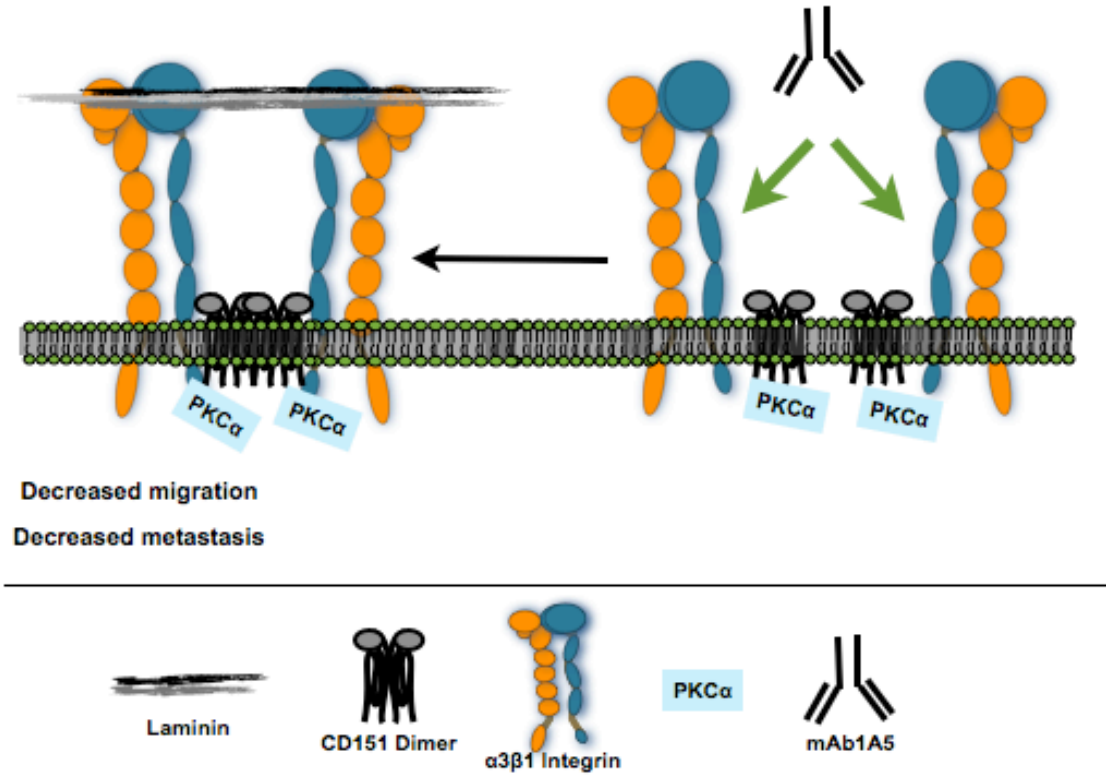

Fig. 5. Hypothesis: CD151 controls migration via the laminin binding integrins. On the tumor cell surface CD151 forms a complex with a3b1 integrin. I hypothesize that the ability of CD151 to inhibit migration and metastasis is due to its association with α3β1 integrin. Furthermore, I hypothesize that CD151 clustering of CD151 promotes its association with α3β1 integrin and facilitate adhesion to laminin.

Summary

We have previously demonstrated that a CD151-specific monoclonal antibody (1A5) can inhibit tumor cell migration *in vitro* as well as metastasis *in vivo* (24). It was subsequently demonstrated that this antibody inhibits motility and metastasis by preventing de-adhesion at the rear of the cell. Herein, I demonstrate that this antibody represents a class of antibodies that are specific for the integrin-binding site of CD151.

Their specificity for the integrin-binding site makes these antibodies specific for integrin-free CD151 ($CD151^{free}$). I demonstrated that $CD151^{free}$ is clustered by antibodies to the integrin-binding site which leads to a pro-adhesive state of the target cell. In a retrospective study I demonstrate that $CD151^{free}$ recognized by the 1A5 antibody has clinical significance in the progression of prostate cancer (Chapter 4). In chapter 5 I demonstrate that targeting $CD151^{free}$ promotes tumor cell adhesion and inhibit cell motility. I explored the mechanism of action by identifying partners of $CD151^{free}$ using the antibody 1A5 among which was the the novel CD151-associated protein Activated Leukocyte Cell Adhesion Molecule (ALCAM). Together these molecules are able to activate the GTPase Rap1A in a PKCα-dependent manner. Perpetual Rap1A activation is sufficient to prevent migration of tumor cells. Collectively, these data support the role of CD151 as a regulator of not only cell-cell adhesion but cell-matrix adhesion to inhibit tumor cell migration.

CHAPTER II

TARGETING CELL MOTILITY TO PREVENT METASTASIS*

Summary

Mortality and morbidity in patients with solid tumors invariably results from the disruption of normal biological function caused by the physical infiltration and destruction by disseminating tumor cells. Tumor cell migration is under intense investigation as the underlying cause of cancer metastasis. The need for tumor cell motility in the progression of metastasis has been established experimentally and is supported empirically by basic and clinical research implicating a large collection of migration-related genes. However, there are few clinical interventions designed to specifically target the motility of tumor cells and adjuvant therapy to specifically prevent cancer cell dissemination is severely limited. In an attempt to define motility targets suitable for treating metastasis, we have parsed the molecular determinants of tumor cell motility into five underlying principles including cell autonomous ability, soluble communication, cell-cell adhesion, cell-matrix adhesion, and the integration of the previous four on molecular scaffolds. The current challenge is to implement meaningful and sustainable inhibition of metastasis by developing clinically viable disruption of molecular targets that control these fundamental capabilities.

Introduction

Metastatic disease remains the primary cause for cancer-related deaths (25). Whether it is present at the time of diagnosis, develops during treatment, or occurs at the time of disease relapse the dissemination of tumor cells from the primary lesion is the principle reason for mortality and morbidity of cancer patients. Surgical resection of the primary lesion, along with cytotoxic and cytostatic systemic therapy

has been relatively successful in treating benign, localized cancer and preventing its progression to metastatic disease. Metastases however, remain difficult to treat and render the disease incurable. Paradoxically, the more effective cancer treatment is at prolonging life, the greater the risk of metastasis. To combat the risk for eventual metastasis, many patients are over-treated with the intent of preventing dissemination of their disease. Therapies that specifically target the motility of tumor cells could significantly improve cancer treatment by removing the threat of systemic disease and decreasing the dependency on therapeutics with detrimental side effects. For the past 5 decades the processes involved in tumor cell metastasis have been micro-dissected in an attempt to identify therapeutically viable targets. The central, defining process of metastatic disease is its ability to mobilize, invade, and cross normally non-permissive tissue barriers. This has greatly intensified the investigation into molecular mechanisms of motility and their contribution to metastasis (Fig. 6). Here, we provide an overview of these investigations and examine the potential for targeting of tumor cell motility in the treatment of metastasis.

The migration of adherent cells is defined as the translocation of cells from one location to another. Detailed discussion is available from the Cell Migration Consortium on the Cell Migration Gateway (www.cellmigration.org). Typically, migration is parsed into five component processes: polarization, protrusion, adhesion, translocation of the cell body, and retraction of the rear (Fig. 7, [2,(26). Although it is mechanistically convenient and sometimes necessary to define cell migration in this manner, the movement of cells within a living organism is highly complex, tightly regulated, and carefully coordinated. The physiology of cell migration is also very diverse. Some cell types such as activated hemopoietic cells exhibit a highly individualized “ameboid” movement with little adhesion and no matrix remodeling. Fibroblasts, and melanocytes generally migrate in a “mesenchymal” fashion as individual which are highly adherent and require

Annual Publications Evaluating the Role of Migration and Metastasis

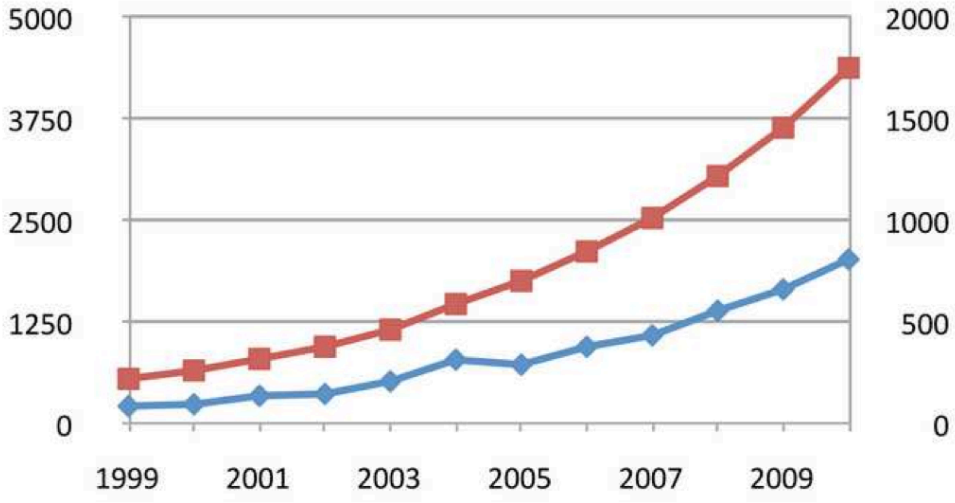

Fig.6. Publications on migration and metastasis: Total and annual number of Pubmed listed publications targeting migration and metastasis. www.pubmed.gov was searched for articles with the keywords "Migration" and "Metastasis" in the title or abstract. The data is presented as the number of publications/year (blue) and the cumulative number of articles in the field up to and including the indicated year.

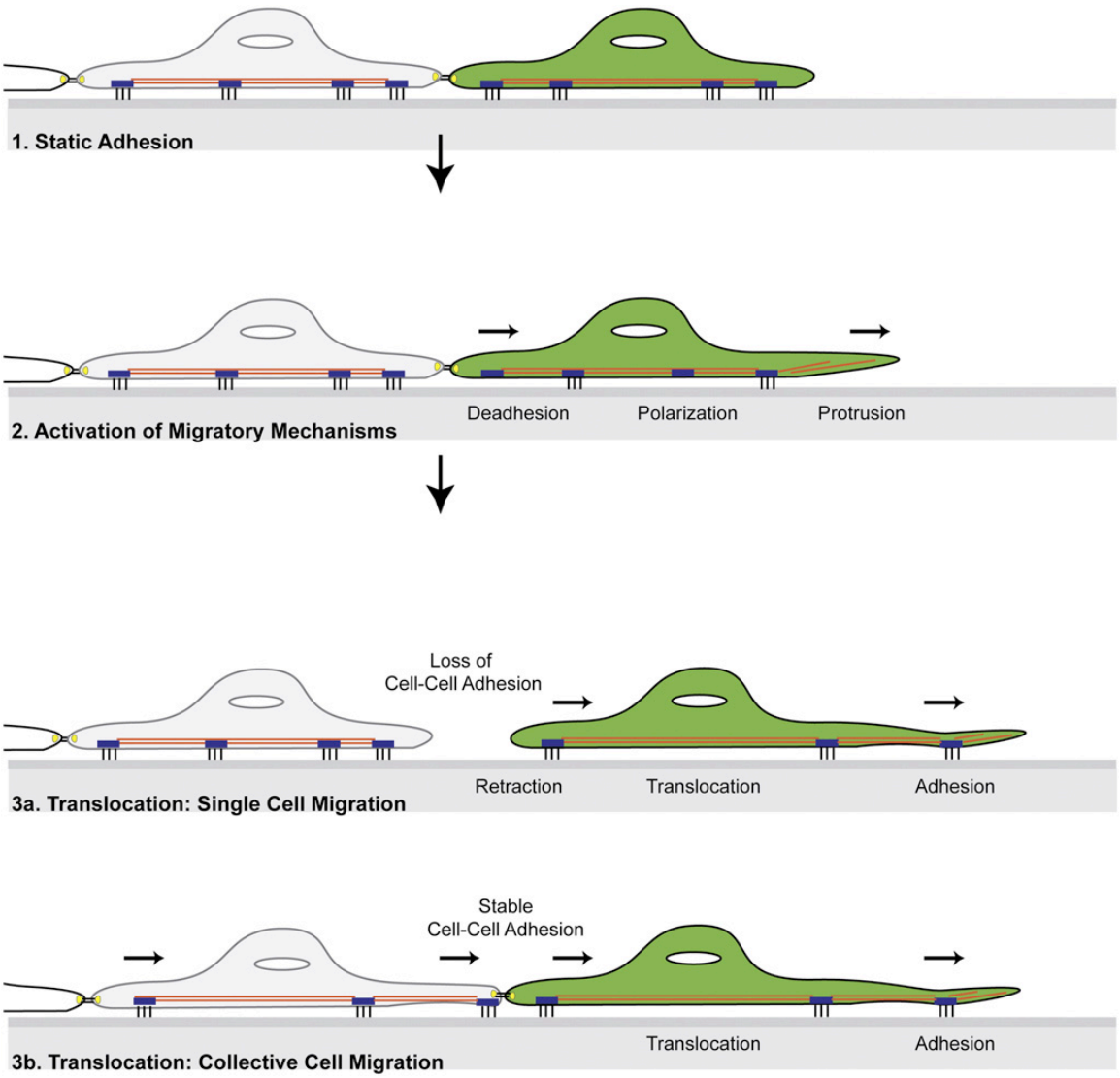

Fig. 7 Cell migration: A basic representation of cellular behavior during migration. Migration is parsed into five components processes: polarization, (de)adhesion, translocation of the cell body, and retraction of the rear. Successful motility can be accomplished as isolated, individual cells (3a) or collectively as a group of cells (3b). This figure modified and adapted from (1)

proteolytic remodeling of the matrix. The migration of neuronal and smooth muscle cells is collective, directionally coordinated, and mechanistically integrated. Epithelial cells, the cell type from which most cancers originate, can exhibit multiple migration phenotypes. While epithelial cells are generally present as stationary, tightly interconnected sheets of cells, they can be mobilized during development, physiological homeostasis, and wound repair. Depending on their developmental differentiation, environmental stimuli, and surrounding tissue architecture, epithelial cells can migrate as collective sheets, clusters, tubular structures, or as individual cells (reviewed by Friedl and colleagues in [4,(27) and Rørth et al. in (28). Interestingly, in patients with malignant disease, tumor cells are found as both individual cells and organized collective sheets or clusters, suggesting that tumor cells *in vivo* exhibit the plasticity to switch between single and collective cell migration.

Tumorigenesis is largely driven by the subversion of normal cellular processes that control cell proliferation and cell death. It is therefore not entirely surprising that molecular mechanisms that control cellular motility in normal physiology reappear in metastatic cancer. However, unlike normal migrating cells, metastatic tumor cells no longer respond to contact inhibition and are capable of crossing barriers that are non-permissive to normal cells.

Tumor cell motility is a therapeutically viable target for the treatment of metastasis

Despite the growing evidence implicating tumor cell motility in metastasis, there remains uncertainty about the viability of targeting motility with the intent of treating metastasis. There is evidence for both active and passive mechanisms of cancer dissemination (29,30). Further-more it has been suggested that the transient contribution of tumor cell motility to metastasis does not make it a suitable clinical target (29). These factors raise the question: can the inhibition of motility contribute therapeutically to the

treatment of metastatic cancer? Here we have synthesized data in the field and suggest that the answer is “yes”.

Experimental, empirical, and clinical findings indicate that therapies targeting motility would be effective at treating metastasis. In the clinic, much of the metastatic risk assessment is based on the potential for the cancer to mobilize and cross non-permissive tissue barriers. In basic research, the migration machinery has been shown to promote dissemination. Most importantly, there is a strong correlation between the molecular mechanisms of migration and the progression to systemic disease (31). Thus, the molecular mechanisms that promote tumor cell motility offer several inroads for novel drug design and clinical intervention designed to limit cancer progression towards overt metastasis and to treat existing metastatic disease. These include:

1. Anti-migration therapy may support active surveillance in the clinic. Cancer patients at risk for developing systemic disease are treated aggressively. This strategy is associated with high morbidity, poor quality of life, and elevated risk of treatment complications. Targeting motility could be implemented as a preventative measure to enable the physician to keep a patient under active surveillance without risking the appearance of systemic disease.

2. Targeting tumor cell motility within the primary tumor could limit local invasion. Invasive neoplasia, such as glioblastoma and pancreatic cancer aggressively infiltrate adjacent tissues and can be incurable even in the absence of overt metastasis to distant organs. Surgical intervention is frequently ineffective and disease often recurs. Targeting motility could improve therapy of these malignancies by preventing further infiltration and expansion into normal tissues.

3. Patients with overt metastases may benefit from treatments that block further dissemination. The paradigm suggesting that dissemination to the lymph nodes is the

first step in a metastatic cascade (32) has been challenged in the clinic by the absence of improved survival after removal of regional lymph nodes (33) In fact, it was demonstrated that occult micrometastases are not indicative of disease free survival (34). These observations suggest that the cells responsible for metastases have yet to be mobilized from the primary site and would therefore remain susceptible to anti-migration therapy.

4. Restricting tumor cell motility may limit “evolution” towards an increasingly metastatic phenotype. Tumor cells exhibit an epigenetic diversity and plasticity that contributes to the selective evolution of metastatic abilities. This is evident from the pro-motility gene expression profiles seen in metastatic cells (35) and the *in vivo* selection of tumor cells with increasing metastatic ability (36),(37). Once tumor cells disseminate away from the primary site, these two elements support the evolution and expansion of metastatic cells.

The role of motility in the evolution of a metastatic phenotype is supported by evidence of the primary tumor re-seeding itself with circulating tumor cells (38). Similarly, existing metastases can reseed to unaffected tissues expanding the burden of metastatic disease (reviewed extensively in (39). Preventing the dissemination of tumor cells may therefore limit the Darwinian evolution of the metastatic phenotype. Anti-motility strategies provide a unique mechanism to prevent the development of systemic disease and limit cancer-related death. Equally important, a successful anti-motility strategy may diminish the need for overly aggressive cytotoxic therapies currently used to avert the risk of metastatic dissemination.

Metastasis and the role of tumor cell motility

In order for a tumor cell to disseminate to a distant site, it must detach from the primary lesion, invade locally, and travel to a distant site where it can survive and proliferate (Fig. 8). Dissemination occurs via three avenues: 1) local invasion of normal tissues adjacent to the tumor, 2) infiltration of the draining lymphatic system, or 3) hematogenous metastasis through the vasculature (29). Although large numbers of circulating tumor cells can be detected in tumor-bearing animals, metastatic colonization is remarkably inefficient (37). The metastatic inefficiency of cancer cells is, in part, because successful metastatic dissemination requires the completion of each step in a complex sequence of events (Fig. 8). The interrelated and sequential nature of this metastatic cascade greatly diminish the probability that any single tumor cell will give rise to a metastatic lesion.

The inefficiency of metastasis has raised the question of whether tumor cells disseminate via passive or active migration-dependent mechanisms (29). Three basic principles appear to determine the efficiency by which clinically overt metastases are formed: 1) Tumor–host inter- actions: tumor–host interactions control metastatic progression as tumor cells depart the primary lesion and enter a new environment, 2) Tissue structure and biomechanics: tissue structure and the biomechanics of the vasculature determine the route and distribution of circulating tumor cells, and 3) Pro-metastatic molecular determinants: the acquisition of pro-metastatic molecular determinants previously absent in the primary tumor. The latter can be acquired through a transient epigenetic plasticity such as the epithelial–mesenchymal transition (EMT) (40,41) or an evolutionary selection of epigenetic and genetic changes. (35)

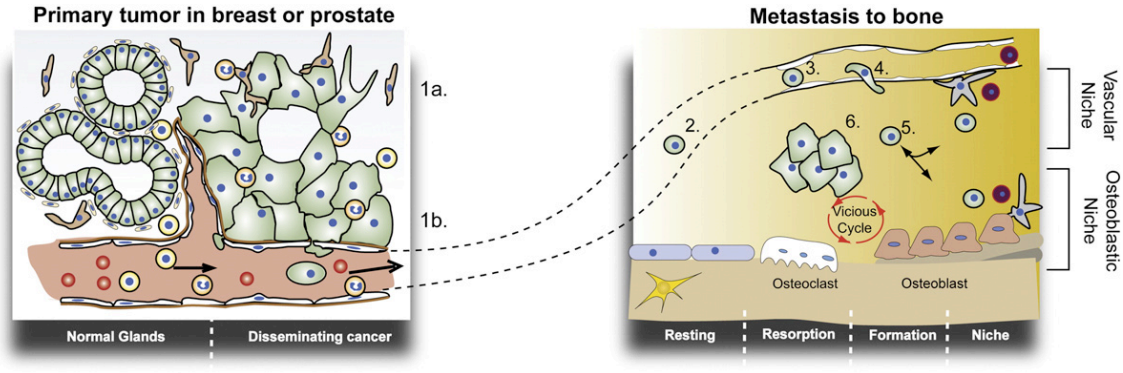

Fig.8. The metastatic cascade: Malignant tumor cells become mobilized and invade the local microenvironment (1a)and intravasate into the tumor vasculature (1b). Circulating tumor cells travel to a secondary tissue (bone) via the blood supply (2) and arrest in the venous supply of the recipient organ (3). Arrested tumor cells subsequently extravasate (4) and invade the bone microenvironment (5). Within the bone, tumor cells can enter a protective niche where it remains as occult or dormant disease. When the tumor cell engages a favorable microenvironment it can engage the osteoblasts and initiates feedforward loop (vicious cycle) that promotes growth and further infiltration of the bone microenvironment (6) This picture modified and adapted from (1).

Tumor–host interactions

In 1889, Paget hypothesized that disseminating tumor cells, which he called seeds, could reach most, if not all, organs but that successful metastasis was determined by selective growth in specific (the soil) (42). This became known as the “seed–to–soil” hypothesis. In subsequent years, extensive interrogation of tissue-specific metastasis validated this hypothesis (reviewed in depth by Weiss (43) and Fidler (36). Reciprocal interactions between tumor and host cells can establish a positive feed-forward loop that supports tumor expansion. Consequently, normal tissue containing tumor cells can support metastatic growth. For instance, bone is a preferred metastatic site for both prostate and breast cancer metastases (44) while lung is preferred by metastatic cells from melanoma and renal cancer. The reciprocal interaction between tumor and host has been investigated extensively in recent years. At the primary site, tumor-associated fibroblasts and inflammatory cells contribute to an environment that supports malignant cancer (45-47). Similarly, reciprocal communication between cancer cells, osteoblasts, and osteoclasts establishes a feed-forward loop in which tumor cells promote the destruction of bone and the release of tumor-supporting growth factors from the bone (44). More recently, it was demonstrated that systemic communication between the primary cancer, the bone marrow, and future metastatic sites can result in the formation of tumor-supportive conditions (pre-metastatic niche) prior to the arrival of metastatic cells (Fig. 8) (48). There is, in fact, increasing evidence to suggest that motility in the local microenvironment and systemically across the circulatory system involves intricate tumor–host communication.

Tissue structure and biomechanics

Systemic dissemination requires that tumor cells access the vasculature and travel via the circulatory system to a distant site where the cells extravasate and establish secondary tumor growth (reviewed comprehensively by Weiss (43) and Fidler (36).

Ewing and colleagues postulated that metastatic inefficiency was related to the location of the tumor within the body, the anatomic structure of the vasculature, and the biomechanics of tumor cell transportation. The contribution of tissue biomechanics to cancer metastasis and to the randomness of colonization were further explored by Weiss (43,49). Indeed, gross anatomical evaluation shows that colorectal cancer metastasizes predominantly to the nearest vascular bed in the liver, pancreatic cancer invades aggressively to adjacent organs and the surrounding viscera, and ovarian cancer cell dissemination is almost exclusively restricted to the peritoneal cavity. While it is certainly true that the capillary bed immediately downstream of the primary tumor is exposed to a greater number of circulating tumor cells, overt metastasis from numerous types of cancer is predominately site specific. This was conclusively, albeit inadvertently, shown in patients with metastatic ovarian cancer (50). These patients received peritoneovenous shunts to alleviate tumor-induce ascites but they developed no overt distant metastases in spite of the circulating, malignant cells. These observations confirm that mere, inadvertent, passive access to the circulatory system is not sufficient to accomplish metastatic growth.

The acquisition of pro-metastatic molecular determinants

The identity of genes responsible for tumor cell metastasis and tissue tropism has been the subject of intense investigation. These studies aim to develop a mechanistic understanding and identify clinically viable targets for the treatment of metastatic disease. Expression analysis of clinical specimens and experimental models with divergent metastatic abilities identified a very large number of genetic determinants that control the metastatic process (35). Furthermore, an evaluation of cancer's epigenetic plasticity revealed an inherent ability of metastatic cells to alter the expression of these determi-

nants in a changing environment. The Epithelial–Mesenchymal Transition (EMT) is an example of cancer cell plasticity in which epithelial-derived cancers adopt a mesenchymal behavior. During EMT, the adhesive repertoire is significantly altered and includes a reduction in E-cadherin, a gain of N-Cadherin, as well as changes in cytoskeletal organizing proteins, and the expression of developmental transcription factors to gain migratory and invasive properties (41). These epigenetic changes often occur transiently in response to growth factor stimuli such as TGFβ and HGF. More permanent changes can clearly evolve within the heterogenous tumor cell population. *In vitro* and *in vivo* selection reveals relatively stable epigenetic changes that support metastatic behavior and tissue-specific metastasis. These changes presumably arise as a consequence of alterations in DNA accessibility through changes in DNA methylation, histone modification, and genomic imprinting (51,52). Unlike the transforming events of carcinogenesis, genetic alterations are relatively rare during metastasis. Instead, metastasis is associated with the accumulation of multiple epigenetic modifications. Many of these modifications drive the mobilization of metastatic tumor cells.

Tumor cell motility

The contribution of tumor cell motility to metastasis is evident in each of the three principles discussed above:

I. Tumor–host interactions can promote tumor cell motility by providing chemotactic stimuli (53), remodeling the tissue to alleviate its non-permissive and motility-suppressive characteristics, and providing guidance cues to promote directional migration (54)

II. The vascular anatomy and architecture of tumor vasculature are responsible for creating hypoxic regions, oxygen, and nutrient gradients as well as preferred points of vascular entry/exit (54),36]. Moreover, recent investigations into mechano-

transduction demonstrate that tissue rigidity in the primary tumor and the bone contributes to the motility of tumor cells (55-57).

III. Molecular determinants that enable cell autonomous motility are seen in almost every expression profiling of metastatic cells. Although a large number of changes occur in the transition from benign to invasive disease, a signature of motility-related genes is evident both in experimental models and patient specimens. In some instances these pro-migratory gene profiles are transiently induced by the environment of the primary tumor or the metastatic site while in other instances the profile is the product of a more persistent reprogramming of the tumor cell (35,54,58-61).

Ultimately, it is the contribution of these three principles that determines the mobility and metastatic ability of a tumor cell. The key to targeting tumor cell motility for therapeutic means lies in the identification of a molecular mechanism required for tumor cell motility that is sufficiently cancer specific to prevent disruption of normal physiology.

Mechanisms that regulate cell motility

Cell migration broadly refers to processes involved in the movement of cells from one location to another. For adherent cells, translocation requires dissociation at the point of origin, physical displacement of the cell body, and re-adherence in another location. While this process appears relatively simple, it is in fact a process of immense biological complexity. Cell migration requires the integration of numerous molecular mechanisms that allow a cell to coordinate the formation of new adhesions while disengaging existing adhesions and simultaneously exerting force to move the cell body. Among the adhesion receptors alone there are four large families: the immunoglobulin superfamily cell adhesion molecules, the integrins, the cadherins, and the selectins (15,62,63).

These adhesion receptors engage immobilized ligands including structural and matricellular proteins of the extracellular matrix or cell-surface ligands on adjacent cells. Scaffolding proteins and signaling molecules mediate intracellular connections of the cytoskeleton with the cytoplasmic tail of adhesion receptors (64,65). Subsequent remodeling of the cytoskeleton transmits force between distant portions of the cell allowing the cell body to migrate to a new location where new adhesions are established. Extracellular stimuli such as cytokines are generally required to initiate and sustain activation of the molecular migration machinery. When a tumor cell encounters a non-permissive barrier such as a basement membrane, the production of proteolytic enzymes, a change in adhesion receptors, and the expression of a pro-migratory matrix can enable a cell to penetrate the barrier and disseminate from the tissue in which it was originally retained (66,67). Close coordination of these adaptations is necessary for successful metastatic dissemination.

Considering the complexity of migration, it is impossible to rigorously review all the molecular determinants of migration and identify all of the potential areas for clinical intervention. Rather than adding increasing layers of complexity to the existing literature we parsed the molecular determinants into five underlying principles that collectively dictate tumor cell motility (Fig. 9).

These include:

1) Cell autonomous ability

2) Soluble communication

3) Cell–cell adhesion

4) Cell–matrix adhesion

5) Integrating mechanisms of migration

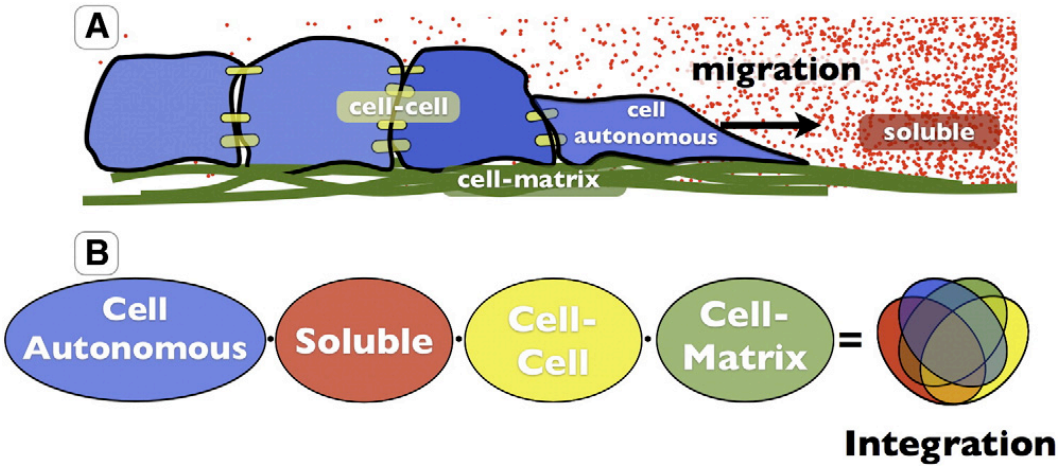

Fig. 9 Classification of cell migration determinants. The innumerable molecular determinants thats contribute to tumor cell motility can be classified in one of five underlying principles. Cell autonomous migratory ability includes the intrinsic determinants that enable a cell to move. Soluble communication includes autocrine and paracrine communication. Cell-cell adhesion includes those determinants responsible for physical cell-cell adhesion and communication. Cell-matrix adhesion includes those determinants responsible for adhesion to and migration on the ECM. Molecular integration includes the molecular scaffolding components that allow for the integration of all molecular determinants of migration. This figure modified and adapted from (1).

The following sections discuss in detail the broad functional contribution of each principle category, we provide representative examples that are possible targets suitable for invention in Tables 1–5, and we provide an extensive overview in Table 6 of ongoing translational work attempting to target tumor cell migration in the clinic.

Cell autonomous ability

Within the human body, each cell type exhibits a distinct autonomous ability to migrate that varies depending upon the molecular mechanisms that remain available to it after developmental differentiation (reviewed extensively by Friedl et al. (27,68) and Table 1). During metastasis, the cell's autonomous migratory ability appears to be driven by the activation of latent mechanisms, the aberrant recruitment of endogenous mechanisms, and/or the ectopic expression of developmentally suppressed migratory mechanisms.

The cell's autonomous migration potential is most apparent in its ability to remodel its cytoskeleton. Of the three cytoskeletal filaments (microfilaments, intermediate filaments, and microtubules) remodeling actin microfilaments is the most studied. This innate, autonomous ability involves hundreds of molecular determinants that control actin polymerization, extension, stabilization, and depolymerization. During migration, these filaments influence cell shape, transmit force, and support cargo trafficking. Key molecular determinants of the endogenous migratory ability include the small GTPases (Rho, Rac, CDC42) (68)] which initiate cytoskeleton remodeling, while members of the Actin Related Proteins (Arp2/3) mediate actin remodeling, and motor proteins of the myosin family control cell protrusion, contraction and cargo motoring. The extent to which autonomous migration contributes to motility was elegantly revealed by Lämmermann and colleagues in a study of 3D-migration of leukocytes from which all integrins were ablated (69). Surprisingly, the integrin-negative leukocytes are perfectly capable of mi-

grating *in vitro* and *in vivo*. These cells migrate by the sole force of actin-network expansion, which promotes protrusive flowing of the leading edge (70). Thus the ability to regulate the cell's cytoskeletal structure can determine its inherent migratory capacity and contribute to the metastatic potential of cancer (Table 1).

The non-receptor tyrosine kinase Src is another example of a cell- autonomous molecular determinant that is frequently and aberrantly activated in malignant cancer. Discovered originally as a viral oncogene(71), Src is over-expressed and ectopically activated in a myriad of cancers (72). Experimental evidence and current clinical trials indicate that this kinase is a fundamental driver of cell motility and tissue invasion. Src influences tumor biology by driving proliferation, altering gene transcription, and regulating adhesion, invasion and motility. Src mediates these functions through activation of a large number of substrates including key components of thefocal adhesion complex, (vinculin, cortactin, talin, paxillin, FAK, tensin, ezrin and p130Cas), junctional proteins, (β - and γ -catenin, ZO-1, occludin, p120ctn, connexin 43, nectin-2) (70,73,74), enzymes involved in phospholipid metabolism, (PLC-γ, p85 subunit of PI3-kinase) and signaling molecules (p190RhoGAP, p120rasGAP, Eps8)(73,75,76).

Unlike its enzymatically active counterparts, the Wiskott-Aldrich Syndrome family of proteins including WASP and WAVE (77) promote actin remodeling through the activation of Actin Related Proteins (ARPs). Together, members of these protein families facilitate the polymerization of new actin strands and promote protrusion of the cell's leading edge. Individual family members are selectively expressed and ectopic expression increases cell autonomous and facilitates metastatic dissemination (12).Although individual molecular determinants are often emphasized, it is increasingly evident that entire pro-migration gene signatures occur in metastatic cells (54).

Table I: Targeting molecular mechanisms that support cell autonomous migration.

Target	Molecular Mechanism
Ras/Rho/ CDC42	Small GTPases involved in the reorganization of actin and microtubulin network formation that controls cell protrusions (lamellipodia and filopodia)
Snail, Twist	Transcription factors that functions as a regulators of the EMT phenotype promote migration and tumor cell motility
SATB1	Transcriptional regulator (chromatin organizer and transcription factor) that integrates higher-order chromatin architecture with gene regulation. Ectopic SATB1 expression promotes aggressive phenotype while its down-regulation promotes E-cadherin expression and inhibits the transcription factors Snail and Twist.
Src	A non-receptor tyrosine kinase that transmits integrin-dependent signals central to cell movement and proliferation
WAVE3	An actin nucleation/polymerizing factor that binds actin and the Arp2/3 complex. It is an effector molecule involved in the transmission of signals from tyrosine kinase receptors and small GTPases to the actin cytoskeleton. Normally expressed in ovary in brain but ectopic expression can occur at high levels in diverse cancers where it promotes motility and metastasis.
miRNA-10b, miR34a	microRNA can regulate expression of a large group of genes that control migration. Both upregulation of migration promoters and down regulation of migration inhibitors has been observed.
Molecules included here are representative molecular determinants of the underlying principle which can be found in (61,71,78-87).	

Transcriptional regulators influence cell autonomous motility by controlling the protein pools available for cytoskeletal rearrangements by regulating their expression. The aberrant and ectopic expression of transcription regulators are recurring themes in cancer metastasis. Snail, Twist (79), and SATB1 (82) reappear in metastatic cells and drive a motility-promoting expression profile that enables tumor cells to mobilize. Conversely, broad regulation of motility genes by microRNA was identified recently (88). Upregulation of the miRNA-10b may reduce expression of motility suppressor genes such as HOXD10 [43] while genomic loss of miRNA101 [66] results in the overexpression of metastasis associated histone methyltransferase EZH2. Similarly, reduced expression of miR34a is apparent in aggressive prostate cancer. This miR has been evaluated preclinically as a therapeutic agent against prostate cancer metastasis [67]. Targeting microRNA may be clinically efficacious because this treatment impacts cellular behavior by altering expression for a group of genes that collectively control migration.

Soluble communication

Soluble mediators, ranging from small molecules to macromolecular protein complexes, enable communication between tumor cells and their environment without direct contact. Mediators produced by tumor and host cells within the local microenvironment provide paracrine and autocrine support for cellular movement (Table 2). Soluble factors produced within the tumor tissue also act systemically to mobilize cells from the bone marrow or to influence host cells in a putative metastatic site. Conversely, the host can provide systemic soluble mediators that influence cellular behavior in the primary tumor and the metastatic lesions.

Tumor-derived mediators

Tumor cells produce a myriad of cytokines that influence the biology of the tumor as well as the local host stroma. EGF, HGF, and generated by the tumor act in autocrine

Table II: Targeting molecular mechanisms that enable soluble communication.

Target	Molecular Mechanism
TGFβ	TGFβ ligands (a 40-member superfamily that incudes TGFβ1, 2, and 3) control cellular growth, differentiation, and motility through heteromeric signaling complexes composed of the TGFβ type I, II, and III receptors. Neutralizing antibodies can diminish metastasis.
VEGF	Ligands that belong to the VEGF-PDGF super family where the VEGF gene yields five isoforms (A-D) that bind to four receptors (Flt-1, Flk-1, neuropilin-1, and Flt-4).
EGF	The EGF family includes ten ligands that bind to dimeric ERBB receptors to induce pro-migratory signaling via receptor tyrosine kinase activity. Currently a target of many anti-cancer therapeutics ,EGF receptor is upregulated in many cancers (breast, colorectal, lung).
SDF-1	A soluble cytokine that binds to its cognate receptor CXCR4. First recognized for its broad impact on immune functions and ability to control homing of bone marrow cells, it is also involved in the homing of tumor cells to distant sites.
TNFα	The most studied member of the tumor necrosis family of cytokines first recognized as a regulator of the immune response. It is produced by a large number of human tumors. The highest levels of TNF-α is secreted by foreign leukocytes present in the tumor microenvironment. Plays a dual role as about both a tumor suppressor and tumor promoter. High serum expression in patients is associated with a poor prognosis.
Molecules included here are representative molecular determinants of the underlying principle which can be found in (53,89-102).	

and paracrine fashion to mobilize both tumor and host cells. In many instances tumor-derived cytokines promote motility indirectly via the host stroma. The exposure of fibro blasts to the pro-inflammatory cytokine TNF-α promotes their differentiation into cancer-associated fibroblasts (CAFs, (102)). CAF promote tumor cell motility through the induced expression of paracrine acting cytokines and by physically encouraging the invasion of tumor cells {144)..Tumor-derived mediators can also have a significant systemic influence on metastasis. Specific cytokines, such as VEGF-A, placental growth factor (PLGF), granulocyte colony stimulating factor (G-CSF), stromal cell derived factor-1 (SDF1) and osteopontin, secreted by the growing tumor can impinge upon the bone marrow (BM) via the peripheral circulation. These factors can switch the quiescent microenvironment in the BM compartment to a highly pro-angiogenic and pro- tumorigenic state that promotes the expansion and mobilization of progenitor cells into the peripheral circulation. These cells engage the primary tumor and develop a favorable metastatic environment in distant organs (premetastatic niche) in response to SDF-1, TNFα, TGFβ, and PLGF (48,103).

Host-derived mediators

Tumors recruit a wide variety of stomal cells including endothelial cells, fibroblasts, inflammatory cells, and bone marrow derived cells such as mesenchymal stem cells. These stomal cells can promote tumor cell motility through the production of a variety of soluble mediators like EGF, HGF, TGFβ, HGF, and CCL5 (103,104). In many instances, a pro-metastatic reciprocal interaction supports paracrine signaling between tumor and host. Tumor cells recruit mesenchymal stem cells which promote tumor cell motility through the production of CCL5 (105). Macrophages are similarly recruited to the invasive front by tumor-derived CSF-1 where they induce tumor cell chemotaxis through the release of EGF (53).

Collectively, host and tumor derived soluble factors enable communication between the cancer and its environment. It is the activation or suppression of the migration machinery in response to these soluble factors that regulates the motility and dissemination of tumor cells.

Cell–cell interactions

While cell–cell interactions generally promote tissue cohesion, adhesive contacts between adjacent cells can both promote and inhibit motility. Cell–cell interactions are controlled at multiple levels, including the expression, localization, and surface presentation of cell adhesion molecules (Table 3). Cell to cell adhesion receptors include members of the immunoglobulin superfamily (IgSF), cadherins, integrins, selectins, ephrins, and the tight junction proteins claudins and occludins. Tissue specific ex- pression of adhesion proteins creates a characteristic expression signature in cancers that reflects their tissue of origin. However, adhesive characteristics change in metastatic disease. A loss of firm adhesion promotes motility and metastasis which is evident in the loss or mis-localization of cadherins and tight junction proteins (63). However, adhesion is rarely lost completely during metastasis, and new adhesive proteins are frequently upregulated or newly expressed to displace previously migration-suppressive adhesions. Such interactions include EpCAM, which can abrogate E-cadherin-mediated adhesion (106). Cadherin switching from E-cadherin to N-cadherin can promote motility during epithelial–mesenchymal transition. The expression of even low levels of Ephrin A2 can promote motility and mediate metastasis by influencing other pro-migratory signaling events such as Src activation (107). Several members of the immunoglobulin superfamily contribute to the motile behavior of tumor cells. ICAM, EpCAM, L1CAM, and ALCAM have all been associated with metastatic behavior (106),75,(108). New understanding of

Table III: Targeting molecular mechanisms that support cell-cell interactions.

Target	Molecular Mechanism
E-Cadherin	*Cell-Cell adhesion molecule* of the cadherin family involved in the regulation of epithelial cell-cell adhesion. Routinely mislocalized or lost in patients with metastatic disease. Experimental over expression can inhibit migration while knock down or cellular relocalization in response to pro-migratory cytokines can promote motility and migration. Generally considered a negative regulator of cell motility.
N-Cadherin	*Cell-Cell adhesion molecule* of the cadherin family involved in the regulation of epithelial cell-cell adhesion. Expressed during epithelial-mesenchymal transition and upregulated in metastatic disease. Upregulation frequently co-incides with the loss of E-Cadherin. Association with FGFR-1 enhances receptor signaling and provides a pro-metastatic mechanism of motility
EpCam	*Cell-Cell adhesion molecule* of the immunoglobulin super family. Can function as a tumor suppressor or oncogene because it inhibit motility but also abrogate E-cadherin mediated adhesion to promote tumor cell motility.
ALCAM	*Cell-Cell adhesion molecule* of the immunogobulin super family. Can inhibit motility when engaged in cell-cell interactions or promote motility when cleaved by ADAM17. Expression is upregulated in prostate, pancreatic, and colorectal cancer but down regulated in breast cancer. Shedding is elevated in all cancers and the shed ectodomain is being explored as a biomarker of metastasis.
Claudin 1, 4, and 7	*Cell-Cell adhesion molecule* of the Claudin family involved in transmitting cell-cell contact to the actin cytoskeleton within the tight junction. Loss of Claudins enhances motility and metastasis.
EphA2	*Cell-Cell adhesion molecule* of the ephrin family capable of promoting motility and metastasis. Integrates with Src, Akt, and HGF-mediated signaling.
Molecules included here are representative molecular determinants of the underlying principle which can be found in (17,41,58,106,107,109-119).	

IgSF shedding suggests that these adhesion molecules are selectively favored in cancer metastasis because their adhesion dynamic is readily augmented by pro-migratory stimuli (62). Thus, changes to cellular adhesion that facilitate cell migration eventually promote cancer metastasis.

Cell–matrix interactions

The dynamic interaction between a cell and its external matrix heavily influences cell migration and the invasive behavior of cancer cells. Cell–matrix interactions are formed between adhesive proteins on the surface of the migrating cell and the structural components of the extracellular matrix. These factors influence both the motility and migratory capacity of normal cells, and the metastasis of tumor cells (Table 4 and (45).

The best studied family of proteins that regulate cell–matrix interactions are integrins, a family of heterodimeric cell-surface proteins, which contain an extracellular alpha and beta subunit. Similar to cell–cell adhesion molecules, some integrins suppress metastasis while others promote dissemination. Integrin α2β1 was recently identified as a metastasis suppressor. It is lost in mouse models of breast cancer and in breast cancer patients (120). Conversely, the integrin αvβ3 promotes cancer metastasis (15,121).Non-integrin receptors are also involved in the migration of metastatic cells. Syndecan-1 is diminished in some cancers and upregulated in others, yet its ability to regulate adhesion and migration is primarily controlled by shedding (122). Conversely, a change in splice variants of the hyaluronic acid receptor CD44 facilitates motility and metastasis by promoting matrix remodeling (123).In addition to changing their adhesion receptor profile, metastatic tumor cells can cross non-permissive tissue barriers by remodeling the matrix that surrounds them.

Table IV: Targeting molecular mechanisms that support cell-matrix interactions.

Target	Molecular Mechanism
Integrin αvβ3	Matrix adhesion receptor of the integrin family involved in the adhesion to a wide variety of matrix components that exhibit the RGD sequence. These include collagen, vitronectin, and fibronectin. This integrin is also extensively expressed by the host vasculature and hemopoietic cells.
Integrin α2β1	Matrix adhesion receptor of the integrin family involved in the adhesion to collagen and a few additional substrates. While the receptor facilitates motility on collagen it actually inhibits migration on non-collagen substrates and suppresses metastasis.
Syndecan-1	Transmembrane proteoglycan of the immunoglobulin super family. Syndecans have previously been considered as ligand gatherers, working as co-receptors in collaboration with signalling receptors but they can also signal independently. Syndecan-1 interacts with the ECM through its glycosaminoglycan side chains. Some reports suggest it can also engage in cell-cell adhesion. Loss of Syndecan-1 is specifically associated with metastasis.
MMP14	Transmembrane metalloproteinase of the MMP family. MMP14 is a collagenase but also capable of cleaving aggrecan, elastin, fibronectin, gelatin, tenascin, nidogen, perlecan, fibrillin, and laminin. It can also cause shedding of Syndecan-1, and betaglycan (TBRIII). It can activate the MMP2 and MMP-13 activation. Its activity can be reguated by ALCAM and CD151. MMP14 is expressed in many metastatic cells and facilitates invasion. However, it is not required for metastasis.
CD44	Transmembrane receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). Splice variant expression is correlated with motility and metastasis. Splice variant-induced changes in adhesion can facilitate migration and metastasis.
Molecules included here are representative molecular determinants of the underlying principle which can be found in (120,124-132).	

Matrix remodeling is accomplished through degrading existing matrix, producing new matrix components, and (re) organizing both new and existing matrix proteins within the existing tissue architecture. Matrix metalloproteases (MMPs), including MMP2, 9, and 14, were among the first matrix remodeling enzymes found to be capable of promoting metastasis by degrading the matrix (66,68,133). Along with serine proteases, MMPs are among the enzymes that enable tumor cells to penetrate the extracellular matrix by cleaving multiple matrix components including collagens, vitronectin, fibrinogen, and laminin. In some instances, cleavage of the matrix protein creates a pro-migratory stimulus (66). This can be seen for laminin-332 cleaved by the serine protease Hepsin (134). Conversely, the expression, deposition, and assembly of new, permissive extracellular matrix by the invading tumor cells that enable their migration further facilitates the metastasis of tumor cells. This is seen in the deposition of tenasin-C isoforms (135,136), fibronectin isoforms (137), collagen (138) and fibrin. Incorporation of new matrix and remodeling of the tumor extracellular matrix also changes the three-dimensional rigidity of tissues, frequently making the tissue less pliable. With this increasing rigidity, the motility of tumor cells can increase (56) leading to increased invasion and cancer malignancy (55). Although the extracellular matrix was initially perceived as a barrier to tumor cell dissemination, it is increasingly evident that the architecture and the matrix composition of the microenvironment can promote tumor cell dissemination.

Molecular integration

Although a large number of molecular determinants influence migration, the contribution of any single component is regulated through its integration into the migration machinery. There are thousands of molecular determinants that can contribute to cell migration. Their integration occurs primarily at the level of molecular scaffolds that allow for a convergence of these individual mechanisms. A molecular scaffold generally consists of an adaptor protein capable of interacting simultaneously with several proteins

which, together, regulate a single biological process. Adaptor proteins are found at every cellular level including the cytoplasm (P130CAS), the membrane (CD151), and the extracellular matrix (SPARC). Among all the therapeutic strategies currently under evaluation (Table 5) there is only one molecular scaffold (FAK) because it is a kinase that is also drug-able. However, the clinical focus on integration mechanisms is likely to intensify as our understanding of molecular scaffolds is rapidly increasing and their uniquely critical participation in migration may improve therapeutic efficacy. To emphasize its importance, four points of integration are presented below.Integration of cell–matrix adhesion with cell-autonomous ability.

Molecular integration of integrins with the cell autonomous migration machinery occurs primarily through Talin and Focal Adhesion Kinase (FAK). Talin is a scaffolding protein that binds the cytoplasmic domain of β integrin subunit and links integrins to the actin cytoskeleton while complexing Vinculin and FAK into a functional complex that controls inside-out activation of the integrin and outside-in signaling resulting from matrix binding. As a non- receptor tyrosine kinase, FAK integrates with integrin associated complexes through the binding of Talin and Paxillin where it engages and controls the activity of a wide variety of cell autonomous signaling molecules including src, PI3K, Shc, PLCγ, RhoGEF, GRB2.

Integration of cell–cell adhesion with cell-autonomous ability

P120 is an adaptor protein of the catenin family and is frequently lost or mutated in cancer. Loss of P120 activity disrupts the function of E-cadherin and facilitates a pro-migratory phenotype. P120 interacts directly with the cytoplasmic tail of E-Cadherin and integrates cadherin, src, and RTK signaling through scaffolding of intracellular signaling molecules (139,140). Its interaction with the zinc-finger
transcription factor kaiso allows P120 to regulate gene transcription (141)

Table V: Targeting mechanisms that support molecular integration.

Target	Molecular Mechanism
CD151	*Transmembrane scaffolding* protein of the Tetraspanin Super Family. CD151 interacts with other tertraspanins as wel as unique partners including integrins α3β1 and α6β1, MMP14, MMP7, PKC, PI4K and other. These tetraspanin-partner dimers are incorporated into macromolecular complexes known as tetraspanin-enriched microdomains through the homo- and hetero-dimerization of the tetraspanins.
Talin-1	*Integrin adaptor protein* that binds to the cytoplasmic tail of the β subunit tail and links the integrin to the actin cytoskeleton and complexes Vinculin and FAK into a functional complex that controls inside-out activation of the integrin and outside-in signaling resulting from matrix binding. Silencing Talin reduces metastasis
FAK	*Non-receptor tyrosine kinase* that binds to Focal Adhesion Complexes by binding Talin and Paxillin. It interacts with a wide variety of signaling molecules including src, PI3K, Shc, PLCγ, P130Cas, RhoGEF,GRB2, and more. Phosphorylation of its partners by FAK regulates motility.
P120 Catenin	*Intracellular scaffolding protein* of the catenin family that stabilizes the formation of E-Cadherin based adhesions and integrates cadherin, src, and RTK signaling through scaffolding of intracellular signaling molecules. P120 is frequently lost or mutated in cancer thereby disrupting the function of E-cadherin and other, currently undefined, signaling components.
Cortactin	*Actin binding protein* that also binds to WASP, MIM, Hax-1, TEM7, Dynamin 2, CD2AP, and Cadherins. Cortactin seems to be important for invasive activity associated with invadapodia present in invasive cells. Cortactin is strongly correlated with cancer progression although its specific role remains unclear.
SPARC	*Matricellular protein* capable of interacting with collagen, fibronectin, EGF, TGFβ, and the β1 integrin. It alters the composition, assembly, and maturation of ECM. SPARC is upregulated in a large number of cancers although its biological contribution to metastasis is very context dependent.
P130 CAS	*Intracellular scaffolding protein* defined as an integrin adaptor molecule. P130cas is capable of binding a very broad range of cytoplasmic signaling molecules including FAK, PYK2, FRNK, PTPNI2, RAPGEF1, CRK, NCK, SFK, Aurora A, and PI3K. It is participates in both transformation and migration.
Molecules included here are representative molecular determinants of the underlying principle which can be found in (24,87,139,140,142-149).	

regulation of GTPase signaling via P120 catenins has been established(150) but the identity of partners is currently unknown.

Integration of soluble communication and cell–matrix adhesion with cell-autonomous ability

P130CAS is a classic molecular scaffold that controls the integration of signaling between integrins, receptor tyrosine kinases (RTK), and Src. p130CAS binds to twelve known partners and is phosphorylated by both Src and FAK. Stimuli from cell–matrix adhesion (integrins), soluble communication (RTK), and cell autonomous ability (Src) each change the phosphorylation, localization, and activity/ availability of P130CAS binding partners. Ultimately, the composition and activity of the macromolecular complex that contains P130CAS determine its ability to influence actin cytoskeleton remodeling, gene expression, and cellular survival (151).

Integration at the membrane with molecular scaffolds

The interactions between the cell periphery and its extracellular micro- environment {92} are organized through macrodomains within the membrane such as focal adhesions (152,153), tight junctions(111), lipid rafts (154), and tetraspanin-enriched microdomains (147,155,156). Together with their partner proteins, membranes scaffolding proteins arrange themselves into organized structures within the plane of lipid bilayer. This high-order structure controls availability as well as activity of partners associated with the scaffolding proteins (157).

Of the membrane scaffolding proteins, the 33-member family of tetraspanins is most prominently involved in cancer biology. Tspan 1, 7, 8, 13, 24, 27, 28, 29, 30, and 31 are involved in tumor progression (156). Metastasis seems to be specifically promoted by Tspan24 (CD151), (156),101) and suppressed by Tspan27 (CD82), (158). Tetraspanins function as membrane scaffolding proteins by organizing a large selection of partner proteins into higher order structures usually referred to as tetraspanin-enriched micro-

domains (TERM (147)). CD151 is an important regulator of tumor cell motility (156). It interacts and organizes integrins (including α3β1 and α6β1), MMPs (including MMP7 and MMP14), tetraspanins (including CD9, CD81, CD82, and CD151), cytoplasmic signaling molecules (including PKC and PI4K) (Reviewed in detail in [101](159)]. Tetraspanin expression is frequently altered in cancer (155) and direct targeting of CD151 can, in fact, inhibit metastasis (24). The ability of CD151 to control motility and metastasis will be the central topic of the remaining chapters of this thesis and it is my hope that it provides some insight into the targeting of molecular scaffolding proteins in the treatment of metastatic disease.

The central organizing function of molecular scaffolds and adaptor proteins makes them very attractive clinical targets. Considering the limited success of cancer therapies that target individual proteases, growth factors, and kinases, altering the molecular integration of such prominent pro-migratory stimuli may prove to be most effective strategy for targeting motility and preventing metastasis.

Therapeutic targeting of tumor cell motility

Therapy that targets tumor cell migration has tremendous potential in the clinic. Ideally targeted therapy limits, intervenes, or disrupts a molecular process that supports the pathology without disrupting normal physiological function. Successful targeting of tumor cell motility with the intent of disrupting metastasis will require the specific targeting of molecular mechanisms involved in metastatic dissemination without disrupting normal migratory processes such as inflammation and wound healing (160).

The principal obstacle to developing therapy that specifically blocks metastasis is the time needed to determine therapeutic efficacy. Current anti-metastasis therapy is tested after first line therapy has failed or as an adjuvant to cytotoxic therapies. For many cancers these patients will remain disease free for several years thereby extending the duration of any trial and requiring a large number of patients. Patients subjected

to anti-metastasis therapy will have to be under continuous treatment because it is currently not possible to predict when a cancer becomes metastatic. This chronic treatment compounds possible negative side effects such as any impact on immunity or wound healing. Lastly, it is not possible to predict if resistance to targeted therapy for motility would develop considering that thousands of molecular determinants are involved in the motility of tumor cells. The main focus of existing pharmaceutical strategies is to design targeted therapies that disrupt molecular processes both central to survival and required in the motility of tumor cells. Consequently, all the drugs that are currently under consideration target not only motility but also influence cell viability and proliferation. Although to date no therapy targeting tumor cell motility has been approved for clinical use, several treatments are promising and under review in ongoing clinical trials. Table 6 provides a comprehensive representation of targeted therapies.

Future directions

In 2010 more than 1300 manuscripts were published on the topic of tumor cell migration and its contribution to metastasis (Fig. 6). Numerous molecular mechanisms involved in this complex process have been described and suggested as potential therapeutic targets, yet only a very small fraction have been evaluated clinically for their ability to limit metastasis, and none are in clinical use.

The difficulty of implementing motility-targeting therapeutics should not come entirely as a surprise considering the complications of determining therapeutic efficacy in the adjuvant setting with chronic treatment of cancer patients. This is further complicated by the central role of motility in normal physiology and therefore the need to determine metastasis-specific therapies. Nevertheless, the proof of principle has been presented several times in the form of reduced metastasis in preclinical animal models and promising results from clinical trials. These trails target Src, VEGF, EGF, and TGFβ which have broad biological impact and inhibit multiple mechanisms involved in cancer progression

to maximize a favorable clinical outcome including the inhibition of both angiogenesis and metastasis or the inhibition of both tumor growth and motility.

Significant progress in the treatment of metastasis is expected to be made as therapies targeting specific migratory mechanisms such as SDF-1, c-MET, EpCAM, and Rho-kinase progress through their clinical evaluation (Table 6 and 7). The difficulty in confirming long-term suppression of metastasis will remain the main obstacle to successful clinical translation of any therapy targeting motility. To accelerate the evaluation of therapies that target metastasis, it may be necessary to take advantage of short, pre-operative neo-adjuvant trials and establish new short-term parameters of clinical success. These parameters could include the detection of changes in circulating tumor cells or a circulating metastasis biomarker.

The integrating function of scaffolding proteins is of particular interest because it brings together multiple molecular aspects of migration and is therefore less likely to fall subject to drug resistance. In addition, it is likely that combination therapies, targeting multiple mechanisms belonging to distinct principles presented here, will be developed to optimize efficacy and diminish drug resistance. In the past two decades many putative targets have been identified. If the first candidates, currently in clinical trials, prove successful, then this approach and a new class of targeted therapeutics will define the future of treating metastatic

Table VI: Migration inhibitors in clinical development

Drug	Target	Company	Clinical Phase
Cell autonomous			
Saracatinib (AZD0530)	Src	AstraZeneca	II
Bosutinib (SKI-606)	Src	Wyeth	II, III
Dasatinib (BMS-354825)	Src	Bristol-Myers Squibb	I,II
Fasudil	Rho kinase	Asahi Kasei	I,II,III
Emodin	Cdc42/Rac1		I
Soluble interactions			
CTCE-9908	SDF-1	Chemokine Thera-peutics	I, II
MetMAB (PRO143966)	Met	Roche/Genentech	II
AMG 208	Met	Amgen	I
GC1008	TGF-β family	Genzyme	I, II
Trabedersen (AP 12009)	TGF-β2	Antisense Pharma	I, II
Infliximab	TNF-α	Centocor	I, II
EGFR	Herceptin^ (trastuzumab)	Genentech/Roche	I,II,III
VEGF	Avastin^	Genentech/Roche	I,II,III
CXCR-4	CTCE-9908	Chemokine Thera-peutics	I,II

Table VI Continued

	Drug	Target	Company	Clinical Phase
Cell-cell interactions				
	IGN-101	EpCAM	Aphton	I, II
	Exherin (ADH-1)	N-cadherin	Adherex	I, II
Cell-matrix interactions				
	Cilengitide (EMD121974)	αvβ3 and αvβ5 integrins	EMD/Merck KGaA	II, III
	Volociximab (M200)	α5β1 integrin	PDL/Biogen Idec	II
	Etaracizumab (Abegrin)	αvβ3 integrin	MedImmune	I, II
	ATN-161	Integrins	Tactic Pharmaceuticals	I,II
	BMS-275291#	MMPs	Bristol-Myers Squibb	I,II,III
	Endostatin	MMPs	Alchemgen Therapeutics	III
	Curcumin	MMPs	Sabinsa Corporation	I, II
	Tigapotide (PCK3145)	MMP9	Ambrilia Biopharma	II
	A6	CD44	Angstrom Pharmaceuticals	II
	Mesupron (WX-671)	uPA	Wilex	I,II
	ATN-658	uPA	Tactic Pharmaceuticals	I
	Tempostatin (Halofuginone hydrobromide)	Stroma	Collgard Pharmaceuticals	II
	PI-88	Heparanase	Progen	I,II, III
	Vitaxin	αvβ3	MedImmune	II
Molecular integration				
	CFAK-C4	FAK	CureFAKtor Pharmaceuticals	I
	PF-562271	FAK	Pfizer	I

References for these indicated studies can be found in (161-178) .
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Chapter III

"CD151"

MEMBRANE ORGANIZATION THROUGH MOLECULAR SCAFFOLDING

Summary

Tetraspanin proteins represent an interesting class of cell surface molecules that are able to control numerous cellular functions and processes. Unlike your traditional cell surface proteins they do not posses any catalytic activity or functional domains but rather act as scaffolding proteins that are able organize cell surface partner proteins into macromolecular signaling complexes. CD151, is potentially the most well-studied member of the tetraspanin family. This chapter will serve as an introduction to tetraspanin proteins while primarily focusing on CD151. I will discuss much of what is known about CD151 including; its functions, defining characteristics, and its role in regulating cellular behaviors. Finally, I will introduce work from our laboratory using a specific anti-CD151 monoclonal antibody that served as a basis for this thesis project.

Introduction

CD151, one of 33 members in the mammalian Tetraspanin (Tspan) family, regulates diverse processes associated with adhesion including cell migration, platelet aggregation, tumor cell invasion and metastasis. Structurally, CD151 contains 4 transmembrane spanning domains that form 2 extracellular loops leaving two short intracellular C and N terminal tails in the cytoplasm. The largest loop is thought to be important for interactions with its transmembrane and membrane-proximal partners while the transmembrane and cytoplasmic domains engage in interactions with cytoplasmic sig-

naling molecules such as PKC. Like most Tspans, CD151 functions as a membrane scaffolding molecule by promoting the assembly of itself along with its partners into macromolecular protein complexes within the lipid bilayer of membranes. These complexes are known as Tetraspanin Enriched Microdomains (TERMs). CD151 interacts with a variety of molecules include integrins (α3 and α6), proteases (MMP14 and MMP7), signaling molecules (PKC and PI4Kinase), adhesion molecules (E-cadherin) and other Tspans including itself. It is widely accepted that the Tspan partner incorporated into the TERM contributes to the functional role of the tetraspanin in biological systems. Together these partner-Tspan interaction which form the TERM are macro molecular complexes that regulate complex cellular behavior but are physically and functionally distinct from other signaling structures such as lipid rafts and adhesion complexes. One of the key differences between TERMs lipid rafts is the fact that lipid rafts are disrupted in cholesterol depletion experiments while TERMs are maintained(22). CD151 it is almost ubiquitously expressed but is predominantly found on epithelial cells, endothelial cells, and platelets. While its loss in humans and experimental mouse models leads to phenotypes related to basement membrane failures including skin blistering and renal failure, CD151 is frequently upregulated in cancer. In summary, CD151 participates in regulating adhesive functions of normal and tumor cells by organizing partner proteins actively involved in the adhesive process.

Protein Function

CD151 functions principally as a transmembrane scaffolding protein. Like most tetraspanins, CD151 interacts with other transmembrane or membrane-proximal proteins through its LEL and short cytoplasmic tails. Multimerization of the Tspan and its partner proteins leads to the formation of large multi-molecular complexes within the membrane. These complexes were originally referred to as the "tetraspanin web" (179-181), however, the most current literature refers to them as Tetraspanin Enriched Microdomains

(TERM or TEM), (147,155). The large extracellular loop (LEL) contains the majority of amino acids important for interactions with transmembrane and membrane proximal partners while the transmembrane regions and cytoplasmic tails are thought to be required for intracellular interactions with Tspan-associated cytoplasmic signaling molecules. Specific partners include α3β1, α6β4, PI4K, and PKCα. The molecular mechanism(s) by which this Tspan conveys biological activity remains under investigation. CD151 participates in a wide variety of normal physiological processes as well as pathologies including kidney function, angiogenesis, platelet activation, inflammation, wound healing, tumor invasion, and metastasis. ;(23,24,180,182-187). It is generally considered to be a pro-migratory molecule and is routinely upregulated at the invasive front of metastatic tumors. *In vitro* the forced expression of CD151 is able to promote migration while in cells that express high levels of CD151 the knockdown of CD151 has been shown to decrease migration.

CD151 Expression

CD151 was originally identified as a platelet cell surface antigen (188), (189) it is expressed nearly all tissues. It is abundantly expressed in many cells of the vasculature system including endothelial cells, megakaryocytes and platelets. It is highly expressed in ductal epithelial cells of the breast, colon and prostate. It is also expressed in the glomeruli and tubules of the kidney, the basal epithelia of the skin, cardiomyocytes, and Schwann cells of the brain. CD151 is also expressed in most solid tumors and upregulated in many aggressive neoplasia. In contrast, it is distinctly absent from lymphoma. In normal tissues and confluent endothelial or epithelial cell cultures CD151 is predominantly localized to the basolateral surface and to areas of cell-cell contact(189-193). In individually cultured cells without cell-cell contact or non-adherent cells, CD151 is frequently internalized into endosomal and lysosomal compartments (21,194) as a compo-

nent of the endocytic pathway. In contrast, tumor cells frequently exhibit general cell surface distribution and localization in cytoplasmic vesicles. In epithelial cells CD151 is also seen in hemidesmosome-like structures where it associates with α6β4 (184,187). More recently CD151, along with other tetraspanins, have been shown to be enriched in exosomes. (182,147).

Interactions with ligands and other proteins

Presently, there is no specific ligand known for CD151. CD151 interacts with a large variety of proteins including integrins, other tetraspanins, proteases, and signaling molecules. It is has been suggested that Tspans such as CD151 can interact with more than 100 proteins (147,156,195). The most well studied CD151 interactions are those that involve the laminin binding integrins α3β1 and α6β1(196), these CD151-integrin complexes can regulate cell morphology and migration by controlling adhesion and adhesive strengthening (197) and cytoplasmic signaling . The nature, stoichiometry, and affinity of tetraspanin-partner interactions have been difficult to assess quantitatively. Direct partners of Tspans are identified through a combination of protein cross-linking, cell-surface biotinylation, differential detergent extraction, and reciprocal immunoprecipitation(179,190,198-200). The α3β1 CD151-integrin complex is one of the best defined interactions. This complex can be immunoprecipitated under both mild and stringent detergent extraction conditions indicating a strong, primary interaction that is defined by the specific QRD binding sequence (196,201). Furthermore, the interaction is highly stoichiometric using reversible crosslinking, biotinylation, and reciprocal immunoprecipitation of the Tspan and its partner (202). Furthermore, the loss of CD151 leads to migration deficiencies in laminin-rich matrixes (203-205). In addition to manipulating the activity of its integrin partners directly, evidence has been presented that the tetraspanin

can regulate partner trafficking (194), glycosylation (206). Together these observations provide strong support for both a physical and functional role for CD151 in α3β1 biology. The integrin α6β1 and α6β4 are also partners of CD151 although the interaction is not as stable as the CD151-α3 interaction(184,207). Disruption of CD151-α6 interaction does not alter integrin cell surface expression or cellular adhesion to laminin, however, it does diminish alpha 6 dependent cell spreading and integrin-mediated adhesive strengthening (197). It is important to note that the regulation of integrin function has been demonstrated biochemically and functionally only for laminin-binding integrins α3 and α6). The molecular mechanism by which CD151 controls migration remains unknown. *In vivo* work with anti-CD151 antibody suggests, however, that CD151 can have a broad impact on all matrix-binding integrins (24)

CD151 association with intracellular signaling molecules

CD151 also associates with signaling molecules including PKCα and PI4Kinase type IIα (192,200,202). The importance of these interactions are not fully known however it reasonable to speculate that the CD151 interactions with these signaling molecules could influence intracellular signaling by incorporating them into the TERM. This is particularly likely for PKCα which only binds to CD151 in its active form. More recently an interaction with the E3 ubiquitin ligase GRAIL was identified however, a functional role of this interaction has not been explored (208). Several Matrix Metalloproteinases have been associated with CD151 including MMP7(209) and MMP14 (23). These studies suggest that the tetraspanin may control activation and localization of these proteases. The incorporation of CD151 into tetraspanin-enriched microdomains will most likely allow it to interact directly and indirectly to a large number of proteins and regulate their function. The extent to which to this activity influences biological function will have to be determined for each individual biological system.

Regulation of Activity

The activity of CD151 is primarily regulated post-translationally through modifications of its subcellular distribution or the stability of its interaction with membrane partners. The most common post-translation modification involves the palmitoylation of conserved cysteine residues in the transmembrane domains by the palmitoyl-acyl transferase (PAT) DHHC2, one of 23 known mammalian PATs (210). Palmitoylation of Tspan proteins such as CD151 affects the assembly and maintenance of TERMS while also affecting tetraspanin subcellular distribution, stability during biosynthesis, cell signaling, motility, and morphology. It has also been shown that palmitoylation of CD151 is involved in the regulation of Tspan-Tspan interaction because palmitoylation-deficient CD151 is unable to complex with other Tspan family members (CD9, CD63, CD81) (180,211-213). The importance of this modification has been confirmed both by siRNA mediated knockdown of expression of DHHC2 as well as the expression of palmitoylation-deficient CD151 mutants. In addition to palmitoylation on conserved cysteine residues CD151 is also routinely glycosylated in its extracellular domain. Glysosylation of CD151 regulates the CD151/alpha 3 beta integrin association while also modulating integrin-mediated adhesion and spreading (206). Tspan clustering is the primary mechanism that can influence their biological activity. Clustering antibodies for CD151 and other tetraspanins have demonstrated dramatic phenotypes including complete immobilization of tumor cells (24). These experiments demonstrate that mechanisms capable of regulating Tspan clustering are potent regulators of Tspan activity.

Clustering of CD151 using monoclonal antibodies

As I alluded to above monoclonal antibodies have been indispensable to our understanding of not only tetraspanin functions but also in the identification of tetraspanin associated partner proteins. (214,215). Our laboratory has demonstrated that clustering of CD151 using the specific anti-CD151 antibody 1A5 promotes tumor cell immobiliza-

tion and inhibits metastasis *in vivo* (24). Monoclonal antibody (mAb) 1A5 was isolated in a screen for metastasis inhibitory antibodies and was subsequently shown to inhibit metastasis by inhibiting tumor cell intravasation (216). Although the head and neck small squamous cell carcinoma cell line (HNSCC) HEp3 served as the immunogen in the subtractive immunization approach used to generate 1A5 clustering of CD151 was able to inhibit *in vivo* metastasis of the fibrosarcoma cell line HT1080. *In vitro* treatment of tumor cells with 1A5 inhibits migration by promoting an increase in adhesion, which inhibits cell migration on all matrices tested including laminin, collagen, fibronectin and vitronectin(24). Collectively, this demonstrated that the clustering of CD151 controls motility by promoting a pan-integrin adhesive phentoype. Many of the functions and mechanisms of action related to clustering of CD151 suggested the involvement of the laminin binding integrins particularly α3β1 However,herein we present data to suggest that integrin independent mechanisms of CD151 exist. Interestingly, integrin free CD151 ($CD151^{free}$) is potentially involved in prostate cancer progression. The expression of CD151free is also a predictor of poor overall survival in prostate cancer This work will be presented in the following chapter.

CHAPTER IV

INTEGRIN-FREE TETRASPANIN CD151 CAN INHIBIT TUMOR CELL MOTILITY UPON CLUSTERING AND IS A CLINICAL INDICATOR OF PROSTATE CANCER PROGRESSION

Summary

Normal physiology relies on the organization of transmembrane proteins by molecular scaffolds, such as tetraspanins. Changes in their organization or expression are commonly associated with oncogenesis. The tetraspanin CD151 is thought to contribute to cancer progression through direct interaction with the laminin-binding integrins α3β1 and α6β1. However, this interaction cannot explain the ability of CD151 to control migration in the absence of these integrins nor its regulation of migration on non-laminin substrates. We demonstrate that CD151 can regulate tumor cell migration without direct integrin binding and that integrin-free CD151 ($CD151^{free}$) is clinically correlated with tumor progression and metastasis. Clustering of $CD151^{free}$ through its integrin-binding domain promotes its stable accumulation in areas of cell-cell contact leading to enhanced cell adhesion and subsequently inhibition of tumor cell motility *in vitro* and *in vivo*. $CD151^{free}$ clustering is a strong regulator of motility even in the absence of α3 expression but requires PKCα suggesting that CD151 can control migration independent of its integrin associations. The histological detection of $CD151^{free}$ in prostate cancer correlates with poor patient outcome. When $CD151^{free}$ is present, patients are more likely to recur after radical prostatectomy and progression to metastatic disease is accelerated. Multi-variate analysis identifies $CD151^{free}$ as an independent predictor of survival. Moreover, the detection of $CD151^{free}$ can stratify survival among patients with elevated PSA. Cumulatively these studies demonstrate that a subpopulation of CD151 exists on the surface of tumor cells that can regulate migration independent of its integrin partner. The clinical correla-

tion of CD151^{free} with prostate cancer progression suggests that this tetraspanin subpopulation may contribute to the disease and could be used to predict cancer progression.

Introduction

Cancer metastasis typically involves the migration and invasion of disseminating tumor cells. The activity of cytokine receptors, adhesion receptors and proteases responsible for this migration, is frequently controlled through their organization within the cell membrane. Tetraspanins are key membrane scaffolding proteins responsible for this organization. Although the tetraspanins lack enzymatic activity and canonical signal-transducing capacity, their ability to organize macromolecular structures is their primary mechanism of action (1). Of the 33 tetraspanin family members, 6 have been implicated in cancer (2, 3) and CD151 has been shown to be a particularly important regulator of tumor cell motility (4) and metastasis (5, 6). CD151 interacts with several partners including MMP14, cadherins, immunoglobulin proteins, and integrin subunits α3 and α6, as well as other members of the tetraspanin family (7-9). Consequently, further elucidation of tetraspanin function requires investigation of its molecular organization and the role of specific partners. The interaction between CD151 and the laminin binding integrins α3β1 and α6β1 has been investigated in detail (reviewed in (1) and (10)). Loss of CD151(11) diminished migration on laminin while disruption of the integrin-binding domain limits spreading on laminin and tubule morphogenesis as well as tumor growth in 3D laminin-containing matrixes (12, 13). Recently the CD151-integrin-laminin axis has been shown to facilitate EGF signaling (14-16). However, a number of biological activities have been associated with CD151 that are likely to involve different molecular mechanisms. These include migration on non-laminin substrates, platelet function and cell-cell adhesion. Effective approaches to investigating these functions have included genomic ablation (16-18), mutational analysis (12, 19, 20) and CD151-specific antibod-

ies (6, 21-24). These studies suggest that both integrin-dependent and integrin-independent roles exist for CD151 in tumor progression.

CD151 can be targeted to inhibit tumor cell motility and metastasis using the antibody mAB 1A5 (6). Treatment with mAB 1A5 prevents de-adhesion at the rear of migrating cells resulting in their immobilization and subsequent inhibition of metastatic dissemination(6). MAB 1A5, like most anti-CD151 antibodies, was generated in mice against a human antigen(25, 26). Consequently these antibodies primarily recognize antigenic differences between human and mouse CD151. Some CD151 antibodies exhibit differential staining patterns in human tissues most likely because certain epitopes are masked by protein-protein interactions of the CD151 complex(12, 22, 27-29). Epitope mapping allows these antibodies to identify the composition of a CD151 complex. Yamada et al., (23) used flow cytometry to map epitope binding of CD151 antibodies on CD151 mutants with substitutions at amino acid residues that are not conserved between human and mouse CD151. These studies demonstrated that several antibodies recognize an epitope on CD151 required for binding integrins including α3β1 (12). Interestingly the knockdown of α3 reduced the overall level of CD151 but did not diminish the level of CD151 recognized by antibodies to the integrin-binding domain. This observation suggests that distinct populations of CD151 are maintained on the cell surface. One of those populations is associated with integrins while the other is not. We will refer to these populations as $CD151^{ITGA}$ and $CD151^{free}$ respectively.

Considering the epitope-specificity of antibodies, it is likely that antibodies which recognize distinct CD151-partner complexes can provide insight into the function and clinical relevance of that CD151 subpopulation. We hypothesized that the inhibition of motility in response to mAB 1A5 was due to its ability to target a specific subpopulation of CD151 and affects its activity. Since a monovalent fragment of mAB 1A5 was unable to inhibit tumor cell motility, we predicted that this antibody mediated clustering of all, or a specific sub-population of CD151. Live-cell imaging was implemented to investigate

clustering in response to antibody binding. Using the flow cytometry approach developed by Yamada et. al. we determined that mAB 1A5 recognized the integrin-binding domain on CD151 and is therefore unable to interact with integrin-bound CD151. The analysis of tumor cell migration *in vivo* was used to investigate the ability of mAB 1A5 to control cell motility independent of integrin α3. These investigations were extended to other antibodies that recognize the same integrin-binding domain (8C3 and 14A2.H1) or bind an unrelated site (11G5A). Finally, we determined if the presence CD151 not bound by integrins was relevant in prostate cancer progression by histological analysis of $CD151^{free}$ in tissue from two cohorts of prostate cancer patients.

Materials and Methods

Cell culture, reagents and antibodies

HEp3 cells are perpetually maintained on the chick chorioallantoic membrane to retain metastatic and migratory potential (43-45). All cell lines were grown in media supplemented with pen/strep, sodium pyruvate, non-essential amino acids and 10% fetal bovine serum and cultured at 37℃ in 5% CO2 incubator and passaged every 2-4 days. HEp3, NIH3T3 and HT1080 cells were maintained in DMEM. A549 cells were maintained in RPMI. The CD151 plasmids in the eGFP-N1 vector (Clontech) were received from received from Dr. Kiyo Sekiguchi (University of Osaka, Japan). Transfections of all cells were performed using Extreme Gene HD (Roche). The mAB 1A5 and the control antibody 29-7 was generated as described previously (46).The anti-CD151 antibodies 11G5A and 14H2.1 were purchased from Abcam. Anti-C151 antibody 8C3 was generously provided by Dr. Sekiguchi. Anti-ITGA3 antibodies were purchased from Santa Cruz Biotechnology (P1B5) and Millipore. The smart pool RNAi specific to α3, PKCα, and the control siRNA was obtained from Dharmacon.

Tumor cell motility

In vitro cell migration

HEp3 and HT1080 cells were seeded in 6-well plates and allowed to attach overnight in DMEM containing 10% FBS on the following day the cells were switched to serum free/ insulin free media for an additional 24 hrs. On the day of the assay the confluent monolayers were scratched with a pipet tip in order to create a uniform wound after which the cells were washed with PBS to remove any floating cells. Cultures were returned to full medium and the wound was documented at 0 hrs and 16 hrs post-scratch using a light microscope TMS-F (Nikon) equipped with a D90 SLR camera (Nikon). Wound closure (% surface area) was determined using T-scratch image analysis software (47, 48).

In vivo cell motility

Assays were performed as previously described (6). Briefly, cells to be injected were washed 2 times with PBS and detached with 2mM EDTA. The cells were resuspended in PBS and injected IV into Day 12 chick embryos. Four days post-injection the disseminated colonies were photographed using a Lumar V12 stereomicroscope (Zeiss) equipped with a Retiga Exi camera and controlled with Volocity image acquisition software (PerkinElmer). Antibody treatments were introduced by intravenous injection one day after tumor cell injection. For visualization of the vasculature rhodamine-conjugated dextran was injected intravenously and allowed to circulate for 15 minutes prior to tissue collection. “Non-motile” colonies were defined as colonies comprised of 5 or more cells where individual cells remained in direct contact. Such non-motile colonies are compact while “motile” colonies contained a migratory cell populations dispersed in the CAM. Data is represented as the % of colonies within a single animal that demonstrated a motile phenotype.

Flow cytometry

Standard flow procedure

Cells to be used in flow cytometry experiments were trypsinized with 0.25% Trypsin-EDTA and resuspended in cold Milytenyl FACs buffer (2mM EDTA, 0.5%BSA, PBS). For the analysis of cell surface expression of specific antigens the cells were washed 2 times with FACs buffer and then stained with the specific primary antibodies for 1hr on ice. Following incubation with the primary antibody the cells were washed 2 times with cold FACs buffer and then incubated with species-specific, fluorophore-conjugated secondary antibody.

Epitope mapping using flow cytometry

NIH 3T3 were transfected with the CD151 human/mouse GFP substitution mutants {Yamada, 2008, I92090} using Fugene HD (Roche). Transfected cells were prepared for flow cytometry as described above 24 hr after transfection. Cells were stained with anti-CD151 antibodies (mAB 1A5, 8C3, 14A2H.1 and 11G5A) on ice for 1 hr followed two washes and incubation with Alexa 647-conjugated secondary antibody (1 hr on ice). The stained cells were washed twice with cold FACs buffer resuspended and analyzed by two-color flow cytometry. Untransfected controls and empty vector (pEGFP N1) transfections were used as a no color and GFP only positive controls respectively. An isotype IgG was used for as a control for gating purposes. In order to analyze the GFP-positive and Alexa 647 double-positive populations, the cells were gated for GFP expression to identify GFP expressing cells which were subsequently analyzed for their binding of Alexa 647-conjugated antibody (which represents binding of the respective anti-CD151 antibody). The data is presented as dot plots with the detection of GFP graphed on the X-axis and the detection of Alexa 647 plotted on the Y-axis.

Immunoprecipitation, immunoblotting, and immunodepletion

Immunopreciptation

HEp3 cells and A549 cells were lysed in either 1% (vol/vol) Triton X-100 lysis buffer or 1% (vol/vol) Brij-99 lysis buffer on ice for 30 minutes. The samples were cleared by centrifugation (14,000 rpm, 15 min) and cleared lysates were transferred to new tubes and stored at 4˚. Protein concentrations were determined by BCA (Pierce). Immunoprecipitations were performed by incubating 2 µg of the antibody with 1 mg total cell lysate over night at 4° with end-to-end rotation. The following day the protein/ antibody complexes were bound to Protein A/G sepharose beads (Fisher Scientific) for 4 hours at 4° with end-to-end rotation. Beads were collected by centrifugation (8000 rpm, 30 secs) washed with lysis buffer and stored together with unbound material for further analysis. Immunoprecipitated complexes were eluted in 100 µl Lamelli sample buffer and evaluated by immunoblot analysis. For each sample equal amounts of protein were loaded into SDS-PAGE and blotted onto polyvinylidene fluoride membranes (PVDF, Millipore). Protein detection was performed with enhanced chemiluminescence (ECL, Pierce) after blocking (5% non-fat milk from powder in PBS, 0.05% Tween-20) and incubation (O/N 4˚C) with antibodies prepared in blocking buffer.

Immunodepletion of mAB 1A5-bound CD151: 1) HEp3 or A549 cells were lysed in 1% Triton X-100 lysis buffer and incubated with 2 µg of antibody for two hr at 4˚ with end-to-end rotation. The immunecomplexes were then captured with Protein-G sepharose beads for 1hr at 4˚. This process was repeated 3 times using the unbound lysate. Each immunodepletion was evaluated by immunoblotting as described above. 2) For immunodepletion of mAB 1A5 bound to live cells HEp3 and A549 cells were treated overnight with mAB 1A5 or control antibody at a concentration of 2 µg/ml. Antibody-bound cells were lysed 24 hr later and antibody-bound CD151 was immunodepleted with protein-G sepahrose beads. The unbound material was depleted 2 additional times with

protein-G beads and bound as well as unbound material was evaluated by immunoblotting.

Cell Surface Biotinylation: For cell surface labeling confluent HEp3 cells were treated with antibody for 1 hr on ice and then washed 3 times with cold PBS. Cultures were subsequently biotinylated with sulfosuccinimidyl-6-[biotin-amido]hexanoate using the EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Scientific) according to manufacturer's instructions. Cells were lysed in either 1% Brij 99 followed by extraction of the insoluble material with RIPA buffer Immunoprecipitation was performed as described above. Biotinylated proteins were detected with peroxidase-conjugated strepavidin.

Live cell imaging and immunofluorescent staining

In order to visualize CD151 localization HEp3 cells expressing GFP-tagged CD151 were imaged with a fully automated microscope (BX61, Olympus) equipped with a digital camera (Orca ER, Hammamatsu) every 5 minutes for 1hr. Cells were subsequently treated with Alexa 647-conjugated mAB 1A5 and imaging continued for an additional 3 hours. Movies were analyzed and complied using Volocity Image Acquisition and Analysis Software (PerkinElmer).

Immunofluorescent detection of anti-CD151 antibodies bound to cultured cells was accomplished by staining paraformaldehyde fixed cells with Alexa 546-conjugated secondary antibody. Fibronectin fibrilogenesis was detected on paraformaldehyde fixed cells by immunofluorescent staining with rabbit anti-fibronectin antibody in conjunction with Alexa 546-conjugated anti-rabbit secondary antibodies. Images were captured with a fluorescent microscope using Volocity Image Acquisition and Analysis Software (Perkin-Elmer).

Platelet aggregation assay

Platelet aggregation assays were performed as previously described (49). Blood to be used from donors was collected and mixed 1:10 with 3.8% Sodium Citrate Buffer and spun at 160 X g for 30 minutes. The platelet rich plasma (PRP) was counted and resuspended at a concentration of 2-4 $X10^8$ cells/mL. Aggregation of PRP was performed on a BIO/DATA Corporation PAP-4 aggregometer at 37℃ with stirring (1200rpm). Individual treatments were added as outlined in the text and turbidity analyzed compared to the aggregation of platelet poor plasma (PPP).

Cell clustering assay

Suspension cultures of Jurkat and U937 cells were treated with control antibody or mAB 1A5 overnight using serum free culture medium. Clustering formation was documented at 100X magnification with a digital camera (Nikon D90) mounted to a phase-contrast microscope.

Collagen contraction assays

HEp3 cells and their treatments were mixed with neutralized collagen solution (1 mg/ml in 1X cell culture medium) at a concentration of 0.4 million cells/ml. The collagen/cell mixture was placed in the wells of a 24-well plate, allowed to solidify at 37˚C and cultured for 48 hrs before analysis. The collagen was subsequently released from the wall of the culture well using a metal spatula and contraction of the collagen plug was documented 1 hr after release. The collagen plugs were documented at 100X magnification with a digital camera (Nikon D90) mounted to a phase-contract microscope.

Patients and samples

Two retrospective cohorts of patients with PCa were utilized in this study. The first cohort (cohort #1) of 99 cases underwent radical retropubic prostatectomy (RRP)

between 1994-1998 at the London Health Sciences Centre with pathological stage pT2 - pT3 PCa (Supplemental Tables 1 and 2). A second cohort (cohort #2) of 38 cases was comprised of diagnostic biopsy specimens from PCa patients at the same center who did not undergo RRP and developed metastatic disease during follow-up (Supplemental Table 3). Pathological staging was re-evaluated according to TNM criteria (AJCC 2002). Patient characteristics and disease-related outcomes were obtained from a review of the medical records (Supplemental Tables 1-3). This study obtained approval from an independent local Ethics Committee (UWO REB #15084E) and obtained the informed consent from all patients for the research use of their tissues.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks were cut into 4 μm sections and mounted onto positively charged glass slides. The tissue was deparaffinized and rehydrated using the xylene, graded ethanol, and water method. A two-step epitope retrieval was performed whereby tissue sections were boiled in the microwave in 10 mM Sodium Citrate, 0.05% Tween 20 pH 6.0. The slides were then allowed to cool down, washed with PBS 0.2% Triton-X (PBST), and incubated with pre-warmed pepsin (digest-all™ 3, Invitrogen) for 3 minutes. After washing with PBST, the specimens were stained using the UltraVision Detection System (Thermo-Scientific). CD151 mAb 1A5 was diluted 1:800 in 1% BSA in PBS, CD151 mAb 11G5a (Abcam) was diluted 1:100 in 1% BSA in PBS and α3β1 antibody P1B5 was diluted 1:250 in 1% BSA in PBS. Samples were then counterstained with Hematoxylin and mounted with Cytoseal (Thermo-Scientific). Negative controls were prepared using the identical treatment with omission of the primary antibody. Non-immune mouse IgG was used as an additional negative control.

CD151 expression analysis

CD151 expression in the RRP and biopsy cohorts detected by immunohistochemistry was categorized as negative (score=0), weakly positive (score=1), moderately positive (score=2) or strongly positive (score=3) independently by two pathologists who had no knowledge of the patient's clinical status. A consensus was reached where any discrepancy was identified. To determine the statistical significance of mAb 1A5 immunoreactivity, specimens were divided into negative (score=0) and positive (score=1-3). CD151 protein expression analysis was performed in cancerous areas as well as, benign tissue adjacent and distant from areas of tumour. Biochemical recurrence after RRP was defined as two consecutive PSA measurements greater than 0.2 ng/mL. Bone metastasis was defined as presence of metastatic lesions on a bone scan.

Profiling of CD151 mRNA expression in normal and prostate cancer tissue was accomplished using publicly available data obtained from the NCBI Gene Expression Omnibus (GEO) as well as The Cancer Genome Atlas (TCGA) via The cBio Cancer Genomics Portal (217). These included GDS2545 (n=171) (50), GDS1439 (n=19) (51), GSE6099 (n=102) (52) and the prostate adenocarcinoma dataset from TCGA. Datasets were categorized according to their clinical diagnosis and analyzed by ANOVA with post-test analysis for linear trend. CD151 expression levels were extracted from publicly available expression dataset GDS3113 (n=3/tissue) (53) to demonstrate relative levels of CD151 expression in prostate compared to other tissues.

Statistical Analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC), SPSS 20 (IBM), and Graphpad Prism (Graphpad software, La Jolla, CA). Experimental groups in migrations assays were compared to the control groups using a non-parametric Mann-Whitney test. The primary endpoint for cohort #1 is biochemical recurrence-free survival and the primary endpoint for cohort #2 is metastasis-free survival. Correlations between mAB 1A5 immunoreactivity and biochemical recurrence-free

survival in malignant areas compared to benign areas were analyzed by McNemar's chi-square test. Kaplan–Meier plots were used to assess biochemical recurrence-free and metastasis-free survival. Group comparisons were made using the log-rank test. The Cox proportional hazard model was applied for multivariable analysis. In all statistical analyses, a two-sided,$p<0.05$ was considered statistically significant. Graphical representation of statistical significance included * ($p< 0.05$), ** ($p< 0.01$) and *** ($p<0.001$).

Image Processing and Analysis

Included images are representative of at least three replicate experiments. Fluorescent images are false-colored to represent specific labels. The brightness of images captured of *in vivo* experiments was uniformly adjusted for purposes of clarity. Where indicated the images were cropped and scaled without change in resolution. Quantitation of signal was performed on raw image files in Volocity Image Acquisition and Analysis Software (PerkinElmer) prior to creating the figures.

RESULTS

CD151 clusters and accumulates at areas of cell-cell contact in response to mAB 1A5 binding

Given that tetraspanins facilitate macromolecular organization within cellular membranes, we hypothesized that the ability of anti-CD151 antibodies to influence migration involved changes in CD151 localization. This possibility was evaluated by analyzing changes in CD151 surface distribution in response to antibody binding (Fig. 10). NIH3T3 cells transfected with human CD151-GFP exhibited a uniform surface distribution of CD151 when cultured at subconfluent densities (Fig. 10A and Bi/i'). Upon antibody binding CD151-GFP re-organized into punctate structures on the cell surface (Fig 10A and Bii) and accumulated at areas of cell-cell contact (Fig. 10A and Biii). To confirm that antibody-bound CD151-GFP complexes were on the cell surface, a TRITC-

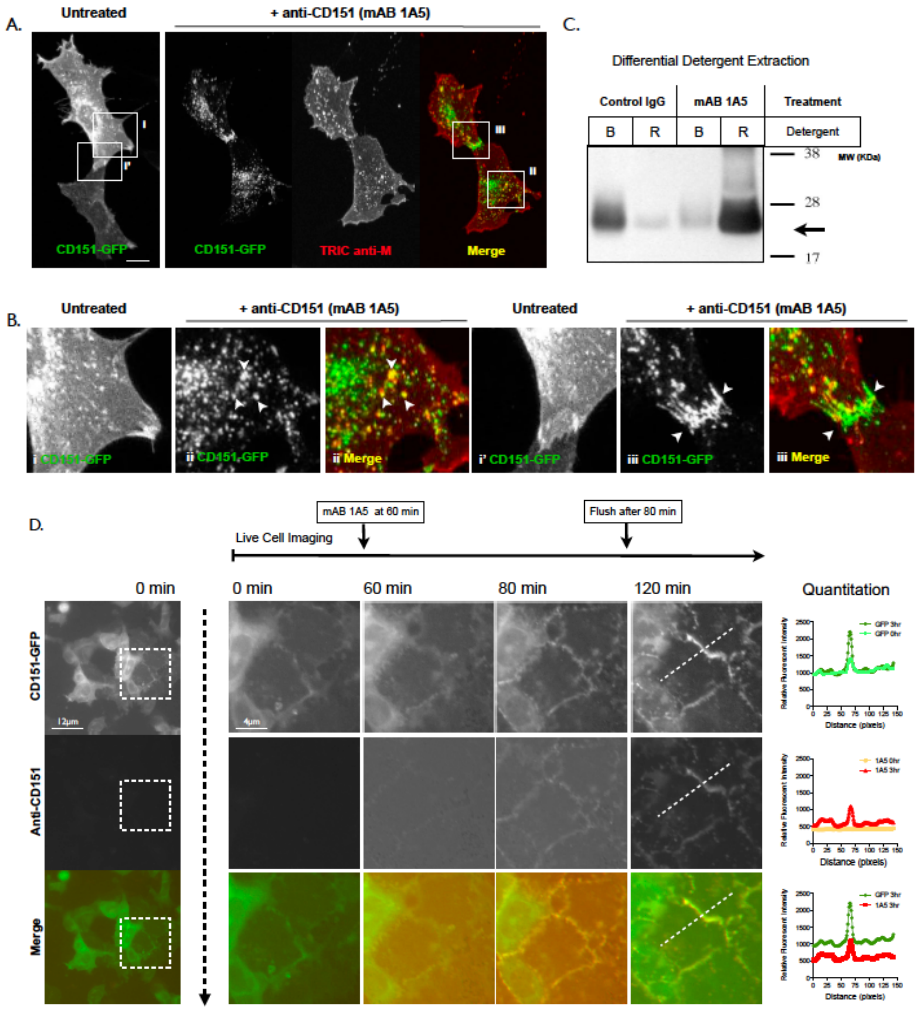

Figure 10. Cell surface clustering of CD151 in response to mAB 1A5. The cell surface distribution of CD151 was visualized by fluorescent microscopy in NIH3T3 cells after transfection with CD151-GFP and culture in the absence or presence of mAB 1A5 (2μg/ml) for 2hr. (A) CD151-GFP visualized in the absence (left panel) or presence of mAB 1A5 (right panels, 2μg/ml for 2hr). Surface bound antibody was detected using TRIC-conjugated anti-mouse IgG post fixation. Insets in A emphasizing general cell surface (i and ii) v.s. areas of cell-cell contact (i' and iii) are magnified in (B). (C) Surface CD151 was detected in sequential Brij99 and RIPA extractions with avidin-HRP after biotinylation of surface proteins and immunoprecipitating the tetraspanin from HEp3 cells cultured in the presence of a control IgG or mAB 1A5 for 24hr. (D) Live cell imaging was used to evaluate changes in CD151 distribution in response to mAB 1A5 binding. A549 cells transfected with CD151-GFP (green) were imaged every 5 min for 2 hr with the addition of Alexa 546-conjugated mAB 1A5 (red) added at t=60 min and flushed from the system at t=80 min. Data is representative of 3 independent experiments. Fluorescence quantitation across the indicated white dashed line was performed in Volocity and calculated as the mean fluorescence in intensity minus background. Quantitation is representative of ≥5 cells per experiment.-

conjugated secondary antibody was used to detect mAB 1A5 on the cell surface of intact cells fixed 2 hr after antibody treatment. Similar observations were made with HELA, HEp3, HT1080 and A549 cells. This data suggests that mAB 1A5 ligation of CD151 promotes the formation of macromolecular (clustering) on the cell surface. Clustering of tetraspanins is known to cause a change in detergent solubility (1). To determine if antibody-mediated clustering induced a change in solubility we performed cell surface biotinylation and differential detergent extraction of HEp3 cells treated with mAB 1A5 (Fig. 10C). Indeed, cell surface CD151 (biotinylated-CD151, arrow) became resistant to extraction by Brij99 and was solubilized by subsequent RIPA extraction. The increase in relative abundance of biotinylated CD151 in the RIPA re-extraction confirms that the clustered tetraspanin accumulated in detergent-resistant complexes on the cell surface.

When cell densities were sufficient to allow for cell-cell contact, antibody-binding induced clustering of CD151 at areas of cell-cell contact (Fig. 10Biii) rather than at randomly distributed surface aggregates. To further assess the recruitment of CD151 to areas of cell contact, live-cell microscopy of densely cultured A549 cells expressing CD151-GFP was performed during the addition of Alexa 546-conjugated mAB 1A5 (Fig. 10D). A549 cells have intact cell-cell contacts (30) where CD151 is readily visible. Addition of mAB 1A5 caused rapid accumulation of both labeled antibody and CD151-GFP in areas of cell-cell contact (Fig. 10D “0 min” v.s. “120 min”). The accumulation of CD151 at areas of cell-cell contact was quantified by mapping pixel intensity from time 0 to 120 min. in the GFP channel (CD151-GFP) and the Alexa 546 channel across a reference line. Although A549 cells have well established cell-cell contacts, similar observations were made for epithelial-derived cancer lines that lack stable cell-cell contacts such as the HNSCC HEp3 during *in vitro* culture (Fig. 11) and *in vivo* tumor growth (Fig. 12). Thus clustered CD151 accumulated at areas of cell-cell in all models and experimental settings.

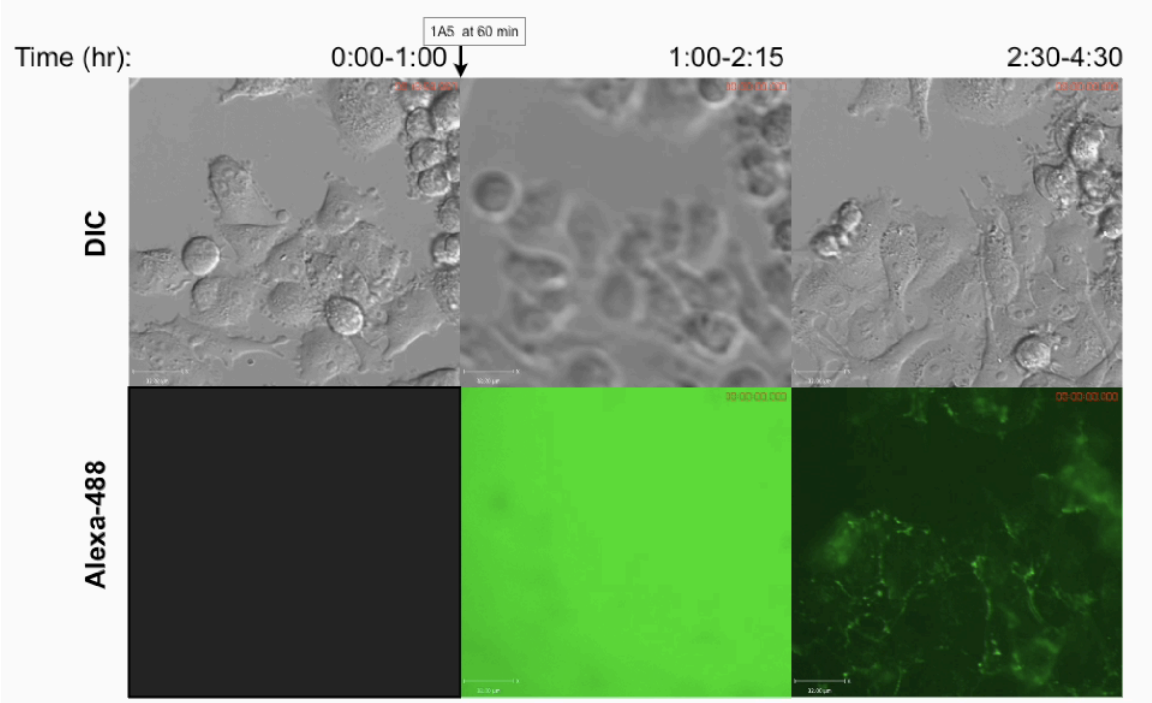

Fig. 11.Screen shot of movie depicting CD151 clustering at areas of cell-cell contact in HEp3 tumor cells. HEp3 cells cultured to 80% confluency were monitored by time-lapse microscopy before, during and after treatment with 1µg/ml Alexa 488-conjugated mAB 1A5. Cell behavior was monitored by differential interference contrast (DIC) imaging while antibody localization was monitored by fluorescence microscopy.

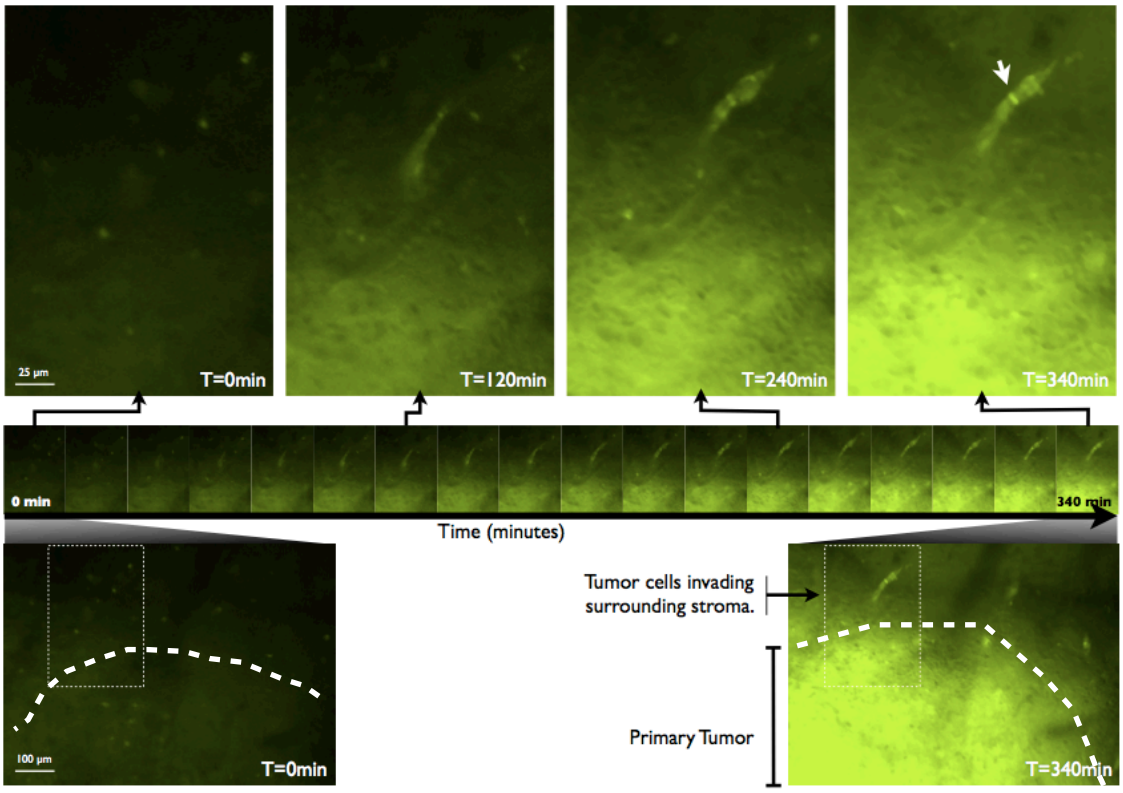

Figure 12. **CD151 clustering at areas of cell-cell contact *in vivo*.** Chick embryos bearing a tumor composed of HEp3 cells were treated with Alexa 488-conjugated mAB 1A5 (5 µg/animal). Accumulation of fluorescently-labelled antibody in the tumor was document with time-lapse imaging of Alexa 488 fluorescence. The bottom panels represent the full micrograph at 0 and 340 minutes with the primary tumor outlined by the white dashed line. The central panel shows a full time-lapse of the cropped region (dashed white lines). The top panels are magnified to reveal accumulation at intermediate time points. A white arrow highlights accumulation of mAB 1A5 at the area of cell- cell contact between two tumor cells invading stroma adjacent to the tumor. Demonstrating that in migrating cells in vivo CD151 localizes to the areas of cell-cell contact in the presence of 1A5. Data is representative of 3 independent experiments >5 cells were tracked in each experiment.

Antibody-induced clustering promotes an adhesive phenotype

We previously observed that mAB 1A5 prevents detachment at the rear of migrating cells thereby inhibiting tumor cell migration and metastasis *in vivo* (6). Evaluation of HEp3 migration at increasing concentrations of mAB 1A5 demonstrated a dose-dependent inhibition of migration *in vitro* (Fig. 13A) and *in vivo* (Fig. 13B). In the presence of control antibody, GFP-expressing HEp3 form metastatic colonies comprised of widely spaced individual cells that are disseminating freely into the surrounding tissue. In contrast, treatment with mAB 1A5 limited cell motility resulting in compact colonies at concentrations as low as 0.5μg antibody/animal (Fig. 13B, upper right panel). The ability to immobilize tumor cells *in vivo* as such low antibody concentrations together with previous observed inhibition of detachment (6) suggests that the tetraspanin clustering mediates a gain of adhesive function.

To confirm that CD151 clustering in response to mAB 1A5 induces adhesion, we evaluated a range of cellular behaviors dependent upon adhesion including collagen contraction, fibronectin fibrilogenesis, platelet aggregation and clustering of non-adherent cells. Collagen contraction assays performed in the presence of mAB 1A5 demonstrated that CD151 clustering promotes collagen contraction when compared to cells treated with the control antibody (Fig. 13C). This contrasts with the inhibition of collagen contraction by agents that inhibit matrix remodeling by MMPs (GM6001) or block integrin-mediated adhesion to collagen (anti-α2β1 integrin). Fibronectin fibrilogenesis, an integrin-dependent process (31, 32), was evaluated in HT1080 cells. These cells exhibit very limited fibril assembly during routine culture but treatment with mAB 1A5 resulted in the formation of visible fibrils compared to the control IgG treated cells (Fig. 13 D). Platelet aggregation is an integrin-dependent process stimulated by collagen binding or thrombin-mediated protease activated receptors (PARs) (33). Platelets express an abundance of CD151 (34) where it contributes functionally to platelet aggregation via αIIbβ3 (17). Treatment with mAB 1A5 dose-dependent platelet aggregation which could

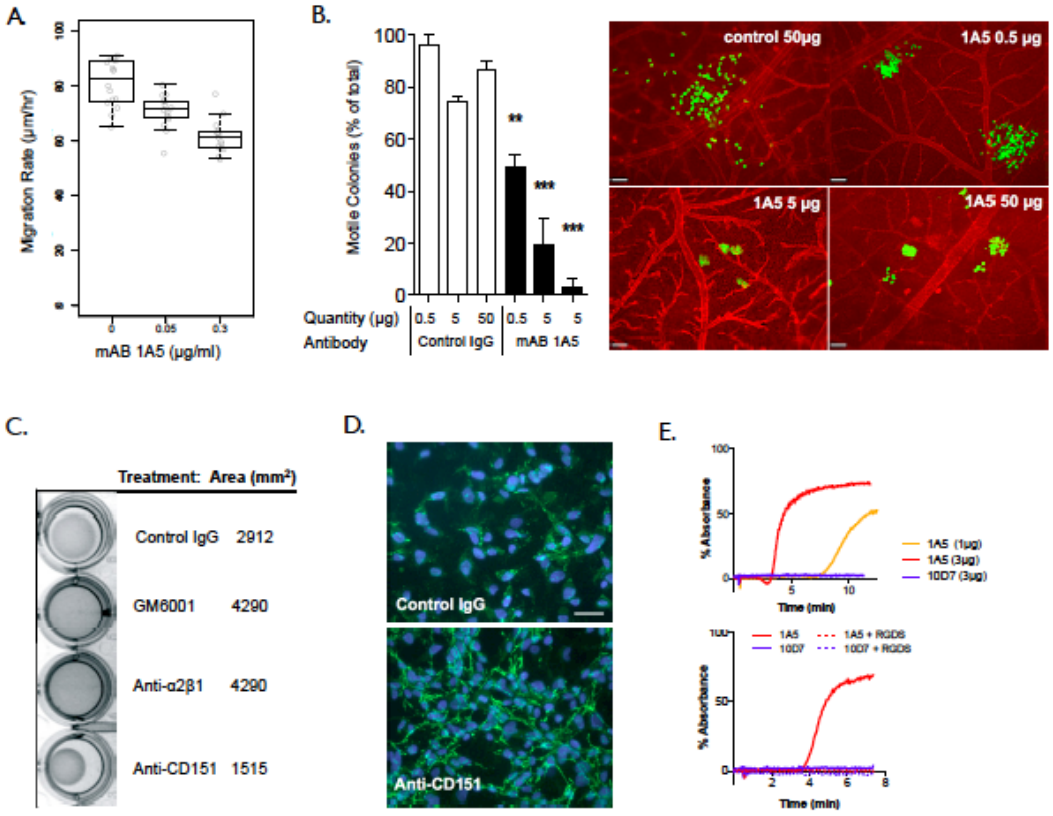

Figure 13. Antibody-mediated clustering promotes an adhesive phenotype. (A) Dose-dependent inhibition of HEp3 cell migration in response to mAB 1A5 using an *in vitro* wound healing assay. (B) Dose-dependent inhibition of HEp3 cell migration in the CAM of chick embryos in response to mAB 1A5 treatment. (C) Collagen contraction by HT1080 cells in response to mAB 1A5 when compared to control IgG, inhibition of MMP activity (GM6001) and blocking of collagen binding (α2β1). (D) Fibronectin fibrilogenesis in response to mAB 1A5 or control IgG treatment as detected by immunofluorescent staining of fibronectin fibrils. (E) Platelet aggregation in response to mAB 1A5 treatment in the presence or absence of RGDS peptides to block integrin-mediated adhesions.Data is represented as SEM * ($p<0.05$), **($p<0.01$), *** ($p>0.001$). Data is representative of 3 independent experiments. For in vivo colonization in B data is representative of $n\geq5$ for each experiment.

be prevented with integrin-blocking cyclic RGDS peptide suggesting ligand-specific adhesion through integrins (Fig. 13E). Lastly, antibody-binding promoted clustering of lymphoma (U937) and leukemia (Jurkat) cells (Fig. 14A and B). Staining of suspended cells with mAB 1A5 after fixation demonstrates uniform surface distribution of CD151 (post-fixation, . Fig. 14B). In contrast, treatment of the cells with mAB 1A5 prior to fixation induced clustering of the cells and resulted in accumulation of the antibody-CD151 complex at areas of cell-cell contact similar to what was observed for adherent cells (Fig. 10). These observations consistently demonstrate the clustering of CD151 to promote cell adhesion.

MAB1A5 binds CD151 not associated with integrin α3 ($CD151^{free}$)

The ability of CD151 to contribute to adhesion and motility on laminin through direct interaction with the laminin-binding integrins α3β1 and α6β1 is well established(11, 12). However, much of the adhesive behavior described above did not involve laminin and adhesion to collagen, assembly of fibronectin, and the aggregation of platelets (Fig. 13 C-E) does not involve integrins that contain the α3 or α6 subunits. These observations suggest that CD151 clustering and biological activity in response to mAB 1A5 was independent of its association with laminin-binding integrins. Yamada et. al. (23) demonstrated that several CD151 antibodies recognize the integrin-binding domain of CD151 and only bind CD151 that is dissociated from its integrin partners ($CD151^{free}$). Together, these observations suggest that an integrin-independent mechanism might be involved in the regulation of adhesion by CD151.To determine if mAB 1A5 selectively recognizes $CD151^{free}$ vs $CD151^{ITGA}$, its ability to co-immunoprecipitate an integrin partner was evaluated.. A comparison was made with an antibody specific for the integrin binding domain which cannot precipitate thetegrins (8C3) and an antibody that binds outside this domain and readily precipitates the integrin (11G5A) (23). Since our principle tumor

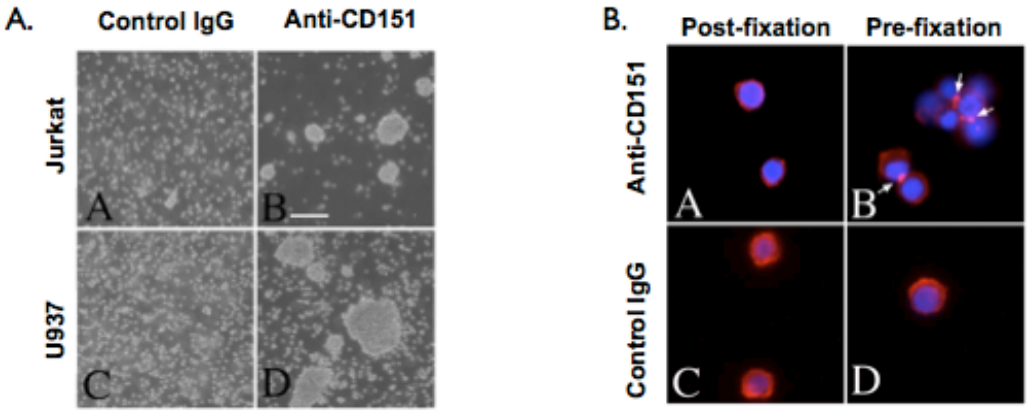

Figure 14. Cell-cell adhesion in response to CD151 clustering. (A) Jurkat and U937cells cultured in the absence or presence of either control IgG or mAB 1A5. (B) MAB 1A5 (A and B) or control IgG (C and D) were added to Jurkat cells after (A and C) or 1 hr before fixing the cell with paraformaldehyde. The antibody location was detected using Alexa 546-conjugated anti-mouse IgG. Data is representative of 3 independent experiments with >5 cells imaged per experiment in B.

model (HEp3) is nearly devoid of α6, we evaluated integrin interactions primarily by monitoring α3. Immunoprecipitates were evaluated for the presence of α3 by immuno munoblotting (Fig. 15A). The integrin subunit α3 co-immunoprecipitated with CD151 bound by mAB 11G5A but not 1A5 or 8C3 (Fig. 15A). Similar observations were made in immunoprecipitations from HEp3 cells (. Fig. 16). To determine if mAB 1A5 bound the CD151/α3β1 complex in intact cells, we treated A549 cells overnight with mAB 1A5 (2μg/ml) and lysed the cultures in Triton X-100 lysis buffer after removing all unbound antibody. The cell lysate was immuno-depleted with protein-G sepharose beads in three sequential incubations and the presence of α3 and CD151 was subsequently evaluated in both the bound and unbound fractions. Antibody-bound CD151 was removed in the first round of immuno-depletion while α3 remained in the unbound fraction. To confirm that the CD151 which remained in the unbound fraction remained associated with α3, we immunoprecipitated the integrin with mAB P1B5 and evaluated both bound and unbound fractions for α3 and CD151 (Fig. 15C). The majority of CD151 in the lysate co-immunoprecipitated with α3. Since mAB 1A5 failed to co-precipitate α3 and the integrin remained associated with CD151 not bound by the antibody we conclude that mAB 1A5 only binds CD151 that is not engaged with α3.

Monoclonal antibody 1A5 specifically recognizes the integrin-binding domain of CD151

The integrin-binding domain of CD151 includes the ^{194}QRD^{196} sequence of its large extracellular loop (12). Anti-CD151 antibodies that are unable to precipitate the CD151/α3β1 complex frequently bind this domain (35). Yamada and colleagues used 2-color flow cytometry to map epitope-binding of human-specific anti-CD151 antibodies according to their reactivity with CD151 mutants in which twelve residues across the large extracellular loop (LEL) of the human protein were substituted with their mouse counterpart (23)(Figure 17A and B). We implemented a similar approach to determine which residues of CD151 are involved in recognition by mAB 1A5.

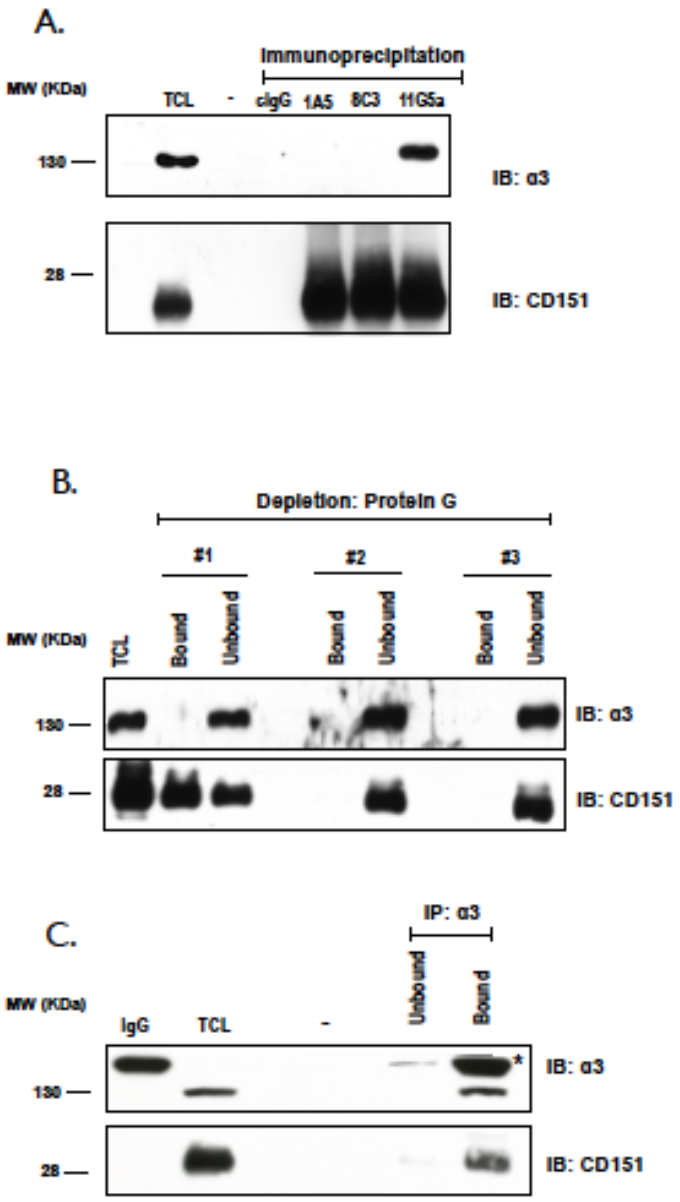

Figure 15. MAB 1A5 binds to CD151 not engaged with integrin α3. (A) Immunoblot of α3 and CD151 after immunoprecipitation of the tetraspanin from A549 cell lysates (Triton X-100) using non-specific mouse antibody (cIgG) and three distinct antibodies against CD151 (1A5, 8C3, and 11G5a). (B) A549 cells were culture in the presence of mAB 1A5 for 24 hr. Cell lysates were generated with Triton X-100 and antibody bound CD151 was depleted from the lysate in three sequential incubations with immobilized Protein-G. Bound and unbound fractions were analyzed for α3 and CD151 by immunoblotting. (C) The integrin subunit α3 was immunoprecipitated from the unbound fraction of the 3rd depletion from (B) with anti-α3 (P1B5) and this sample was evaluated for the presence of α3 and co-precipitated CD151 by immunoblotting. “TCL” refers to Total Cell Lysate while IgG refers to normal mouse included as a reference for the IgG band (*). Data is representative of 3 independent experiments.

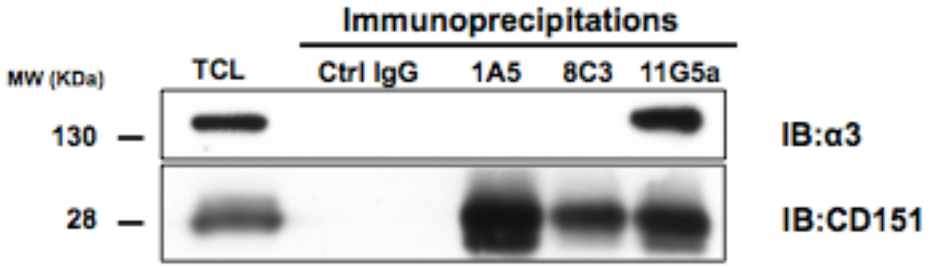

Fig. 16.MAB 1A5 binds to CD151 not engaged with α3 integrin in HEp3 cells. CD151 was immunoprecipitated from HEp3 cell lysates using three distinct anti-CD151 antibodies (1A5, 8C3 and 11G5A). The precipitated CD151 and the co-immunoprecipation **α**3 was detected by immunoblotting. Data is representative of 3 independent experiments.

Monoclonal antibody 1A5 specifically recognizes the integrin-binding domain of CD151

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Antibodies that recognize the integrin-binding domain of CD151 can inhibit tumor cell motility in vivo

Yamada et. al. (23) stratified anti-CD151 antibodies according their epitope specificity. MAB 1A5 belongs to “group I” which contains antibodies that specifically re

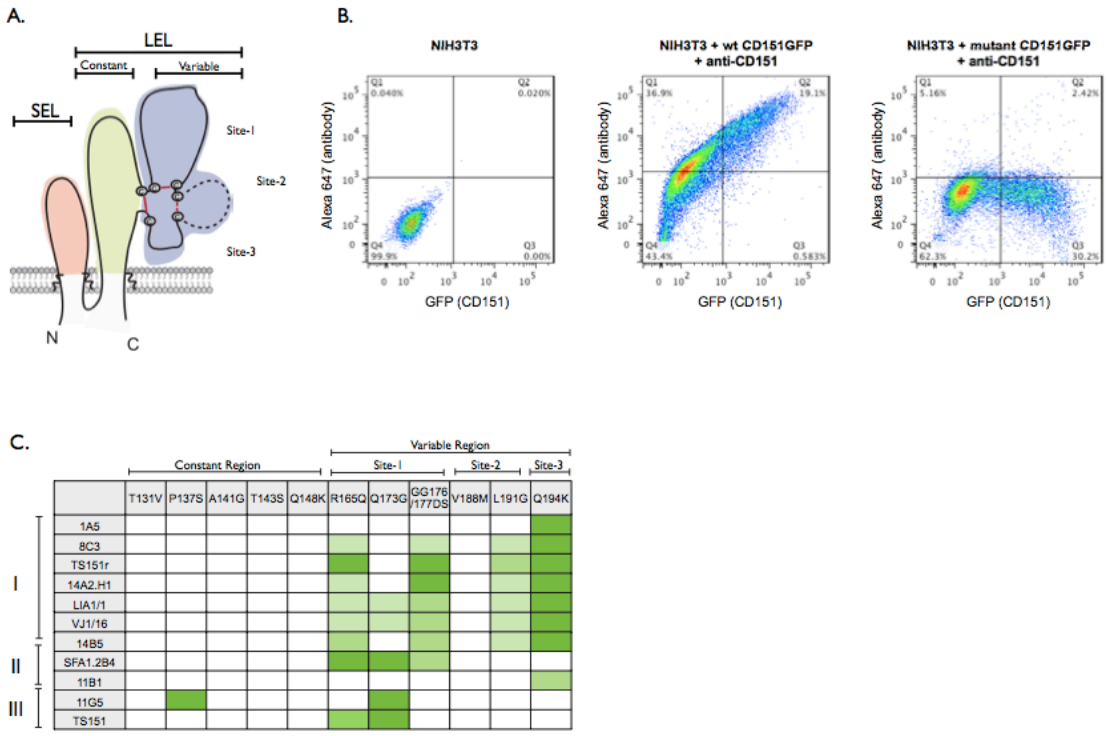


Figure 17.MAb 1A5 recognizes the integrin-binding domain of CD151. (A) Schematic of CD151 highlighting the extracellular domains of the protein including the small extracellular loop (SEL) and the large extracellular loop (LEL). The LEL as depicted is divided into 2 regions a constant region and a variable region. The variable region can be further divided into 3 subdomains denoted site 1, site 2 and site 3. (B) Representative flow cytometric dot plot of NIH3T3 cells gated for GFP and Alexa 647. The three panels show NIH 3T3 cells the were mock transfected, transfected with a wildtype CD151-GFP, or transfected with mutant CD151-GFP not recognized by the antibody. (C) Table adapted from Yamada et. al (201) grouping the various anti-CD151 monoclonal antibodies including mAB 1A5 according to their abilities to bind to the CD151-LEL mutants as demonstrated by two-color flow cytometry. Data is representative of >3 independent experiments.

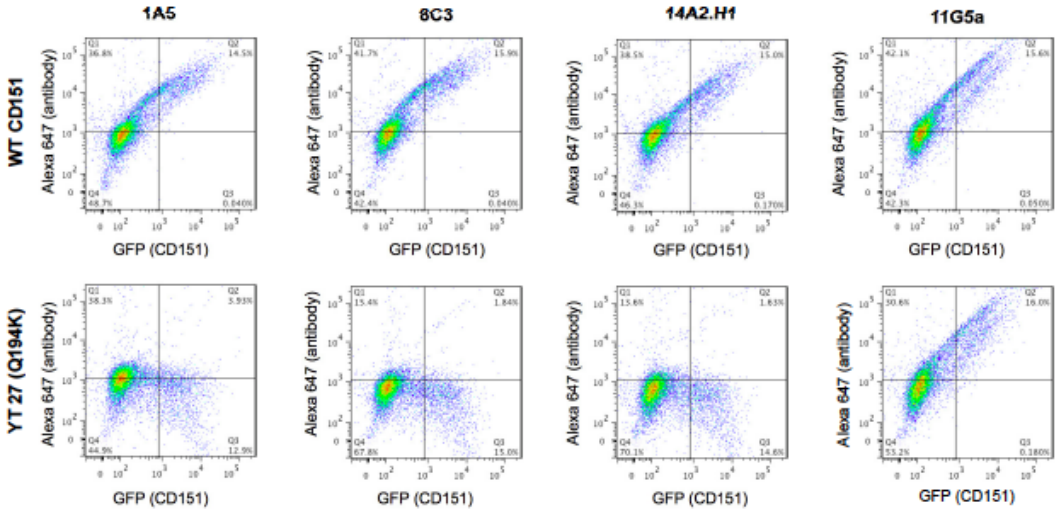


Fig. 18. MAb 1A5 recognizes the integrin-binding domain of CD151. Two-color flow cytometry of the CD151 human/mouse substitution mutants was used to evaluate what epitope on CD151 is required for mAB 1A5 binding. NIH3T3 cells were transiently transfected with WT or mutant CD151-GFP constructs. Transgene expression was detected using GFP while antibody binding was detected using an Alexa 647-conjugate anti-mouse IgG. A comparison is made between WT CD151 and the YT 27 (Q194K) mutant which disrupts binding with the α3 integrin subunit. mAb 1A5 is compared to two antibodies known for their inability to bind this mutant (8C3 and 14A2.H1) and one antibody whose binding is unaffected by this mutation (11G5A). A loss of antibody binding is represented by a decrease in the percentage of GFP and Alexa 647 double positive cells in quadrant (Q2). Data is representative of greater > 3 independent experiments.

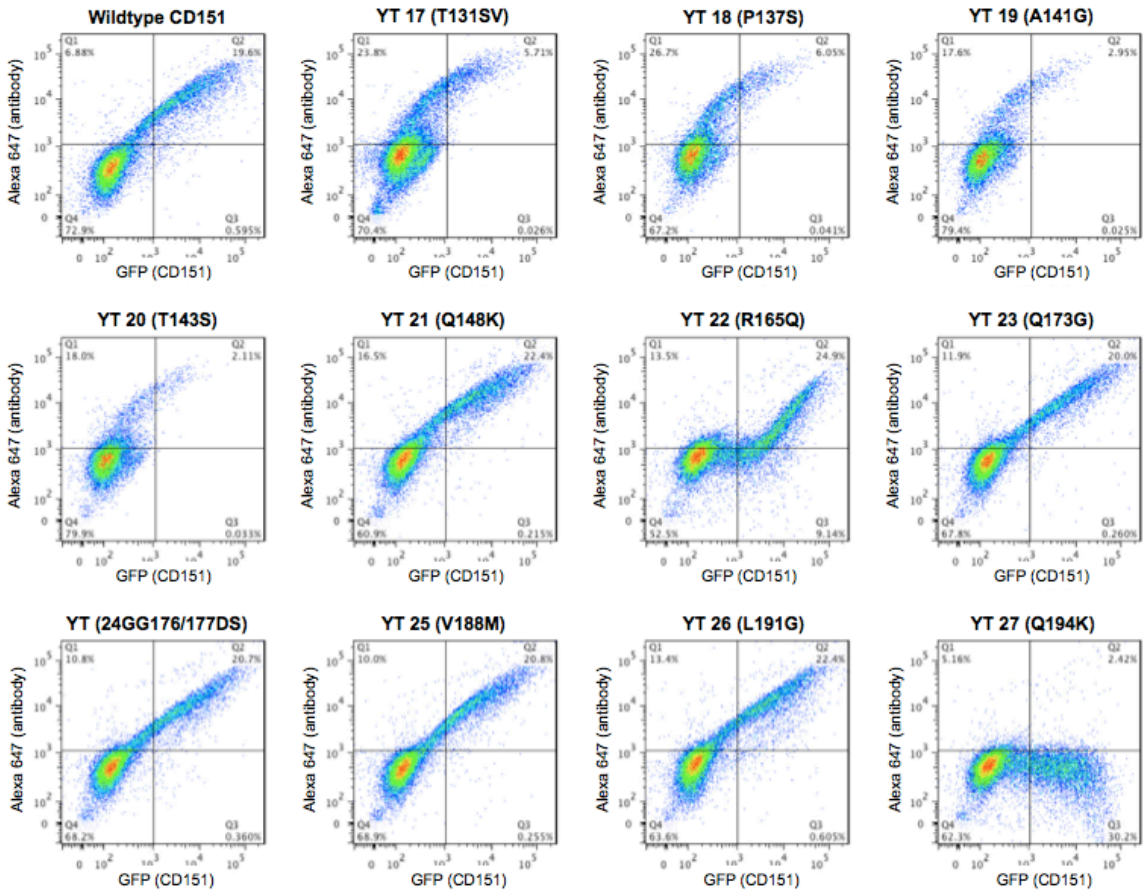


Fig. 19. MAb 1A5 recognizes the integrin binding domain of CD151. The remaining human/mouse substitution mutants were used in 2 color flow cytometry with 1A5.Data is representative of >3 independent experiments.

cognize CD151^{free} because they bind the integrin binding domain and fail to immunoprecipitate α3. Since mAB 8C3 and 1A5 share specificity we hypothesized that they might also share the ability to cluster the tetraspanin at areas of cell-cell contact. Live-cell imaging of A549 cells expressing CD151-GFP confirmed that mAB 8C3 but not 11G5A can cluster the tetraspanin (Figure 20). Since antibodies in "Group I" share both domain-specificity and the ability to cluster CD151, we explored the possibility that these antibodies specific for CD151^{free} can control tumor cell migration. To achieve this, HEp3-GFP cells injected I.V. into chick embryos were treated with antibodies specific for CD151^{free} (8C3, 14A2.H1 and 1A5) and an antibody that binds outside this domai(11G5A) or a control antibody (Fig. 21A). Metastatic colonies were allowed to form for 3 days before their morphology was documented and the number of colonies with a motile phenotype was quantified (Fig. 21B). At 0.5-5 μg/animal only mAB 1A5 inhibited tumor cell motility *in vivo*. However, at 20 μg/animal tumor cell motility was inhibited by 8C3 and 14A2.H1 but not 11G5A or the control antibody. These data demonstrate that antibodies specific for CD151^{free} are capable of inhibiting tumor cell migration by clustering CD151 not associated with integrins.

Tumor cell immobilization in response to CD151 clustering requires PKCα but not integrin α3

To determine if α3 is required for the immobilization mediated by CD151*, in vivo* migration assays were performed following α3 knockdown. HEp3 cells were transfected with control or α3-specific siRNA and a ≥90% reduction in α3 expression after siRNA delivery was confirmed by immunoblotting (Fig. 22A). HEp3 cells bearing control or α3-specific siRNA were subsequently injected into chick embryos and allowed to form metastatic colonies in the presence of mAB 1A5 or a control antibody (29-7). Knockdown of α3 did not impair the migration of tumor cells as evidenced by the absence of coloni- with an immobilized phenotype (Fig. 22B and C). Moreover, in the presence of mAB 1A5

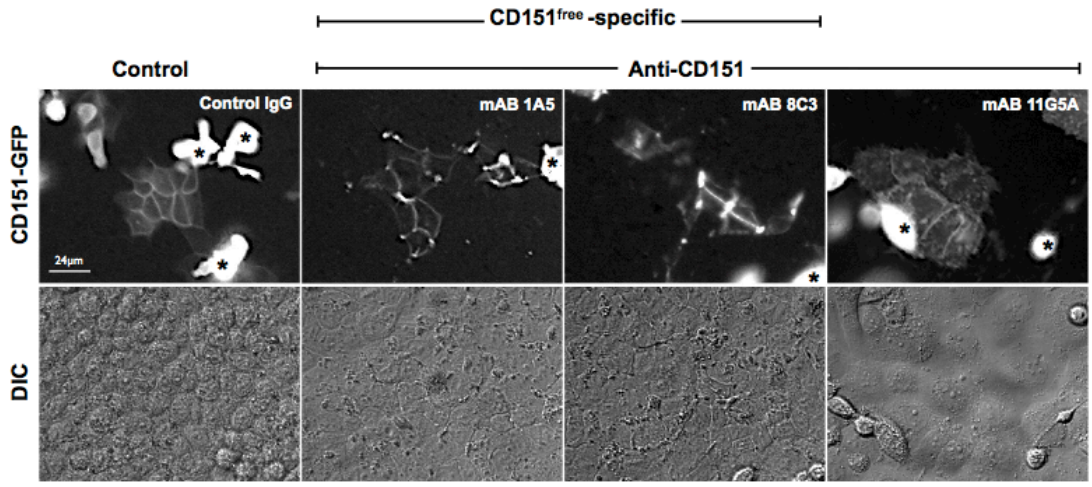


Fig 20. Antibodies that recognize the integrin-binding domain of CD151 mediate clustering at areas of cell-cell contact. A549 cells transiently transfected with CD151-GFP were treated with the control IgG or the anti-CD151 antibodies 1A5, 8C3 or 11G5A at 5 µg/ml. Localization of the tetraspanin was documented by fluorescent microscopy. *refer to those cells that are expressing high levels of CD151GFP. Data is representative of 3 independent experiments with >5 cells imaged per experiment.

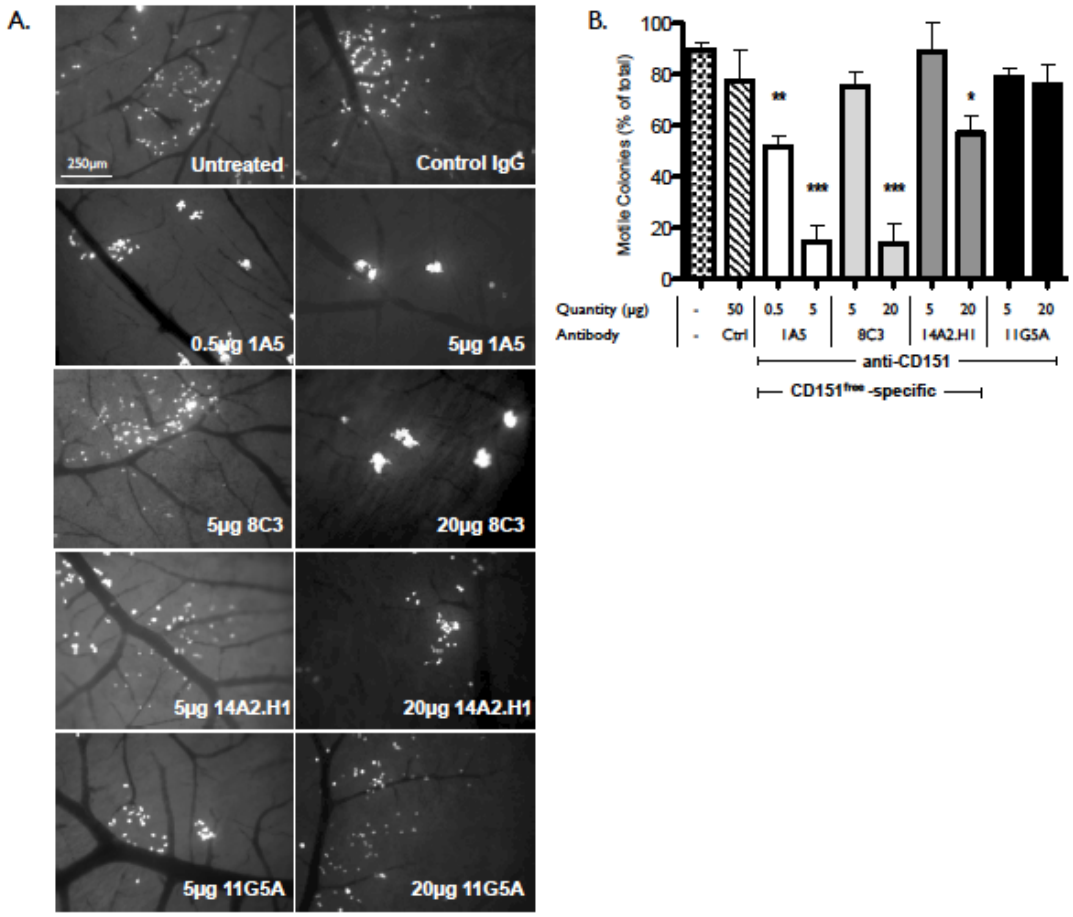


Fig. 21. Clustering of CD151 through antibodies specific for the integrin-binding domain inhibits tumor cell motility *in vivo*. Changes in tumor cell motility in response to anti-CD151 antibodies were determined by analyzing the formation of metastatic colonies in the chorioallantic membrane. (A) The inhibition of tumor cell dissemination in the presence or absence of anti-CD151 antibodies (1A5, 8C3, 14A2.H1, and 11G5A) was compared to the dissemination in the absence of tumor targeting antibodies (untreated) or a control IgG. Images were taken after 4 days of treatment with 0.5, 5, or 20µg or antibody (A) and the number of colonies with motile tumor cells were quantified (B). Data is represented as SEM *($p<0.05$), **($p<0.01$), ***($p<0.001$). Data is representative of three experiments with $n \geq 5$ for each experiment.

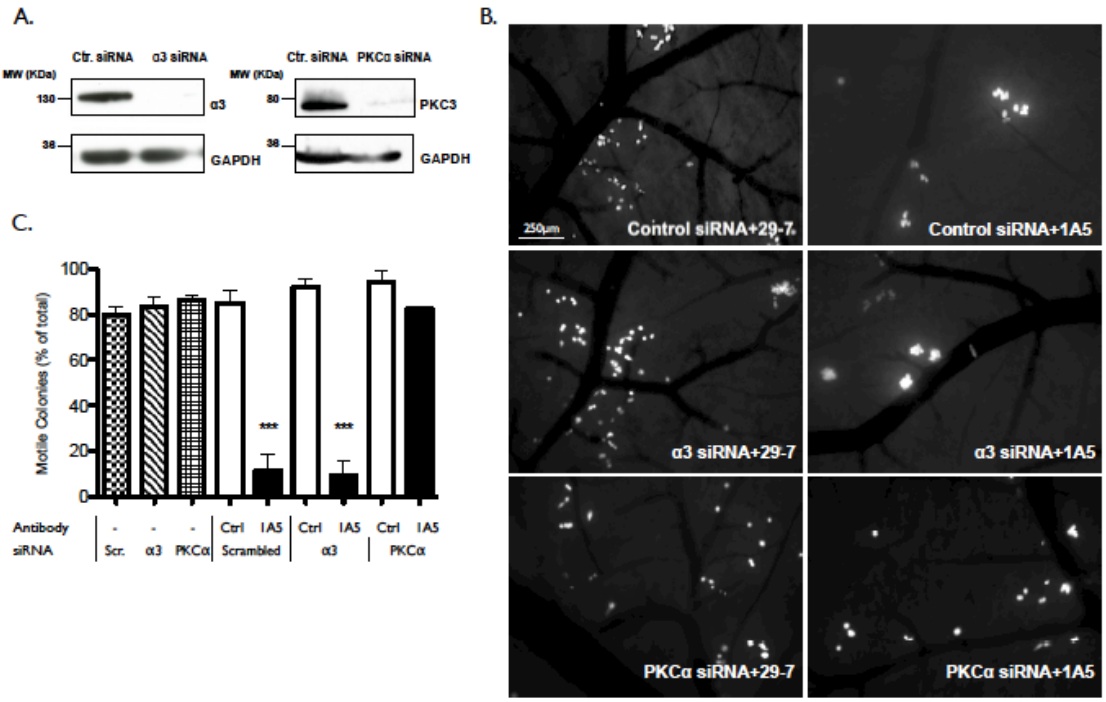


Fig. 22. PKCα but not integrin α3 is required for the regulation of motility through CD151^{free}. (A) Immunoblotting of Triton-X 100 lysates from HEp3 cells transfected with the control, integrin α3-specific or PKCα-specific siRNA to evaluate the efficacy of target gene knockdown. GAPDH is included as a loading control. (B) The analysis of tumor cell motility *in vivo* after delivery of control, integrin α3 or PKCα siRNA. Tumor-bearing animals were treated with control IgG or mAB 1A5 (5μg) 1 day after tumor cell injection. Images of metastatic colonies were taken 2 days after antibody injection. The immobilization of tumor cells in response to mAB 1A5 was quantified in (C). Data is represented as SEM *($p<0.05$), **($p<0.01$), ***($p<0.001$). Data is representative of three experiments with $n \geq 5$ for each experiment.

both HEp3 control- and α3-siRNA transfected cells form compact colonies demonstrating that the absence of α3 does not alter the ability of CD151-clustering to inhibit motility and colonization *in vivo*. PKCα is a known partner of CD151. To determine if PKCα is required for the inhibition of tumor cell motility upon CD151 clustering, *in vivo* migration assays were performed following PKCα knockdown (Fig. 22). The knockdown of PKCα did not affect tumor cell motility but did prevent immobilization in response to mAB 1A5 induced clustering of CD151.

The integrin-binding domain of CD151 is detectable in prostate cancer and correlates with poor patient outcome

Antibodies specific for integrin-binding domain of CD151 define a subpopulation of this tetraspanin ($CD151^{free}$) that is distinct from the tetraspanin bound to its integrin partner ($CD151^{ITGA}$). The immobilization of tumor cells through clustering of $CD151^{free}$ (Fig. 21 and 22) and the subsequent inhibition of metastasis (6) demonstrates that $CD151^{free}$ may be relevant in the malignant progression of cancer. Recent work demonstrated a specific role for CD151 in the progression of prostate cancer (36). We hypothesized that detection of $CD151^{free}$ is altered during tumor progression and correlates with patient outcome. This relationship was evaluated in prostate cancer by comparing the detection of $CD151^{free}$ to biochemical recurrence (for patients who underwent radical retropubic prostatectomy (RRP): cohort #1, N=99) or metastasis (for patients who did not undergo RRP: cohort #2, N=38). See tables 7-9 for patient information.

Histological detection with the mAB 11G5A (which binds both $CD151^{ITGA}$ and $CD151^{free}$) demonstrates abundant expression of CD151 in both normal and malignant prostate tissues. Conversely, mAB 1A5 (which binds only $CD151^{free}$) exhibited weak staining of normal prostate glands but elevated detection of $CD151^{free}$ in tissue from prostate cancers (Fig. 23A). CD151 gene expression is relatively abundant in normal adult prostate tissue (. Fig. 24A). An analysis of CD151 mRNA expression in prostate

Table 7 Demographic information of patients that underwent RRP at the London Regional Cancer Program between 1994 and 1998 (*n*=99) data unpublished collected by the laboratory of Dr. John Lewis

Variable	**Value (SD)**
Age (years)	62.4 (5.7)
PSA (ng/mL)	9.7 (5.2)
Follow up (years)	12.1 (1.6)
Clinical Stage	**# patients (% of total)**
T1	36 (36.4%)
T2	55 (55.5%)
T3	2 (2.0%)
Unknown	6 (6.1%)

Table 8. Pathological and clinical outcomes of patients that underwent RRP at the London Regional Cancer Program between 1994 and 1998 (*n*=99) data unpublished collected by the laboratory of Dr. John Lewis.

Variable	# patients (% of total)
Pathological stage	
pT2	60 (60.6%)
pT3	35 (35.4%)
pT4	4 (4%)
Gleason	
≤6	26 (26.2%)
7	38 (38.3%)
≥8	27 (27.3%)
Unknown	8 (8.0%)
Margins	
Negative	76 (77.6%)
Positive	23 (23.2%)
Seminal vesicles	
Negative	87 (87.9%)
Positive	12 (12.1%)
Positive Nodes	0
Biochemical failure	35 (35.4%)
Hormone resistance	1 (1%)
Bone metastasis	2 (2.1%)
Solid organ metastasis	2 (2.1%)

Table 9.Demographic information of patients that developed metastasis during follow up at the London Regional Cancer Program between 1994 and 1998 (*n*=38) data unpublished collected by the laboratory of Dr. John Lewis.

Variable	**% (SD)**
Age (years)	66.7 (9.76)
Clinical Stage	**# patients (% of total)**
T1	6 (15.7%)
T2	19 (50%)
T3	10 (26.3%)
T4	1 (2.6%)
Unknown	2 (5.2%)
Gleason	
≤7	19 (50%)
≥8	19 (50%)
Hormone resistance	31 (81.5%)
Bone metastasis	34 (89.4%)

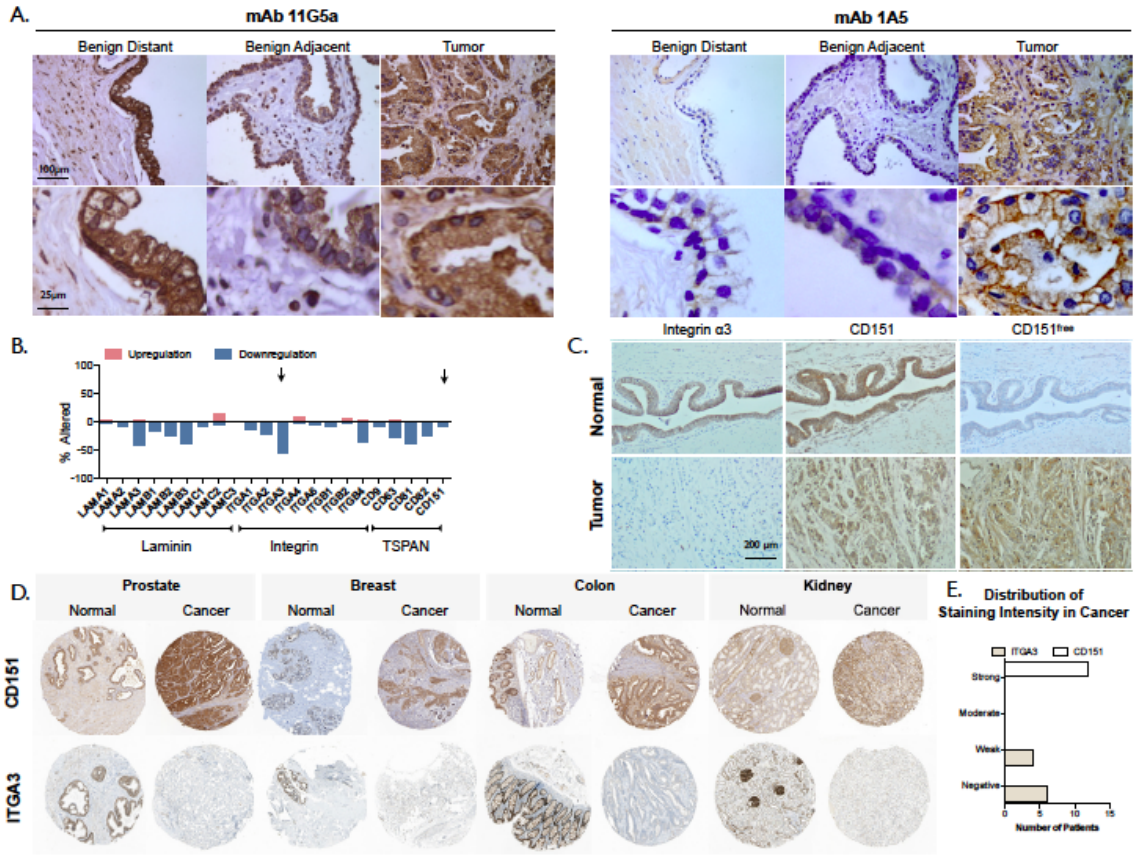


Fig. 23. Detection of CD151^{free} in prostate cancer corresponds with poor patient outcome. Immunohistochemical detection of CD151 was performed on two cohorts of prostate cancer patients (see methods). General expression of CD151 was detected with mAB 11G5A which recognizes all CD151 complexes while mAB 1A5 was used to detect CD151 not engaged with α3 (CD151^{free}). (A) Micrographs of CD151 immunohistochemistry with mAB 11GA or 1A5 on adjacent benign, adjacent normal and tumor tissue. human prostate cancer tissue while 1A5 does not. In the primary tumor however, similar to 11G5A, 1A5 positively stains the primary tumor. (B) Evaluation of gene expression in a TCGA data set of 216 patients with prostate adenocarcinoma. Arrows indicate integrin α3 (ITGA3) and CD151. (C) Immunohistochemical detection of integrin α3 (P1B5, left) CD151 (11G5A, middle), CD151^{free} (1A5, right) and in normal (top) and tumor tissue (bottom). (D) Immunohistochemical detection using polyclonal antibodies for CD151 (top row) and integrin α3 (bottom row) in normal and tumor tissue of the prostate, breast, colon and kidney obtained through ProteinAtlas.org. Each image is representative from a collection of 10-12 patients. (E) quantitation of staining intensity of prostate cancer patients in D.

cancer studies available through The Cancer Genome Atlas (TCGA; CD151, Fig. 24) and NCBI GEO (. Fig. 24B-C) revealed no significant changes in CD151 gene expression. This observation is in accordance with histological assessment of CD151 protein detection with 11G5A (Fig. 23A). In contrast, 55% of the 216 prostate adenocarcinoma patients analyzed in the TCGA exhibited reduced expression of integrin α3 (ITGA3, Fig. 23B). This observation suggested that the appearance of $CD151^{free}$ might coincide with reduced expression of integrin α3. Indeed, paired histological analysis of advanced prostate cancer tumors reveals that CD151 is readily detected in the tumor tissue, adjacent normal and distant normal tissue. In contrast, integrin α3 expression is reduced in the tumor relative to normal tissue and $CD151^{free}$ is detectable only in the tumor (Fig. 23C). Independent histological evaluation of integrin α3 and CD151 available through the Human Protein Atlas confirms reduced expression of the integrin in tumor tissue compared to normal tissue not only in prostate cancer (Fig. 23D quantified in E) but also in cancers of the breast, colon and kidney (Fig. 23D).

To evaluate the correlation of $CD151^{free}$ with patient outcome in prostate cancer, we compared the levels of $CD151^{free}$ to biochemical recurrence (for patients who underwent radical retropubic prostatectomy (RRP): cohort #1, N=99) or metastasis (for patients who did not undergo RRP: cohort #2, N=38). See tables 8-10 for cohort description. First, we dichotomized patients that underwent RRP (cohort #1) based on mAB 1A5 staining (positive vs. negative). $CD151^{free}$ staining on benign tissue adjacent to the tumor (Fig. 25A) and normal tissue distant from the tumor (Fig. 25B) failed to demonstrate an association with biochemical recurrence. In contrast, detection of $CD151^{free}$ (mAB 1A5 positive) in tumor tissue corresponded to a significantly reduced time-to-biochemical recurrence compared to tumor tissue with no detectable levels of $CD151^{free}$ (mAB 1A5 negative, Fig. 26A; mean survival = 10.3 vs. 13.5; Log-Rank, p=0.023). Moreover, the detection of $CD151^{free}$ further stratified patients that were already stratified by high (≥8) and low (<8) baseline PSA levels in regards to biochemical recurrence (Fig.26B). Bio

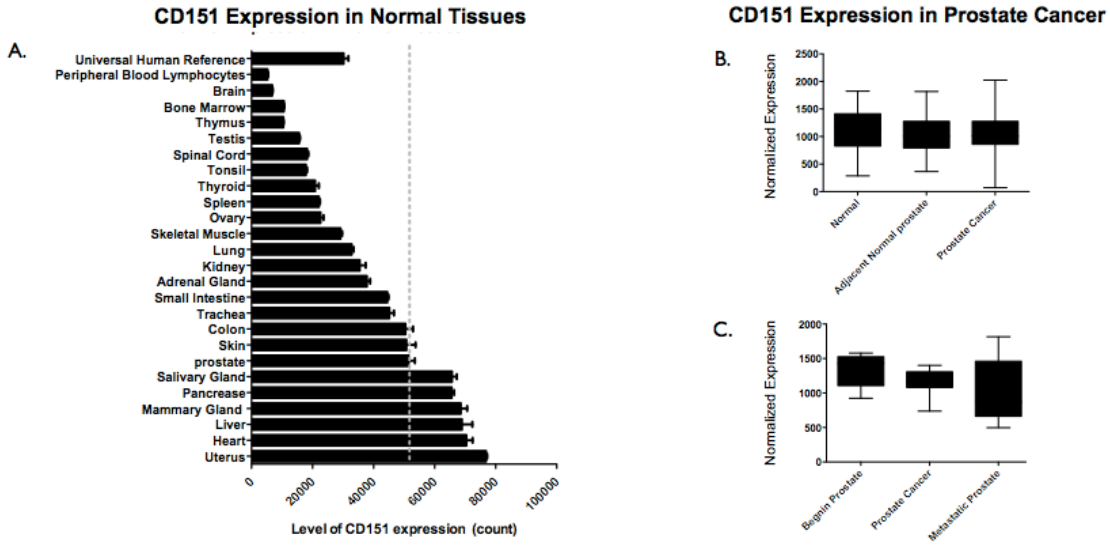


Fig. 24 Evaluation of CD151 expression in prostate tissue using the publicly available GDS3113 (n=3/tissue). Tissues are arranged according to expression levels following the universal human reference. The dashed line corresponds to the level of expression found in normal prostate. A comparison of CD151 expression in prostate cancer vs. normal or benign tissue was accomplished using GSE2545 (B, n=171) and GSE6099 (C, n=102) respectively.

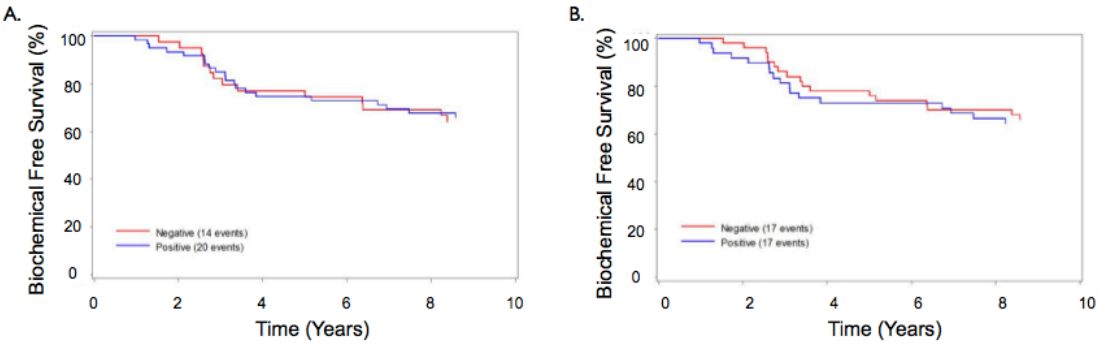
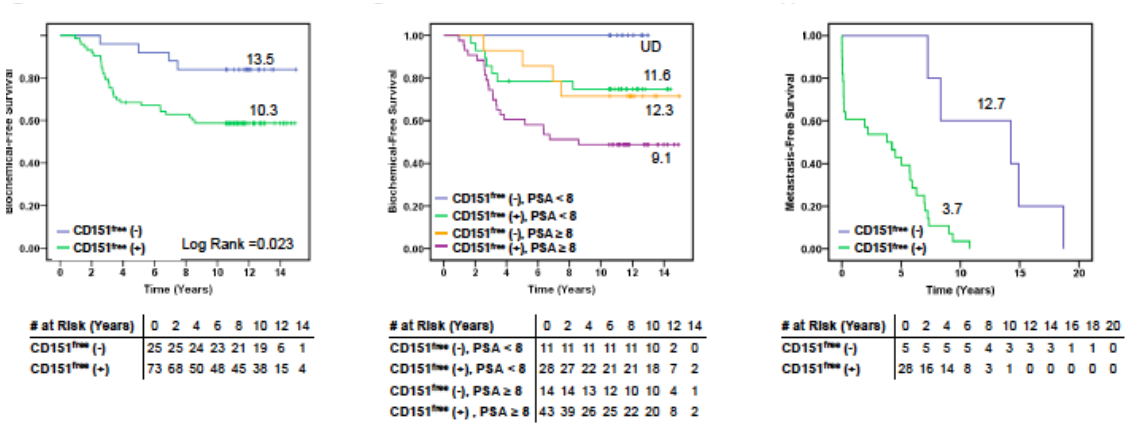


Figure 25.CD151^{free} detectable in benign and normal prostate tissue does not correspond with patient outcome. Kaplan-Meier curves of recurrence-free survival generated using the CD151^{free} immunoreactivity in tissue sections of prostate tissue obtained from patients that received a RRP after diagnosis whom were monitored for biochemical recurrence (cohort #1, N=99). A analysis of CD151^{free} staining on adjacent benign (A) and distant normal tissue (B).

chemical recurrence-free survival is reduced in patients that are singly positive for either CD151^{free} or high PSA compared to those negative for both (mean survival = 11.6 and 12.3 years vs. no; Log-Rank, p=0.068) Furthermore, singly positive. patients have significantly greater biochemical recurrence-free survival compared to patients that are double positive(mean survival = 11.6 and 12.3 years vs. 9.1 years; Log-Rank, p=0.017). Most importantly, in patients with high PSA, CD151^{free} (positive vs. negative) is an independent predictor of biochemical recurrence-free survival after adjusting for tumor stage (adjusted hazards ratio = 13.5; 95%CI 1.2-148.1; p=0.033). To determine if the presence of CD151^{free} in the primary tumor could be indicative of future metastasis, we evaluated biopsy specimens from prostate cancer patients that did not undergo RRP and eventually developed metastasis (cohort #2). Patients were dichotomized according to absence/presence of mAB 1A5 staining and its relation to metastasis-free survival was visualized using Kaplan-Meier analysis (Fig. 26C). Patients with mAB 1A5 positive biopsies at time of diagnosis exhibited a greatly reduced metastasis-free survival (mean survival = 3.7 vs. 12.7; Log rank p=0.001). These observations indicate that CD151^{free} is evident only in tumor tissues where its appearance coincides with reduced integrin α3 expression and its detection is an independent negative predictor of biochemical-free as well as metastasis-free survival.



# at Risk (Years)	0	2	4	6	8	10	12	14
CD151free (-)	25	25	24	23	21	19	6	1
CD151free (+)	73	68	50	48	45	38	15	4

# at Risk (Years)	0	2	4	6	8	10	12	14
CD151free (-), PSA < 8	11	11	11	11	11	10	2	0
CD151free (+), PSA < 8	28	27	22	21	21	18	7	2
CD151free (-), PSA ≥ 8	14	14	13	12	10	10	4	1
CD151free (+) , PSA ≥ 8	43	39	26	25	22	20	8	2

# at Risk (Years)	0	2	4	6	8	10	12	14	16	18	20
CD151free (-)	5	5	5	5	4	3	3	3	1	1	0
CD151free (+)	28	16	14	8	3	1	0	0	0	0	0

Fig. 26. The expression of CD151free is a negative prognostic marker of metastasis free survival and recurrance Kaplan-Meier curves of recurrence-free survival were generated using the CD151^{free} immunoreactivity in tissue sections of prostate tissue obtained from patients that received a RRP after diagnosis whom were monitored for biochemical recurrence (cohort #1, N=99). Log-Rank test-p=0.023.

CONCLUSIONS

The tetraspanin CD151 is an established regulator of cell adhesion, migration and tumor cell metastasis (5, 14, 25, 36, 37). Its interactions with the integrin subunits α3 and α6 have been shown to be important for adhesion, migration and morphogenesis on laminin (12, 13, 15, 20, 38). However, using immunoprecipitation and flow cytometry we demonstrate that there is a subpopulation of CD151 on the surface of tumor cells that is not associated with its integrin partner ($CD151^{free}$). This population of $CD151^{free}$ can be detected with antibodies specific for the integrin-binding domain of CD151, including mAB 1A5, 8C3 and 14A2.H1.

We previously demonstrated that anti-CD151 mAB 1A5 potently inhibits tumor cell migration and metastasis (6) by preventing de-adhesion at the rear of the cell. The inhibition of motility required a bivalent antibody suggesting that mAB 1A5 could mediate its activity by clustering the tetraspanin. Indeed, mAB 1A5, along with other antibodies specific for the integrin-binding domain of CD151 cluster the tetraspanin. Clustering of $CD151^{free}$ promotes cell adhesion and results in tumor cell immobilization, enhanced collagen contraction, greater fibronectin fibrilogenesis, improved platelet aggregation and elevated cell-cell adhesion. Although many of these activities are integrin-dependent, they do not involve adhesion to laminin nor association with the laminin-binding integrins that are established integrin partners of CD151. Indeed, the immobilization of tumor cells in response to the clustering of $CD151^{free}$ does not require α3 expression (Fig. 22). We have previously demonstrated that the loss of CD151 does not lead to tumor cell immobilization (6), thus the inhibition of tumor cell motility associated with the clustering of $CD151^{free}$ is likely a distinct mechanism for the control of cellular adhesion and migration.

These observations raise a number of questions including: a) what mechanism creates $CD151^{free}$? b) does $CD151^{free}$ occur in normal tissue? c) is the mechanism by which $CD151^{free}$ controls motility distinct from the mechanism of integrin-associated

CD151? and d) is CD151^{free} a tumor promoter? While fully answering these questions will require further investigation, significant insight is available from these and published studies. CD151^{free} could become available when the stoichiometric balance with its integrin partners is altered. This alteration is evident when the expression of integrin α3 is significantly reduced in tumor relative to normal tissue (Fig. 7) but could also occur when CD151 expression is upregulated as reported for hepatic carcinomas {{Ke:2009be}. However, CD151^{free} is also likely to occur in normal tissues when expression of the laminin-binding integrins is reduced or absent. This is evident in cardiac tissue which exhibits little α3 and α6 expression but an abundance of CD151 that is detectable by antibodies specific to the integrin binding epitope (11B1, 14A2.H1 (27). Even more evident is the presence of CD151^{free} in the bone marrow, circulating hematopoietic cells and platelets where the laminin-binding integrins have low expression and the surface expression of CD151^{free} is readily detectable with any of the antibodies specific for integrin-binding epitope The ability of mAB 1A5 to promote adhesion in platelets and suspended hematopoietic cell lines (Fig. 1) is perhaps the first and most obvious evidence that CD151^{free} can control an adhesive mechanism distinct from the laminin-binding integrins. The ability of CD151^{free} clustering to inhibit tumor cell migration after the knockdown of α3 (Fig. 22) suggests that CD151^{free} can also regulate adhesion and migration of epithelial-derived tumor cells through a mechanism independent of laminin-binding integrins. A preliminary evaluation of the CD151 signaling mechanisms revealed that neither Rac nor Rho activity was altered upon CD151^{free} clustering. However, the canonical signaling partner of CD151, PKCα, was required for CD151^{free} clustering to inhibit tumor cell motility. Although PKCα is generally thought of as a promoter of migration, PKCα was identified as an inhibitor of cell migration by the Brugge laboratory during an *in vitro* screening assay of normal epithelial migration (218).

We hypothesize that the distinct mechanisms by which CD151 can regulate cell adhesion and migration are determined primarily by the nature of the associated partner

and the cellular context in which they function. In the context of a transformed cancer cell the CD151-integrin-laminin axis ("$CD151^{ITGA}$", 15, 16) participates in promoting tumor cell invasion and metastasis (11, 12, 14, 38, 39) while in normal, quiescent epithelia the association of CD151 with the laminin binding integrins is required for cohesion and (non-motile) epithelial integrity (183,219). Clearly the loss of CD151 diminishes both normal epithelial integrity and cancer metastasis suggesting that both phenomena, which are on opposing ends of the motility spectrum, utilize the adhesion facilitated by CD151. Conversely, $CD151^{free}$ present on migratory/metastatic cells as well as non-adherent cells (hemopoietic cells & platelets) can initiate/promote adhesion resulting in aggregation (platelets, Fig. 13; non-adherent cells, Fig. 14) or inhibition of migration (tumor cells, Fig. 13 and (24) when clustered.

Cell motility is directly linked to dynamic cycling of adhesion/de-adhesion (220). Firm adhesion as seen in normal epithelial cells on laminin-containing basement membranes limits cell motility but promotes epithelial integrity (220-222). Uncoupling CD151 from the laminin binding integrins would promote a less adhesive, more motile phenotype. This is not only supported by the reduction of integrin α3 in tumor cells but also the loss of the integrin during *in vitro* selection for matrix invasion (223). Considering the immobility of firmly anchored cells in normal epithelia, the creation of $CD151^{free}$ during tumorigenesis is a simple mechanism to promote motility. Furthermore, $CD151^{free}$ can be engaged (clustered) to stimulate adhesion (Fig. 13, l. Fig. 14 and (24) suggesting that the creation of $CD151^{free}$ would bias tumor cells towards a more dynamic cycling of adhesion in favor of migration and thereby promote tumor metastasis. What remains to be determined is the identity of partners that control the switch of $CD151^{free}$ to its adhesion-promoting state.

In conclusion, our findings demonstrate that a subpopulation of CD151 exists on the surface of tumor cells that is not associated with integrin partners ($CD151^{free}$) that regulate tumor cell migration (Fig. 27). Clinical correlation of $CD151^{free}$ with prostate can-

cer progression demonstrates that it has independent prognostic value and suggests that this tetraspanin subpopulation may contribute to the disease and could be used to predict cancer progression (

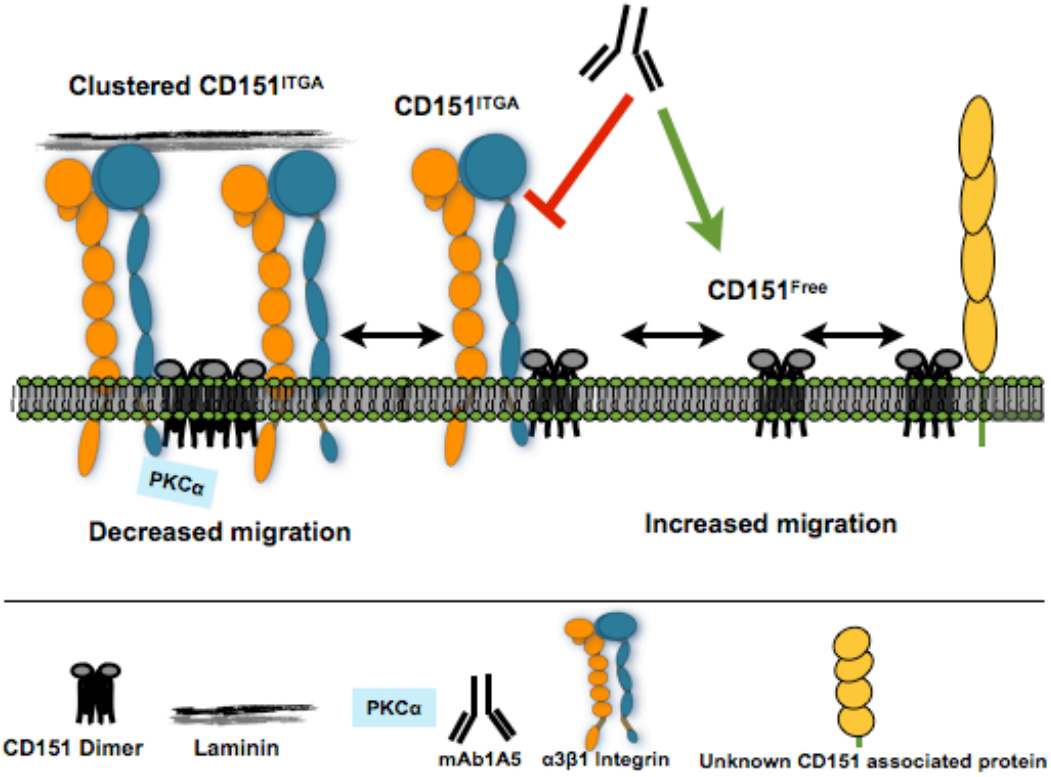


Fig.27. Summary two populations of CD151 exist on the cell surface. IA5 binds to the integrin QRD motif of CD151 and is unable to co-precipitate the CD151/integrin α3 complex from tumor cells. This allowed us to determine that there are two pools of CD151 on the cell surface integrin α3 associated $CD151^{ITGA}$ and integrin α3 non-associated $CD151^{Free}$. $CD151^{ITGA}$ inhibits migration by promoting laminin based adhesion. The appearance of $CD151^{Free}$ is due to the loss of α3 expression in tumor cells and tissues. $CD151^{Free}$ is potentially a pro-migratory molecule and forms a complex with an as of yet unknown cell surface protein.

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Chapter V

CD151 AND ALCAM CONTROL MOTILITY VIA PKCα MEDIATED ACTIVATION OF RAP1

Summary

The tetraspanin CD151 is a membrane scaffolding protein that forms macromolecular complexes with several transmembrane partners and is an important regulator of tumor cell motility. Much of the tetraspanin's ability to regulate migration was initially linked to its association with laminin binding integrins. Recent work revealed that integrin-free CD151 ($CD151^{free}$) is capable of promoting adhesion across many biological systems. In tumor cells this adhesion inhibits migration leading to reduced invasion and metastasis. Using an antibody specific for $CD151^{free}$ we have investigated partners that associate with integrin-free CD151 as well as the signaling responsible for immobilizing tumor cells upon clustering of this tetraspanin. Integrin-free CD151 was found to interact with Activated Leukocyte Cell Adhesion Molecule (ALCAM) and this association was required for $CD151^{free}$ to inhibit cell motility. Engaging the CD151/ALCAM complex through clustering with CD151 antibody or through ALCAM-ALCAM mediated adhesions promoted the activation of the GTPase Rap1A in a PKCα-dependent fashion. Finally, we demonstrate that Rap1A activation is sufficient to inhibit tumor cell motility *in vitro* and *in vivo*. These investigations demonstrate that CD151 can mediate signaling that controls tumor cell motility without direct integrin association by controlling the activation of the GTPase Rap1A. Together these observations suggest that CD151 signaling may switch to integrin-independent mechanisms for control of cell motility during tumor progression.

Introduction

While clinical progression to metastasis is a lethal aspect of cancer, the speed at which the malignancy progresses is very much dependent on the contribution of many individual molecular processes. This contribution is in large part dependent on the molecular integration of many individual mechanism with the biology of the cell and the neoplastic milieu (1, 2). Tetraspanins such as CD9 and CD151 are membrane scaffolding proteins that are important regulators of cancer progression (3-5). They organize macromolecular complexes known as Tetraspanin-Enriched Microdomains (TERM) by facilitating interactions between themselves and their non-tetraspanin partners (6,7).

CD151 has been shown to promote tumor cell motility and metastasis. Genetic ablation of this tetraspanin diminishes cancer metastasis in multiple models (8-10). Conversely, genetic loss of CD151 also disrupts epithelial integrity leading to physiological malfunction in kidney, lung, and the endothelium. CD151 has been proposed to facilitate the biological function of its integrin partners through interactions that ultimately regulate adhesion and migration (11, 12). Several CD151-specific antibodies have demonstrated the ability to augment cell migration (13-16). Recently we demonstrated that these migration-inhibiting antibodies engage integrin-free tetraspanin through its integrin-binding domain, cluster the tetraspanin, promote adhesion and subsequently prevent metastatic dissemination by inhibiting migration (70). These observations demonstrate that CD151 can control migration through a mechanism distinct from its integrin partners by targeting an integrin-free population of CD151 ($CD151^{free}$).

We set out to determine what mechanism was employed by the clustering of $CD151^{free}$ to promote adhesion and prevent metastasis. To accomplish this goal we used mass-spectometry to identify proteins associated with CD151 immunoprecipitated by the anti-CD151 antibody 1A5 specific for $CD151^{free}$. Molecular and genetic strate-

gies revealed the cell-cell adhesion molecule ALCAM (Activated Leukocyte Cell Ahesion Molecule) to be a novel CD151 partner required for antibody-mediated clustering of CD151 to control cell motility. Further interrogation of this complex revealed downstream activation of the GTPase Rap1A to be responsible for controlling cell migration.

Materials and Methods

Cell Culture, Plasmids, Transfections, Inhibitors, and Antibodies

HEp3 cells are perpetually maintained on the chick chorioallantoic membrane (CAM) to retain metastatic and migratory potential (65, 66). Cell lines were maintained in media supplemented with pen/strep, sodium pyruvate, non-essential amino acids and 10% fetal bovine serum and cultured at 37°C in a 5% CO2 incubator. Plasmids used included untagged wildtype CD151 (Clontech). CD151-GFP and ALCAM-GFP were kindly provided by Dr. Kiyo Sekiguchi (Osaka University) Dr. Josh Weiner (University of Iowa) respectively. HA-Rap1 expression vectors were kindly provided by Dr. Hans Bos (Universitair Medisch Centrum Utrecht, The Netherlands). Transfections of all cells were performed using Extreme Gene HD (Roche), excluding Cho cells which were transfected using polyethylenimine (PEI). Signaling pathways inhibitors included small molecule inhibitors of Rho kinase (Y 297632,), PI3kinase (LY294002), PI4kinase, (Wortmanin), MEK (UO126), PKA (KT5720), PKC (Calphostin) and Rap (GGTI-298) obtained from Calbiochem. Knockdown of gene expression was achieved with siRNAs to PKCα, and Rap1 A and B and the control non-targeting siRNA purchased from Dharmacon. CD151 specific shRNA was obtained from Sigma Aldrich. ALCAM and GFP siRNA were purchased from Invitrogen (Grand Island, NY). Anti-CD151 (1A5) was generated as previously described (67)), ALCAM/CD166 (L50, Abcam and R&D systems). The control antibody 29-7 was generated in the same fashion as 1A5 (67).

Mass Spectrometry

HEp3 and HT1080 cells were lysed in 1% Brij99 lysis buffer as described above. Lysates were incubated with control antibody 29-7 or anti-CD151 antibody 1A5 overnight at 4°C and immunoprecipitated with protein-G sepharose beads. The immunoprecipitated complexes were then resolved by SDS-PAGEG. Each lane was excised and prepared for mass spectrometry analysis by the Vanderbilt University Mass Spectrometry Core. Mass spectrometry analyses were performed with a LCQ-Deca or LTQ-Orbitrap mass spectrometer (ThermoFinnigan). Tandem mass spectra were extracted from raw files and used to search the database. Only proteins identified from two independent matrix preparations were considered as candidate components of the matrix.

Microarray and Gene Ontology analysis

Gene Ontology analysis was performed with microarray data obtained from BLM melanoma cell lines were stably transfected with ΔN-ALCAM, sALCAM, or empty plasmid control as described previously (18). Samples were hybridized to Affymetrix U133Plus 2.0 microarray chip. Intensities were normalized by $\log_2$ transformation. Fold change was determined as compared to endogenous expression of wt ALCAM in BLM cells (empty vector control). P-value was determined using 2-tailed Student's t-test with unequal variances. Fold change and p-value calculations were done in Microsoft Excel. Genes with a fold change of at least 2 and p-value of 0.001 or less were considered differentially expressed. 260 gene probes were differentially expressed. Probe IDs of differentially expressed genes were up loaded to Vanderbilt University's WebGestalt analyzer(19). U133Plus 2.0 reference was selected. Gene ontology analysis was performed with default parameters. Enriched biological processes were highlighted in red and labeled with number of associated genes and adjusted p-value.

Cell Motility Assays

In Vitro Wound Healing Assay HEp3 and HT1080 cells were seeded to confluence in 6-well plates with complete medium. After 24hrs the cells were switched to se-

rum free/insulin free media for an additional 24hrs. At 48hrs after seeding the confluent monolayers were scratched with a pipet tip in order to create a uniform wound after which the cells were washed with PBS to remove any floating cells. Cultures were returned to full medium and the wound was documented at 0hrs and 16hrs post-scratch using a light microscope TMS-F (Nikon) equipped with a D90 SLR camera (Nikon). Wound closure (% surface area) was calculated using Tscratch image analysis software(68, 69).

In Vitro Transwell Migration: Tumor cells in a single cell suspension were plated into the top chamber of 8μm transwell (Costar) inserts in the presence of serum free/ insulin free media. Cell culture media containing 10% fetal bovine serum was placed in the bottom chamber to serve as a chemoattractant. Migration assays were carried out for 12hrs in a 37°C cell culture incubator containing 5%CO. The top surface of the inserts was cleaned with a cotton swab soaked with PBS to remove the non-migrated cells. The transwell inserts were subsequently fixed in methanol and stained with 0.2% crystal violet. The number of migrated cells was counted in 3 independent fields under 20x magnification with a light microscope.

Two-Dimensional Migration: Tumor cell migration on soluble ALCAM-Fc was performed by adhering it to tissue culture plates with protein-G. Briefly, protein-G (2μg/ml) was adhered to the culture plastic followed by rat tail collagen type 1 (100μg/ml). Unbound surface was blocked with BSA (0.5% in PBS) after which the surface was washed and incubated with ALCAM-Fc in 0.5% BSA. Control migration was performed on identical surfaces not incubated with ALCAM- Fc. Tumor cells were seeded at low density and tracked by live cell imaging every 10 minutes over a 6hr period with a fully automated microscope (BX61, Olympus) equipped with a digital camera (Orca ER, Hammatsu). Data acquisition and analysis was performed using Volocity image acquisition software (Perkin Elmer).

In Vivo Cell Motility: Assays were performed as previously described (15). Briefly, cells to be injected were washed 2 times with PBS and detached with 2mM EDTA. The cells were resuspended in PBS and injected intravenously into Day 12 chick embryos. Four days post- injection the disseminated colonies were photographed using a Lumar V12 stereomicroscope (Zeiss) equipped with a Retiga Exi camera and controlled with Volocity image acquisition software (PerkinElmer). Antibody treatments were introduced by intravenous injection one day after tumor cell injection. “Non-motile” colonies were defined as colonies comprised of 5 or more cells where individual cells remained in direct contact. Such non-motile colonies are compact while “motile” colonies contained a migratory cell populations dispersed in the CAM. Assays were performed with 5 animals/ treatment and ≥ 5 fields/animal analyzed for colony formation. Data is represented as the percentage of colonies within a single animal that demonstrated a motile phenotype.

Flow Cytometry

Cells to be used in flow cytometry experiments were trypsinized with 0.25% Trypsin-EDTA and resuspended in cold Milytenyl FACs buffer (2mM EDTA, 0.5%BSA, PBS). For the analysis of cell surface expression of specific antigens the cells were washed 2 times with FACs buffer and then stained with the specific primary antibodies for 1hr on ice. Following incubation with the primary antibody the cells were washed 2 times with cold FACs buffer and then incubated with species-specific, fluorophore-conjugated secondary antibody for 30 minutes on ice. After washing two times the cells were suspended in cold FACs buffer and subjected to flow cytometry.

Immunoblot and Immunoprecipitation Analysis

Immunoblotting: For immunoblot analysis HEp3 , HT1080 or CHO cells were lysed in either 1% (vol/vol) Triton X-100 lysis buffer or 1% (vol/vol) Brij-99 lysis buffer and incubated on ice for 30 minutes. The samples were then cleared by centrifugation at 14,000 rpm for 15 minutes and the cleared lysates were then transferred to fresh tubes.

Protein concentrations were determined by BCA assay(Pierce). Equal amounts of protein were loaded in to SDS-Page gels and subsequently transferred to polyvinylidene fluoride membranes (PVDF, Millipore). The membranes were blocked for 1hr in 5% non-fat dry milk in phosphate buffered saline with 0.05% Tween-20 (PBSt). The membranes were incubated overnight at 4°C with specific antibodies prepared in blocking buffer after which the membranes were washed 3 times with PBSt and incubated with the appropriate species-specific horse radish peroxidase-conjugated secondary antibodies for 1hr at room temperature. After washing, antibody binding was visualized by chemiluminescence.

Immunoprecipitation: 1mg total cell lysate was incubated with 2µg of the immunoprecipitation antibody and the antibody lysate solution was incubated over-night at 4°C with end to end rotation. In order to capture the protein/antibody complexes the lysates were coupled to protein G sepharose beads (GE Healthcare) for 4hr at 4°C with end to end rotation. The lysates were cleared by centrifugation at 8000 rpm for 30 secs and the unbound material saved for further analysis. The beads were washed 3 times with either Triton X-100 or Brij-99 lysis buffer accordingly and the immune complexes were then eluted in 100µl lamelli sample buffer and boiled for 5 minutes. The samples were then prepared for SDS-Page analysis. For GBP immunoprecipitations the lysates were incubated overnight with GBP conjugated beads with end to end rotation overnight at 4°C. After clearing the lysates were subjected to SDS- PAGE as described above.

Cell Surface Biotinylation: For cell surface labeling confluent HEp3 cells were treated with 1A5 or control antibody for 1hr on ice and then washed with cold PBS. Cultures were subsequently biotinylated with sulfoccinimidyl-6-[biotin-amido] hexanoate using the EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Scientific) according to manufacturer's instructions. Cells were lysed in 1% Brij 99 followed by extraction of the insoluble

in 1% Triton X-100. Immunoprecipitation was performed as described above. Biotinylated proteins were detected with peroxidase conjugated streptavidin.

Metabolic Labeling: HEp3 cells were labeled overnight in methionine/cysteine free DMEM containing 35S-label. Cells were washed throughly with PBS and lysed in 1% Brij 99 lysis buffer. The lysates were incubated with control antibody or monoclonal antibody 1A5 and immunoprecipitated as described above. The gels were dried and then exposed to film at -80°C.

Rap1 Activation Assays

Rap1 activation was analyzed using the Rap1 activation kit (Chemicon). All treatments were performed in the presence of serum free/insulin free media. Cells to be assayed were washed with ice cold PBS and lysed in RIPA like Rap1 buffer (10% glycerol, 50mM Tris/HCl, 150mM NaCl, 5mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate). The lysates were pushed through a 25 gauge syringe and cleared by centrifugation at max speed at 4 degrees for 10 min. Equal amounts of lysates were immunoprecipitated with RalGDS RBD conjugated agarose beads for 45 minutes at 4℃. After washing the beads where resuspended with sample buffer and boiled for 5 minutes. The immunoprecipitates were separated by SDS-Page as mentioned above, transferred to PVDF membranes, probed with the anti-Rap1 antibody and horse radish peroxidase-conjugated secondary antibody. After washing the membranes were developed by autoradiography.

Immunfluorescence

HEp3 cells grown to confluency on collagen coated coverslips were incubated with 1A5 and then fixed in 4% formalin in PBS. The coverslips were blocked with 5% BSA in PBS for 30 minutes at room temperature and then stained with 1A5 and ALCAM primary anti-

bodies overnight at 4°C. The coverslips were washed with PBS and then incubated with species specific fluorophore-conjugated secondary antibodies for 1hr at room temperature.). After staining the cells were washed with PBS and then counterstained with DAPI. The coverslips were then mounted onto glass slides with Fluorosave mounting media (Calbiochem). Pictures were taken with a BX 61 fluorescent microscope (Olympus) and analyzed using Volocity Image Acquisition Software (Perkin Elmer).

Statistical Analysis

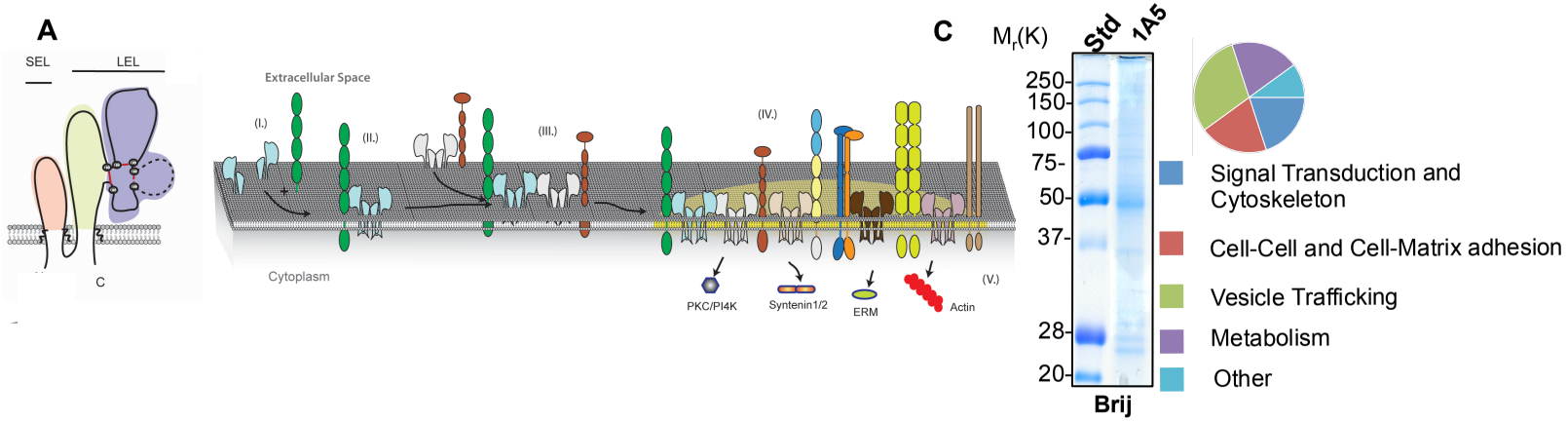
All statistical analysis was performed using Graph Pad Prism Analysis Software (La. Jolla California). For statistical analysis of migration experimental groups were compared to the control groups using non-parametric Mann-Whitney test.

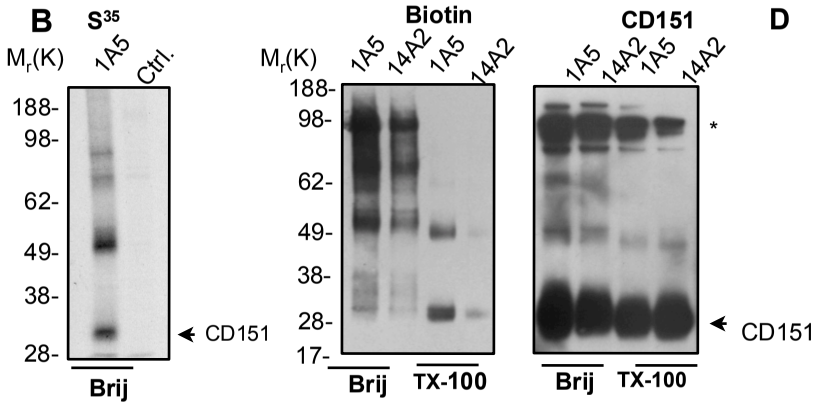
Results

Identification of CD151 associated proteins**.**

The ability of tetraspanins to organize higher-order structures in the membrane leads to complex interactions of variable affinity with a variety of transmembrane and membrane proximal proteins (Fig. 28A, (6)). Immunoprecipitation of CD151 with the motility-inhibiting antibody 1A5 from lysates extracted with mild detergents (Brij) or stringent detergent (TX-100) co-precipitates several proteins (Fig. 28B) visible after metabolic labeling (S35), and cell-surface biotinylation (Biotin). In order to elucidate the components of the CD151-TERM complex responsible for regulating tumor cell motility and metastasis, we used tandem Mass Spectrometry (LC-MS-MS) to identify CD151 partners that co-IP with 1A5 (Fig. 28C). Specifically, the HNSCC cell line HEp3 was lysed with Brij-based lysis buffer to maximize possible protein- protein interactions. Immunoprecipitations were preformed with the $CD151^{free}$-binding antibody 1A5. Control sam

ples included immunoprecipitations with an isotype-matched control IgG or protein-G only. The precipitated complexes were separated by SDS-PAGE, gel pieces were excised and subsequently analyzed by LC-MS-MS using a LCQ-Deca or LTQ-Orbitrap mass spectrometer (ThermoFinnigan). CD151-specific candidates were identified by eliminating targets precipitated with an isotype and protein-G respectively. Candidates identified by two or more peptides in two or more independent analyses were selected as viable targets. A total of 228 proteins were identified of which 51 candidates distributed across five phenotypic subgroups were detected repeatedly (Fig. 28C, pie chart and Table 10). We have recently demonstrated that anti-CD151 antibody 1A5 clusters the tetraspanin and promotes its accumulation at areas of cell-cell contact (70). Based on validated interactions from published studies and the observed cell-cell accumulation, a short list of potential candidates was developed (Fig. 28D, established and putative partners). Nearest neighbor analysis was performed on each putative candidate using the Broad Institute Cancer Cell Line Encyclopedia (http://www.broadinstitute.org/ccle/home, (17)). ALCAM was identified as the nearest neighbor of α3, one of the primary partners of CD151. To evaluate ALCAM's role as a regulator of migration we evaluated changes in gene expression initiated upon expression of a dominant- negative ALCAM lacking the ligand-binding domain (18). Differentially expressed genes were grouped by gene ontology (GO) association using WebGestalt (http://bioinfo.vanderbilt.edu/ webgestalt/ (19) and enriched biological processes were displayed (Fig. 29). The primary processes identified were cell adhesion and migration. In addition to its association with cancer metastasis and migration, ALCAM has also been suggested to interact with PKCα, a key regulator of CD151 activity, supporting its potential as a bonafide partner in the regulation of tumor cell motility by CD151(20). ALCAM has broad expression in epithelial tissues and their corresponding tumors (Fig. 30 A).





D

Targets Identified by Mass Spectrometry

Established Partners	Gene # | Uniprot ID.
CD151 (TSPAN24)	977 | P48509
Integrin subunit α3 (ITGA3)	3672 | P26006
Integrin subunit β1 (ITGB1)	3688 | P05556
MMP14 (MT1-MMP)	4323 | P50281
PI 4-kinase type 2-alpha (PIK42A)	55361 | Q9BTU6
Putative Partners	
ALCAM (CD166)	214 | Q13740
CUB domain-containing protein-1 (CDCP-1)	64866 | Q9H5V8
Claudin domain-containing protein-1 (CLDND1)	56650 | Q9NY35
EGF-like repeat and discoidin I-like domain (EDIL3)	10085 | O43854
CD44 antigen precursor	960 | P16070

Fig. 28. Mass spec analysis and discovery of ALCAM as a CD151 associated protein.(A).(left panel) General tetraspanin schematic with the small extracellular loop (SEL) and the large extracellular loop (LEL) highlighted. (right panel) Schematic representation of tetraspanin partner interaction (I-II), TERM assembly (III-IV) and interaction with intracellular signaling molecules (V). (B) Detection of CD151 and associated proteins by metabolic labeling (S35), cell-surface biotin labeling (biotin), or CD151-specific immunoblot after immunoprecipitation from a HEp3 cell lysate. (C-D) Mass Spec analysis (LC-MS-MS) of CD151-associated proteins after immunoprecipitation by antibody 1A5. (C) SDS-PAGE used for LC-MS-MS and pie-chart representing distribution of identified targets. D). Table of known CD151-associated proteins and putative partners that can potentially control migration.

Table 10. CD151 associated proteins identified by mass spectrometry

Name	Accession number
Signal transduction and cytoskeleton (20 %)	
Alpha-actinin-1	IPI:IPI00013508.5
Alpha-actinin-4	IPI:IPI00013808.1
Calpain small subunit 1	IPI:IPI00025084.3
Cofilin-1	IPI:IPI00012011.6
F-actin capping protein subunit alpha-1	IPI:IPI00005969.3
Phosphatidylinositol 4-kinase type 2-alpha	IPI:IPI00020124.1
Radixin	IPI:IPI00017367.5
Villin 2	IPI:IPI00746388.1
Tropomyosin 1 alpha chain isoform 2	IPI:IPI00000230.6
Guanine nucleotide-binding	IPI:IPI00217906.3
Protein G(i), alpha (isoform 2)	IPI:IPI00033494.3
Cell-cell and cell-matrix adhesion (20 %)	
CD151 antigen	IPI:IPI00298851.4
CD63 antigen	IPI:IPI00215998.5
CD166 antigen (ALCAM)	IPI:IPI00015102.2
CUB domain-containing protein-1	IPI:IPI00290039.5
Claudin domain-containing protein-1	IPI:IPI00072743.3
EGF-like repeat and discoidin I-like domain	IPI:IPI00306046.1
CD44 antigen precursor (isoform 12)	IPI:IPI00297160.4
Integrin alpha-3	IPI:IPI00215995.1
Integrin beta-1	IPI:IPI00217563.3
Matrix metalloproteinase-14	IPI:IPI00218398.5
Plasminogen activator inhibitor 2	IPI:IPI00007117.1
Vesicle trafficking (30 %)	
Calnexin	IPI:IPI00020984.1
Endoplasmin	IPI:IPI00027230.3
Reticulon-4	IPI:IPI00021766.4
Caveolin-1(Isoform Alpha)	IPI:IPI00009236.5
Lysosome membrane protein-2	IPI:IPI00217766.3
Ras-related protein R-Ras2	IPI:IPI00012512.1
Ras-related protein Rab-11B	IPI:IPI00020436.4
Ras-related protein Rab-7A	IPI:IPI00016342.1
Ras-related protein Ral-A	IPI:IPI00217519.3
Serpin B4	IPI:IPI00010303.1
Synaptobrevin homolog YKT6	IPI:IPI00008569.1
Syntaxin-4	IPI:IPI00029730.1
Syntenin-1	IPI:IPI00299086.3
Vesicle-trafficking protein SEC22b	IPI:IPI00006865.3
Secretory carrier-associated membrane protein	IPI:IPI00306382.1
Protein, lipid and carbohydrate metabolism (20 %)	
Cation-dependent mannose-6-phosphate	IPI:IPI00025049.1
Fructose-bisphosphate aldolase C	IPI:IPI00418262.4
Neutral alpha-glucosidase AB precursor (isoform 2)	IPI:IPI00011454.1
Mannose-6-phosphate receptor-binding Protein-1 (isoform A)	IPI:IPI00106668.4
Mannose-6-phosphate receptor-binding Protein-1 (isoform B)	IPI:IPI00303882.2
Pyruvate kinase isozymes M1/M2 (isoform M2)	IPI:IPI00479186.5
Alpha-enolase	IPI:IPI00465248.5
Lactoylglutathione lyase	IPI:IPI00220766.5
Phosphoglycerate kinase-1	IPI:IPI00169383.3
Phosphoglycerate mutase-2	IPI:IPI00218570.6
Unknown (10 %)	
Transmembrane emp24 domain-containing protein 10 precursor	IPI:IPI00028055.4
Transmembrane protein 106B	IPI:IPI00395903.1
Transmembrane protein 109 precursor	IPI:IPI00031697.1
Hypothetical protein DKFZp686I04222	IPI:IPI00413451.1
Isoform 1 of Protein ZNF365	IPI:IPI00007274.6

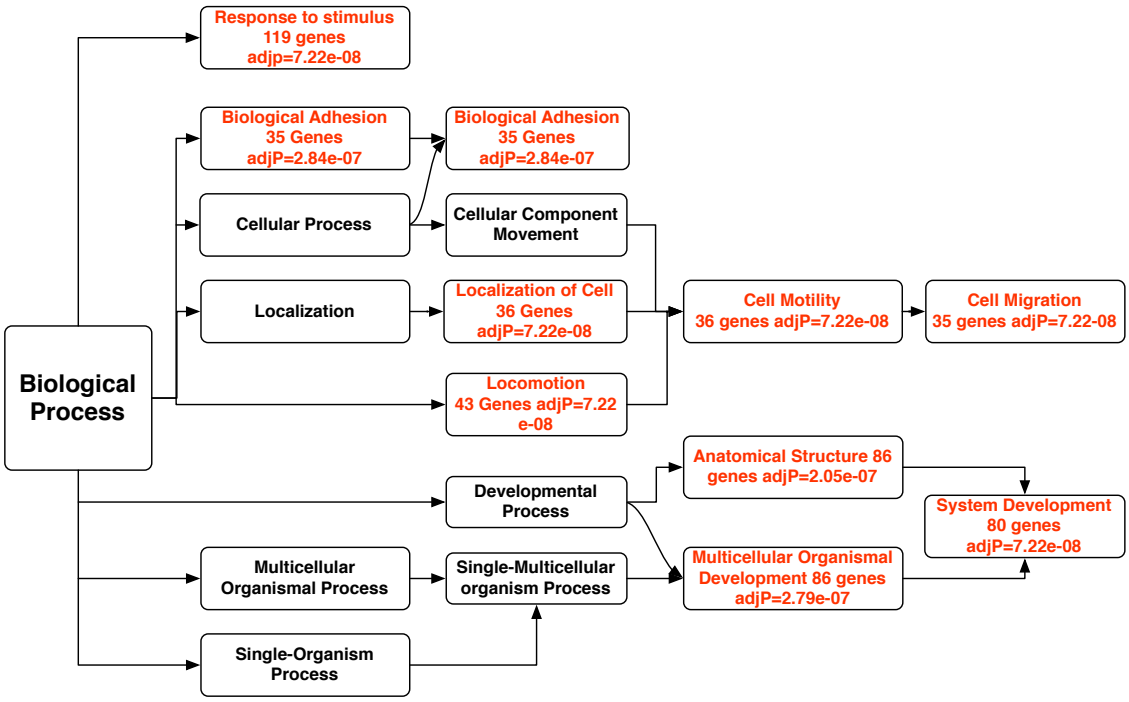


Fig. 29. ALCAM gene signature. Gene Ontology analysis performed with WebGestalt on genes differentially expressed after the introduction of a dominant-negative ALCAM.

Continuous cell lines derived from these tumors continue to express ALCAM both at the level of gene transcription and protein translation (Fig. 30 B and C)

The IgG superfamily member ALCAM is a novel CD151 partner

Clustering of CD151 with 1A5 promotes the localization of the tetraspanin to the areas of cell- cell contact leading to the inhibition of tumor cell migration and metastasis (previously published data (15) and (70). **A**LCAM and CD151 occupy the same areas of cell-cell contact and clustering of the tetraspanin causes ALCAM to co-localize extensively with CD151 (Fig. 31A). To determine if CD151 and ALCAM were physically interacting, the proteins were immunoprecipitated from mild (Brij) and stringent (TX-100) detergent lysates obtained from the HNSCC HEp3 using the antibodies 1A5 and AZL50 respectively and the resulting precipitations were immunoblotted for its possible partner (Fig. 31 B and C respectively). CD151 and ALCAM did indeed co-immunoprecipitate in their reciprocal precipitations. Since 1A5 drives CD151 clustering and promotes an interaction between CD151 and ALCAM, we pursued an independent validation of the interaction between CD151 and ALCAM using affinity purification of GFP-tagged proteins. ALCAM-GFP and CD151-GFP were co-transfected with untagged CD151 or ALCAM respectively into CHO cells. GFP-tagged protein was precipitated from TX-100 lystates using a GFP-binding protein (Fig. 31 D and E). ALCAM indeed co-precipitated with CD151-GFP and CD151 co-precipitated with ALCAM-GFP. These four independent precipitations demonstrate that CD151 forms a complex with ALCAM and that ALCAM is a component of the CD151-TERM. Importantly, this complex is stable under stringent detergent conditions (TX-100) suggesting that the stability of the CD151-ALCAM interaction is similar to those observed previously for CD151-α3.

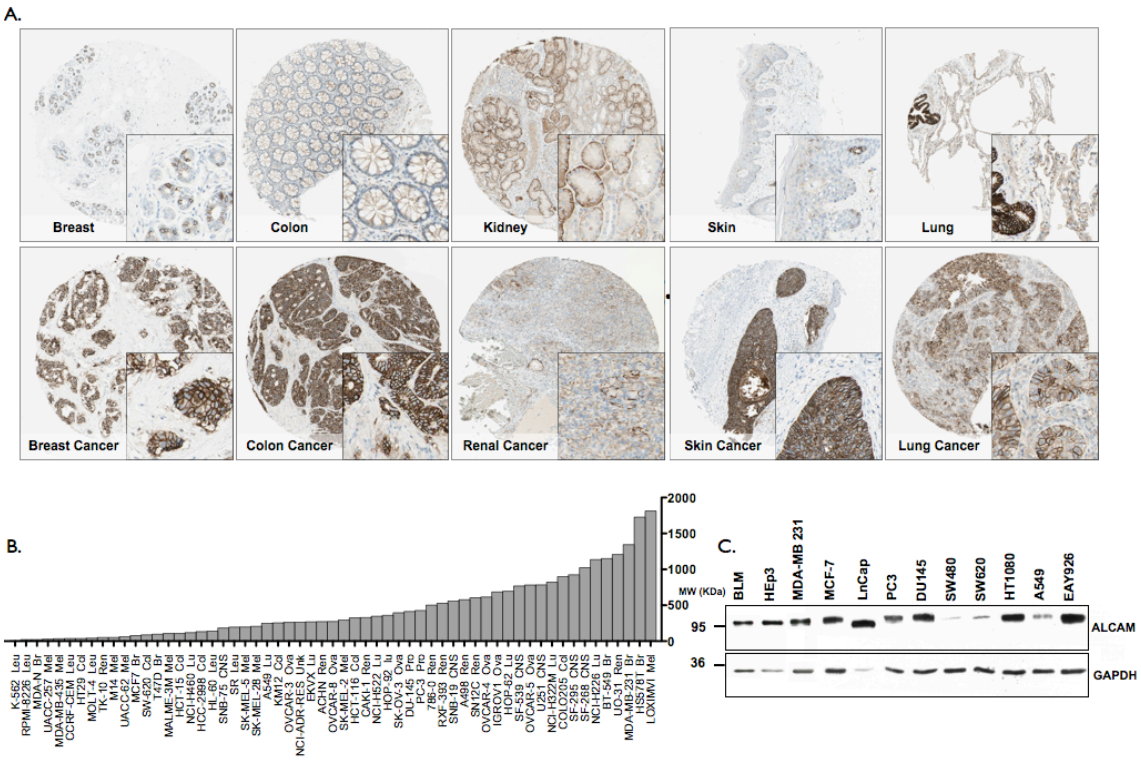


Fig.30. ALCAM expression in cancer and cultured tumor cells. (A) The human protein ATLAS (HPA) was queried for the expression of ALCAM and demonstrates that it is expressed in a number of different tumor tissues **(B)** mRNA expression analysis of the NCI-60 dataset demonstrates that the expression of ALCAM varies, however, epithelial derived cancer cell lines have the highest expression (**C)** Western blot analysis of commonly used cancer cell lines was performed and probed for the expression of ALCAM using the anti-ALCAM antibody and demonstrates that ALCAM is expressed in a number of different cell lines and cell types GAPDH was used as a loading control

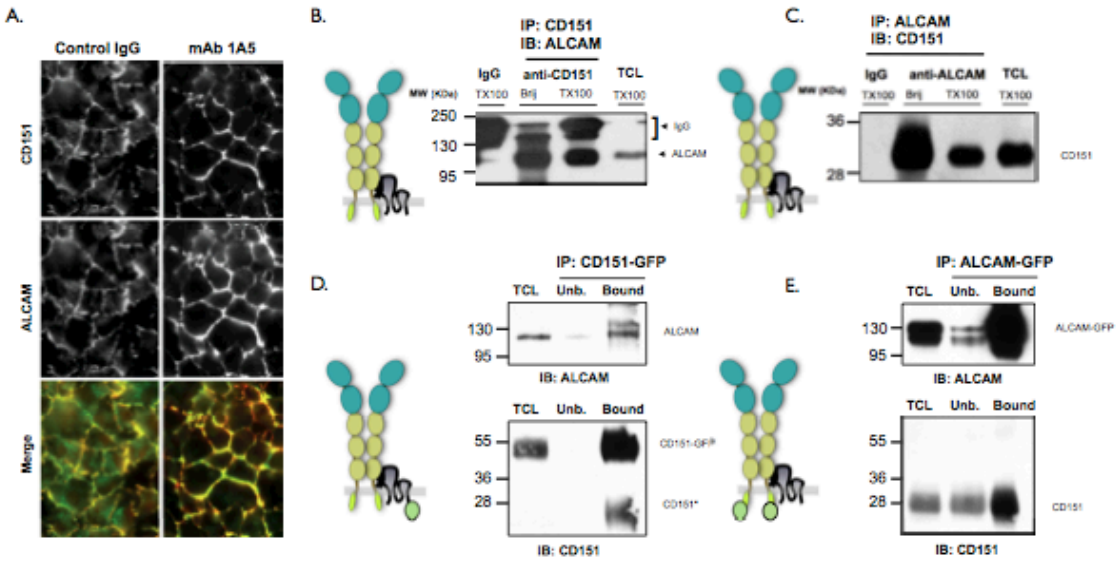


Fig 31.Physical association of CD151 with ALCAM. (A) Immunofluorescent staining of CD151 and ALCAM in HEp3 cells treated with control IgG or 1A5 demonstrates to evaluate their relative distribution. **(B)** Co-immunoprecipitation of ALCAM by anti-CD151 antibody 1A5. Proteins immunoprecipitated from HEp3 cells lysed in either Brij 99 or Triton X-100 containing lysis buffer by anti-CD151 antibody were immunoblotted for ALCAM. **(C)** Co- immunoprecipitation of CD151 by anti-ALCAM antibody. Proteins immunoprecipitated from HEp3 cells lysed in either Brij 99 or Triton X-100 containing lysis buffer by anti-ALCAM antibody were immunoblotted for CD151. **(D-E)** Co-precipitation of ALCAM and CD151 after pull-down of GFP-tagged partner. GFP-tagged CD151 **(D)** or ALCAM **(E)** were transfect into Chinese Hamster Ovary (CHO) cells together with with myc-tagged ALCAM or untagged CD151. GFP- tagged protein was pulled down with GFP-binding protein (GBP) conjugated beads and subsequently evaluated by immunoblotting for CD151 and ALCAM. (CD151*= untagged CD151that co-precipitates with CD151-GFP). Data is represented of 3 independent experiments.

ALCAM and CD151 cooperate in their ability to regulate migration

The physical association of CD151 and ALCAM demonstrates that ALCAM is a component of the TERM complex. To determine the relevance of this interaction in cell motility, the ability of CD151 and ALCAM to regulate migration was evaluated after RNAi-mediated depletion in HT1080 and HEp3 cells (Fig. 32, and Fig. 33). CD151 clustering induced by the antibody 1A5 inhibits HT1080 tumor cell migration (Fig. 32 A). After RNAi-mediated depletion of ALCAM, tumor cells are no longer sensitive to the immobilization by CD151 (Fig. 32 B and C). Engaging HT1080 cells in ALCAM-ALCAM interactions can be accomplished by seeding cells on ALCAM- Fc coated surfaces (Fig. 32 D and (21). Similar to anti-CD151 treatment, engaging ALCAM- ALCAM interactions can inhibit tumor cell motility in a wound-healing assay. The inhibition of cell motility upon ALCAM-ALCAM binding is enhanced by 1A5-induced clustering of CD151 (Fig. 32 E, white bars). Conversely, shRNA mediated knockdown of CD151 in HT1080 cells not only eliminates sensitivity to the anti-CD51 antibody 1A5 but also prevents the inhibition of motility by ALCAM-ALCAM ligation (Fig. 32 E, black bars). Similar observations were made for the HNSCC HEp3 (. Fig. 33.) These data clearly demonstrates that CD151 and ALCAM cooperate with each other to control tumor cell motility.

CD151 and ALCAM control tumor cell migration through PKCα activation of Rap1A

A targeted pharmacological screen was performed in an attempt to identify molecular mechanisms that might contribute to the inhibition of motility mediated by CD151 and ALCAM (Fig. 34 A). Tetraspanins regulate intracellular signaling events by interacting directly or indirectly with key signaling molecules including protein kinase Cα (PKCα) and phosphatidylinositol 4 kinase type IIa (PI4kinase Type IIa) (22-24). Rho and PI3 knase have also been associated with CD151-mediated regulation of tumor cell motility (25). MEK, Erk, and Rap1 were targeted as established mediators of cell motility (26, 27). Transwell migration assays were performed in the presence or absence of each in-

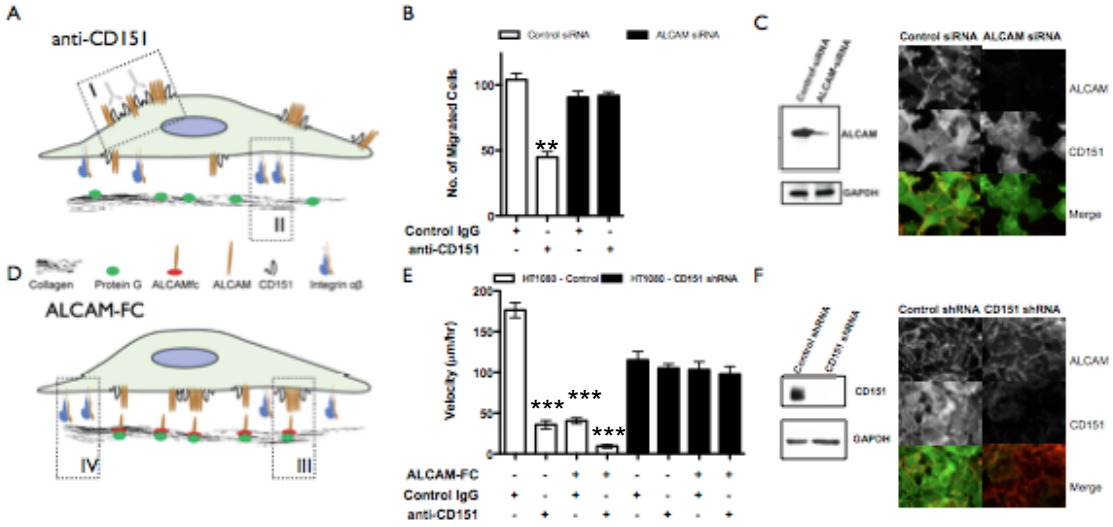


Fig 32. CD151 and ALCAM cooperate in the regulation of tumor cell migration. (A) Schematic representation of migration in response to clustering of CD151 by antibody 1A5 (I) which results in enhanced adhesion (II) and subsequent inhibition of migration. **(B)** Transwell migration of HT1080 cells in the presence or absence of 1A5 after transfection with control siRNA (white bars) or ALCAM siRNA (black bars). **(C)** The knockdown of ALCAM was confirmed by immunoblotting and immunofluorescent staining of cultured cells. **(D)** Schematic representation of migration in response to ALCAM-ALCAM interactions. ALCAM-Fc was bound to protein-G coated plates as described in the materials and methods. ALCAM-Fc engages cell- surface ALCAM (III) which impacts adhesion and subsequent migration (IV). **(E)** Analysis of cell migration on ALCAM-Fc by single-cell tracking. Migration velocity is determined after transfection with control shRNA (white bars) or CD151 shRNA (black bars). The impact of ALCAM-ALCAM interactions was evaluated in the presence of the anti-CD151 antibody (1A5). **(F)** The knockdown of CD151 was confirmed by immunoblotting and immunofluorescent staining of cultured cells.The data is represented as SEM * ($p > 0.05$), ** ($p > 0.01$), *** ($p > 0.001$) Data is representative of 3 independent experiments. Migration assays in B and E performed in triplicate.

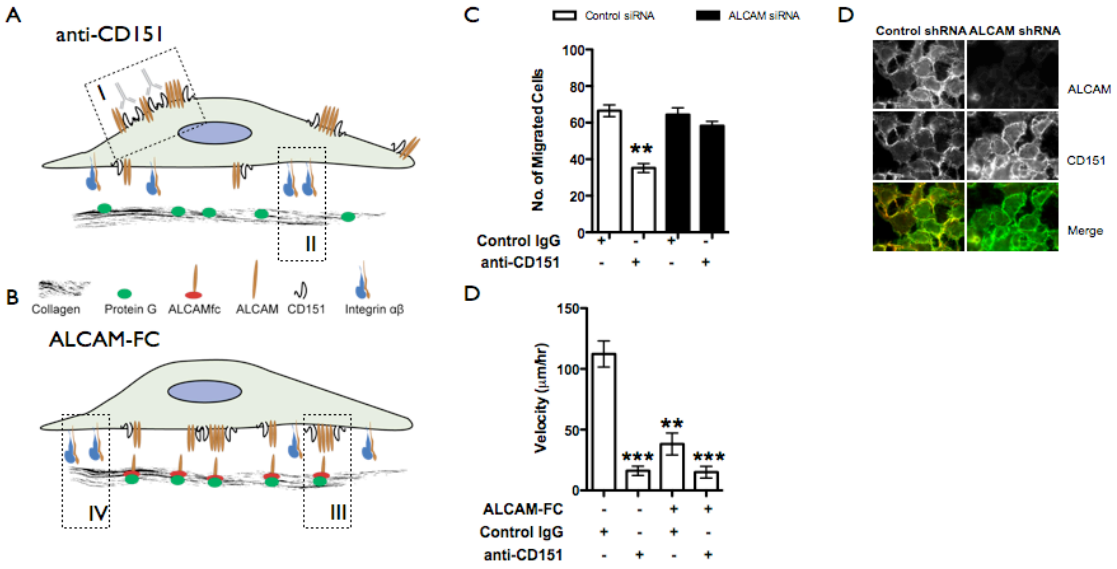


Fig. 33. ALCAM and CD151 cooperate in the regulation of tumor cell migration. **(A)** Schematic representation of migration in response to clustering of CD151 by antibody 1A5 (I) which results in enhanced adhesion (II) and subsequent inhibition of migration. **(B)** Schematic representation of migration in response to ALCAM-ALCAM interactions. ALCAM- Fc was bound to protein-G coated plates as described in the materials and methods. ALCAM-Fc engages cell-surface ALCAM (III) which impacts adhesion and subsequent migration (IV). **(C)** Transwell migration of HEp3 cells expressing the control or ALCAM specific siRNA in the presence of the control antibody or 1A5. **(D)** Immunofluorescence of HEp3 cells expressing the control or ALCAM-specific shRNA grown on glass coverslips. **(E)** Analysis of cell migration on ALCAM-Fc by single-cell tracking. Migration velocity was determined in the presence (white bars) or absence of anti-CD151 antibody 1A5 (black bars). Data represented as SEM * (p>0.05), **(p>0.01)***(p>0.001). Data is representive of 3 independent experiments. Migration assays in C and D performed in triplicate.

hibitor together with or without the anti-CD151 antibody 1A5. The MEK inhibitor (U0126) and PI3 kinase inhibitor (LY294002) inhibited migration independent of anti-CD151 antibody treatment (1A5) while the PI3 kinase inhibitor (Wortmannin) did neither influence the migration of untreated cells nor did it prevent the inhibition of migration by 1A5. Conversely, the PKC inhibitor (Chelerythrin) and the RAp1 inhibitor (GGTI-298) allowed for migration of untreated cells but prevent 1A5 from inhibiting migration. RNAi-mediated ablation (siRNA) of PKCα and Rap1A confirm that these proteins are required for CD151 to inhibit motility (Fig. 34 B). Rap1A is a GTPase well known for its ability to promote adhesion upon activation (28). We hypothesized that Rap1A was activated in response to CD151 clustering as well as ALCAM-ALCAM ligation. To evaluate this possibility, HEp3 cells were treated with 1A5 or seeded on ALCAM-FC for up to 48hrs. Active Rap1A in the cells was evaluated by immunblotting of GTP-bound Rap1A affinity purified from lysates with Ral-GDS beads. Both CD151 clustering and ALCAM-ALCAM binding resulted in rapid and persistent activation of Rap1A (Fig. 34 C). Activation of the GTPase Rac was evaluated as a control to confirm the specificity of Rap1A activation (Fig. 34 D). PKCα is known to associate with CD151 (22) and ALCAM (20). Moreover, it has also been demonstrated to mediate activation of Rap1 (29). Together with the observed contribution of PKCα to the regulation of migration (Fig. 34 A and B), these published reports suggest that PKCα might mediate activation of Rap1A by CD151 and ALCAM. To determine if PKCα signaling is required for Rap1A activation by CD151, GTPase activity assays were performed in HEp3 cells after PKCα was disrupted with Chelethyrine or PKCα-specific siRNA (Fig. 34 E). In control transfected or untreated cells 1A5 promotes activation of Rap1A, however, when PKCα is knocked down by genetic manipulation or inhibited by pharmacological means, 1A5 is unable to promote Rap1A activation. This data clearly demonstrates that downstream of CD151 clustering, Rap1A is activated in a PKCα-dependent manner.

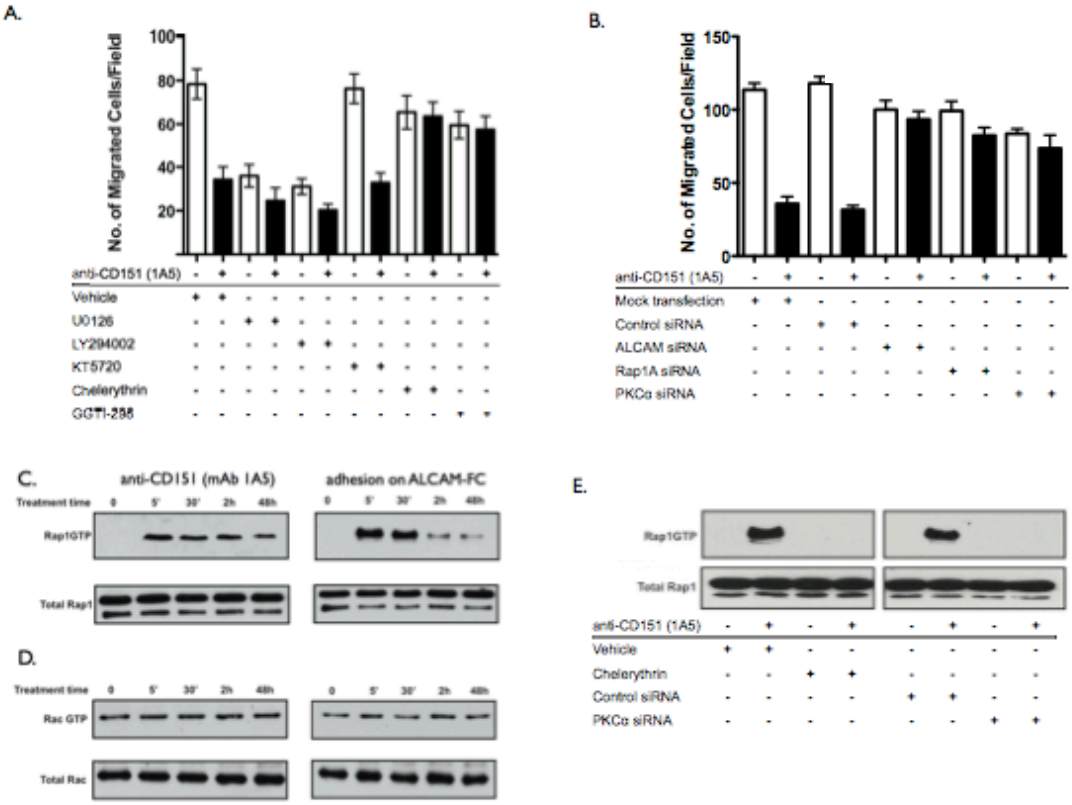


Fig 34. CD151/ALCAM signaling mechanism involves PKC and the small GTPase Rap1. (A) Transwell migration of HEp3 cells in the absence (white bars) or presence of 1A5 Anti- CD151 (black bars) was analyzed in the presence of different pathway inhibitors. The potential pathways that were interrogated for their abilities to regulate cell motility in the presence of 1A5 by chemical inhibition include; MEK (U0126), PI3 kinase (LY294002), PKA (KT5720), PKC (Chelerythin), and Rap1 (GGTI-298). Only the inhibition of PKC and Rap1 rescued the inhibition of migration associated with the engagement of CD151 by 1A5. **(B)** Wound healing migration of HEp3 cells expressing siRNAs specific for ALCAM, PKCα, and Rap1 further demonstrate that the expression of these proteins is required for the inhibition mediated by CD151 clustering. **(C)** Rap1 activation HEp3 cells were serum starved overnight and then treated with 1A5 in culture for the indicated time points. Rap1 activation was determined using a commercial Rap1 activation kit. In untreated cells Rap1 is not activated as demonstrated by the absence of GTP bound Rap1. Within five minutes of treatment however, we observed a robust activation of Rap1 that is sustained for 48hr post treatment. In order to demonstrate the specificity of GTPase activation by 1A5 we also analyzed the activation status of the small GTPase Rac1 which in the presence of 1A5 was not activated above basal levels. **(D)** Plating HEp3 cells on ALCAM-Fc activates Rap1 in a time dependent manner but has not impact on the activation of state of the small GTPase Rac1. **(E)** 1A5 treatment activates Rap1 in a PKCα-dependent manner. Data represented as SEM * ($p>0.05$) ** ($p<0.01$) *** ($p>0.001$) Data is representative of 3 independent experiments. Migration assays in A and B performed in triplicate.

Rap1 activation is sufficient to immobilize tumor cells *in vitro* and *in vivo*

Rap1A serves as a molecular switch capable of regulating cell motility by controlling adhesion (30, 31). We have demonstrated that the inhibition of tumor cell motility in response to CD151 and its partner ALCAM involves the activation of Rap1A. We hypothesized that Rap1 activation was sufficient to inhibit tumor cell motility. To address this question we transfected GFP- expressing HEp3 cells with dominant negative Rap1A (Rap1N17) or the dominant active Rap1A (Rap1V12 (32). The expression of the constructs was verified by western blot analysis (Fig 35 A). Migration assays were subsequently performed in order to determine if Rap1A activity was sufficient to impact on tumor cell migration *in vitro*. Dominant negative Rap1A (Rap1N17) had no significant impact while the dominant active Rap1A (Rap1V12) reduced migration (Fig. 35 B). This observation extended to *in vivo* migration. HEp3-GFP cells transfected with dominant negative Rap1A (Rap1N17) or dominant active Rap1A (Rap1V12) were injected intravenously into D12 avian embryos and metastatic colony formation was analyzed at 4 days post injection. Colony size can reflect tumor cell growth while the local dispersion of metastatic tumor cells reflects on their motility (15). All of the transfected cells demonstrated efficient colony formation. However, HEp3 cells expressing dominant active Rap1A (Rap1V12) formed compact colony indicative of reduced motility while mock and Rap1N17 transfected cells exhibited exhibited a dispersed, highly motile colony appearance(Fig. 35 D) and quantitated in (Fig. 35 C) as the percent (%) motile colonies.

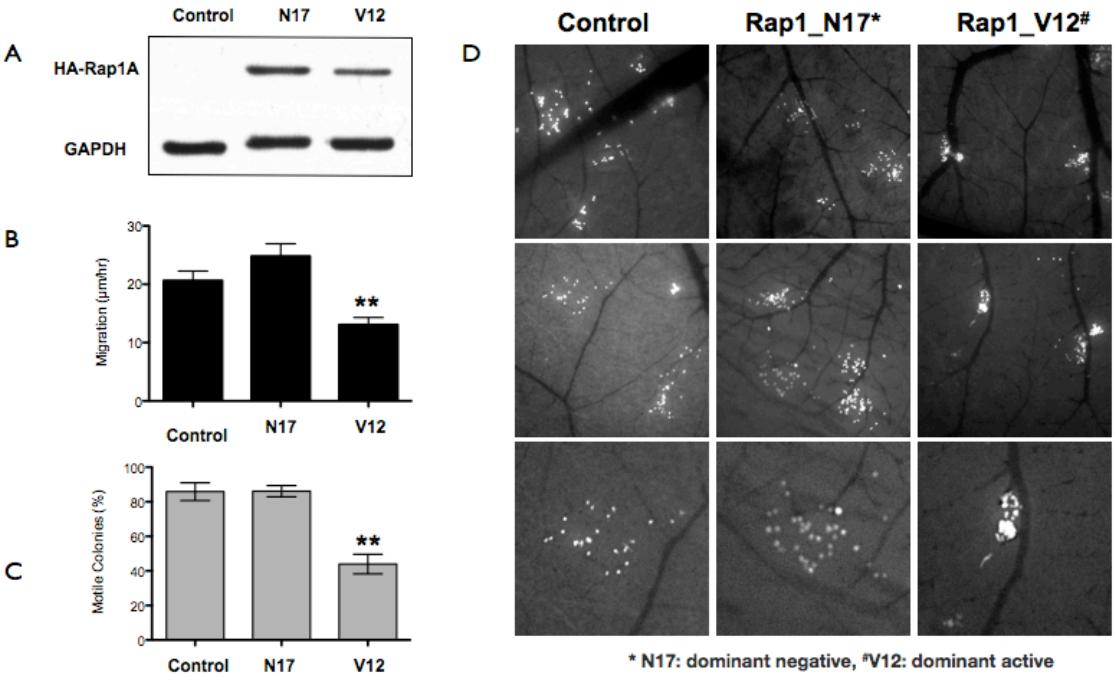


Fig. 35. Rap1 activation is sufficient to inhibit tumor cell motility *in vitro* and *in vivo*. HEp3 cells were transiently transfected with empty vector (Control) dominant negative Rap1 (N17) or dominant active Rap1 (V12). **(A)** Transgene expression was verified by immunoblotting for the HA-tagged protein. **(B)** *In Vitro* cell migration was performed with transiently transfected cells in a wound-healing assay. **(C)** and **(D)** HEp3 cells were injected into 12 day old chicks and the migratory phenotype of metastatic colonies was evaluated (see methods for details). The number of motile colonies was quantified **(C)** from images taken of the chorioallantoic membrane containing metastatic colonies **(D)**. Data is representative of three experiments with n≥5 for each experiment. Data is represented as SEM ** (p=0.01). Migration assay in B performed in triplicate.

Conclusions

The regulation of migration is a complex process that relies on the integration of many molecular processes. Molecular scaffolding through the tetraspanins is one of the means by which signal integration can be accomplished. Many of these mechanisms can both promote as well as inhibit migration. The best example of this is the regulation of adhesion. Too much or too little adhesion can limit migration while an optimal balance of adhesion and de-adhesion promotes motility (Fig.4). There have been several investigations into the molecular mechanism(s) by which the tetraspanin CD151 regulates cell motility (reviewed in (33, 34)and(35)). The characterization of direct interaction with laminin-binding integrins evolved to define the ability of CD151 to promote integrin activity and thereby promote adhesion and migration. Consistent with this hypothesis is the reduction of migration on a laminin substrate (36) and the reduction in metastasis seen with CD151 knockout mice (37). However, recent work has revealed that direct interactions with laminin-binding integrins may not be responsible for some of the molecular activity of CD151 (70). Moreover, the loss of α3β1 in prostate cancer cells promotes, rather than inhibits metastasis (38, 39) suggesting that CD151 may regulate cell motility independent of this integrin. Indeed, α3β1 expression is frequently reduced during carcinogenesis while integrin-free CD151 becomes detectable in the tumor tissue and is prognostic of cancer progression and patient survival (70).

CD151 and ALCAM as partners in the regulation of tumor cell migration.

In the present study we identify ALCAM as a novel CD151 partner that can associate with integrin-free CD151. ALCAM is a cell-cell adhesion molecule belonging to a small subgroup (VVC2C2C2) of the immunoglobulin superfamily (Ig-SF). Though initially identified and primarily expressed in activated leukocytes as the only known ligand for

CD6, ALCAM is in fact expressed broadly in human tissues and cells with predominant expression in neuronal cells, immune cells, epithelial cells, and stem cells of hematopoietic and mesenchymal origin (Fig. 28). It is functionally associated with many cell adhesion events including T-cell activation, endothelial adherence junction, neuronal guidance, and epithelial integrity (40, 41). ALCAM expression and function has been associated with many solid cancers and their progression (40). While the molecular mechanism of action had not been defined, its expression has been broadly recognized to be altered during cancer progression (42, 43)(Fig. 28). We recently demonstrated that the ectodomain of ALCAM is shed during cancer progression and that ALCAM shedding is prognostic of disease progression and patient survival (44). Since ALCAM- ALCAM interactions are responsible for mediating the activation of Rap1A and inhibiting motility, it is reasonable to hypothesize that ALCAM shedding offers a mechanism by which tumor cells can control Rap1A-mediated adhesion and migration.

Molecular integration of CD151, ALCAM, PKCα and Rap1A.

PKCα binding to the C-terminal tail of CD151 allows the tetraspanin to serve as a linker between PKCα and the integrins α3β1 (22) and α6β4 (45) thereby regulating integrin signaling. It appears that CD151 performs the same function for ALCAM but that the ultimate target of this signaling is Rap1A. PKCα has been previously identified as an inhibitor of cell migration by the Brugge laboratory during an *in vitro* screening assay of normal epithelial migration (46). Since PKCα has many substrates, these results suggest that divergent signaling mechanisms may be initiated depending on what partner CD151 interacts with. The elevation of integrin-free CD151 in cancer (70) is likely to divert signaling from integrins to other tetraspanin partners. Both CD151 clustering and homotypic ALCAM-ALCAM interactions mediated promote tumor cell adhesion and control motility by activating the small GTPase Rap1A. Rap1A was first identified in revertants after Ras

transformation of fibroblasts leading to its initial description as Krev (47, 48). Since then many lines of evidence demonstrate that Rap1 can influence cancer progression (49). The most prominent of these may be the identification of SIPA1, a Rap1 GTPase activators (Rap1GAP) as the mediator of differential metastatic ability associated with Polyoma Middle T antigen initiated breast cancer (50-52). Metastatic virulence in breast cancer appears to be associate with specific Single Nucleotide Polymorphisms (SNPs) in SiPA1(53). These lines of evidence suggest that active (GTP-bound Rap1) limits metastatic dissemination. Indeed, our data demonstrates that the activation of Rap1A (generating the GTP-bound state of Rap1A) promotes adhesion and thereby limits dissemination. The activity of Rap1 is regulated by a large number of regulators including Guanidine Exchange Factors (GEF), GTPase activators (GAP) and effectors that mediate the activity of the GTPase (28, 54). While it is extremely challenging to determine the specific molecular entities response for regulating the activity of Rap1A, we demonstrate a requirement for PKCα in the activation of Rap1 by CD151 and ALCAM. In the absence of PKCα, neither 1A5 treatment nor ALCAM adhesion is able to activate Rap1, which suggests that the signaling mechanisms associated with immobility require PKCα to activate Rap1A. Indeed, PKCα can activate Rap1 in platelets and subsequently promote adhesion through integrin activation(29). CD151 was initially identified as a platelet cell surface antigen and its clustering can induce platelet activation (70) suggesting that this mechanism may apply broadly outside the regulation of tumor cell motility.

The ability of Rap1 to control adhesion is seen across many biological systems and adhesive interactions. The GTPase can control adhesion to extracellular matrix (55), cell-cell adhesion in epithelial cells (56, 57), endothelial cells (58, 59) and hemopoietic cells (60) in addition to platelet adhesion (29). Considering the many regulators and effectors of Rap1A, there are multiple mechanisms by which the activity of the GTPase can be regulated. Many of the adhesive properties propagated by Rap1 occur via in-

tegrins but are initiated by non-integrin mechanisms including cytokine stimulation and non-integrin adhesion receptors. Indeed, the IgG superfamily member CD31 can control the activity of Rap1 and thereby regulate leukocyte adhesion via beta1 (VLA-4) and beta2 (LFA-1) integrins (61). Although ALCAM is also involved in leukocyte adhesion (62), it has global expression (Fig. 28) and could convey the ability to control Rap1 activation to epithelial cells.

Tetraspanin interactions with, and organization of, seemingly unrelated and divergent proteins and signaling molecules allow them to function as molecular integrators of cellular behaviors. In the present study we aimed to determine the mechanisms by which the clustering of CD151 is able to promote adhesion and control tumor cell motility. As motility is required for metastasis tetraspanins are considered attractive targets in the design of anti-metastatic treatment (1, 34, 63). The data demonstrates that clustering of integrin-free CD151 leads to the activation of Rap1A which subsequently promotes cell adhesion and inhibits tumor cell migration (Fig. 36). Considering that the regulation of Rap1 is a endogenous mechanism that controls cellular and influences the virulence of metastasis (49, 64), this mechanism is an attractive target for limiting tumor cell dissemination.

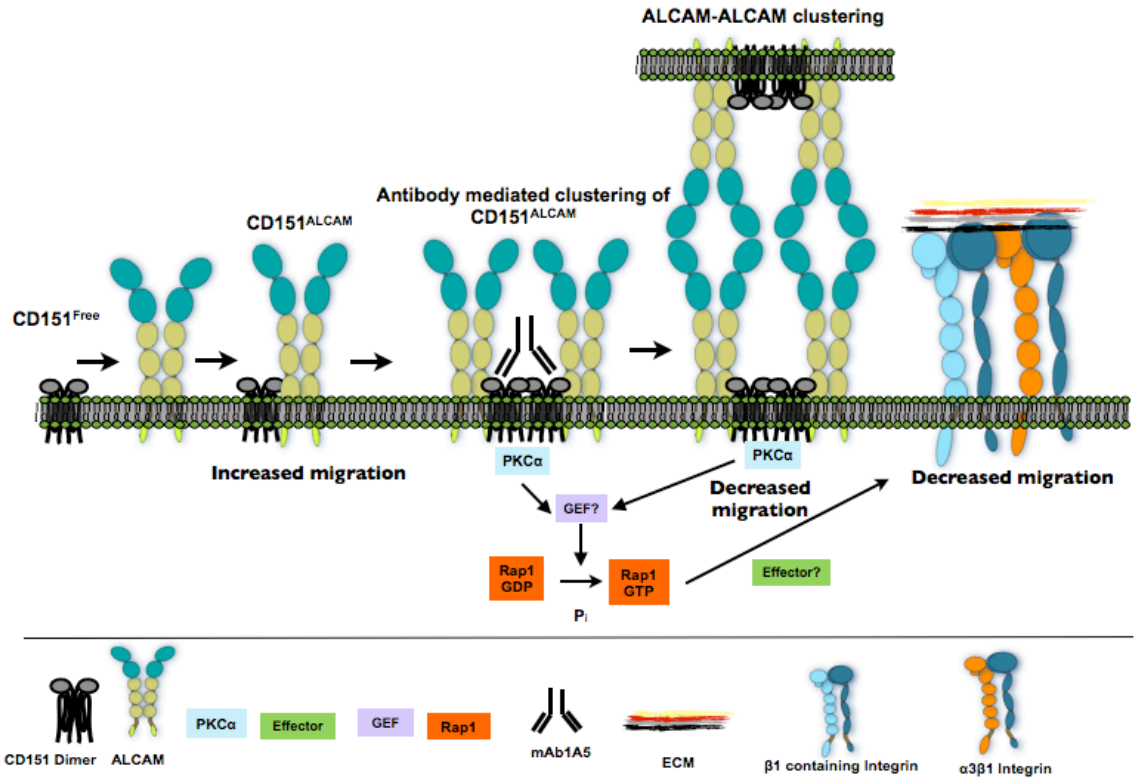


Fig.36. Summary: CD151^{Free} associates with ALCAM/CD166. In the absence of α3β1 integrin CD151^{Free} associates with ALCAM/CD166 (CD151ALCAM) to form a pro-migratory complex. When CD151^{ALCAM} is clustered in the presence of 1A5 cell motility is inhibited in a PKCα Rap1 dependent fashion. Clustering of CD151^{ALCAM} promotes cell-cell adhesion (through ALCAM mediated mechanisms) and cell-matrix adhesion (through Rap1 mediated mechanisms). Rap1 activation regulates the binding of β1 containing integrins to the ECM to inhibit migration, non-integrin proteins could also be involved as well.

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CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Summary

The data presented within this thesis provides insight into the mechanism by which CD151 controls tumor cell motility. CD151 is principally considered a promoter of tumor cell metastasis and dissemination. This is supported by a wealth of data and recently it was demonstrated that the global loss of CD151 diminishes metastasis in a mouse model of prostate cancer (224). I demonstrate herein that clustering of CD151 by the 1A5 antibody is able inhibit migration and metastasis further supporting the idea that CD151 is potentially a pro-migratory molecule. Clustering of CD151 ultimately promotes associations with proteins that are able to inhibit migration. Presently, it is not known if CD151 promotes or inhibits migration. Ultimately, the answer to this question will depend on the cellular context in which CD151 is expressed. As a member of the tetraspanin family, the principal role of CD151 is to organize other tetraspanins and tetraspanin-associated proteins into macromolecular signaling complexes on the cell surface. These complexes known as Tetraspanin Enriched Microdomains or TERMs give tetraspanins the ability to associate with and regulate the activity of cell surface partners and intracellular signaling molecules. In chapters 1 and 2 I presented the idea that there are five key factors that regulate cell motility including; soluble factors, cell autonomous factors, cell-cell adhesion, cell-matrix adhesion, and molecular integration of two or more of the other four factors. In chapter 3 we focused primarily on the roles and functions of CD151. In chapter 4 we presented data to demonstrate that 1A5 binds to the α3β1 recognition epitope of CD151 and therefore; binds to integrin free CD151 ($CD151^{free}$). Furthermore, we reveal that the ability of CD151 to control tumor cell migration through the clustering of CD151 can be independent of α3β1. Using histological staining of prostate cancer tis-

sues with anti-CD151 antibodies we demonstrate that the expression of $CD151^{free}$ is a clinical indicator of prostate cancer progression. Based on our new-found understanding of integrin-free CD151, we demonstrate in chapter 5 that CD151 can function as molecular integrator of migration by promoting the formation of a cell surface signaling complex that triggers both cell-cell and cell-matrix adhesion. This complex contains the novel CD151 associated protein ALCAM and signals to activate the small GTPase Rap1A. We also demonstrate that the expression of Rap1A in tumor cells can inhibit motility *in vitro* and *in vivo*. This work adds clarity to the mechanism by which CD151 can control tumor cell migration and metastasis. These findings not only provide mechanistic insight and possible clinical application, they also provide a basis for us to propose further investigation into the function of tetraspanins and their contribution to cell migration and cancer metastasis.

Therapeutically targeting CD151 in cancer

In recent years, the potential of targeting tetraspanin proteins like CD151 has garnered a lot of attention (155). This is primarily due to the published role of CD151 as an integrator of molecular mechanisms important for tumor cell migration and metastasis. There are essentially three proposed mechanisms by which these antibodies are potentially exerting their effects: 1) by promoting antagonistic effects and preventing tetraspanin association with lateral partners, 2) by promoting agonistic effects and activating membrane associated partner proteins and 3) by mediating antibody-dependent cell cytotoxicity (ADCC). The only way that we can truly figure out the mechanisms of action is to take an approach similar to the one outlined in this thesis. The data that we have collected demonstrates that 1A5 and antibodies like 1A5 can function in multiple ways 1) by antagonizing partner proteins in the case of ITGA3 and 2) by promoting agonistic affects and mediating signal transduction via partner proteins to control cellular processes as we demonstrate for ALCAM/CD166.

Anti-CD151 antibodies that recognize the integrin-binding (QRD) motif can inhibit motility

Our work is the first to demonstrate that anti-CD151 antibodies that bind to the integrin motif are able to control tumor cell migration by clustering the tetraspanin not associated with its integrin partner ($CD151^{free}$). Monoclonal antibody 1A5 is able to inhibit *in vitro* migration and *in vivo* motility at low micro molar concentrations. The other antibodies specific for $CD151^{free}$ can also inhibit motility and cluster CD151 albeit in the presence of much higher concentrations of antibody. Further investigation into the efficacy of 1A5 is necessary to explain these results. It maybe a simple function of affinity but it is also possible that 1A5 recognizes a distinct epitope or structure of CD151 that is functionally important for the activity of clustered CD151. The affinity of individual antibodies could be determined using Biacore analysis with purified CD151. This system would allow us to calculate association constants (Ka) and dissociation constants (Kd) and also rank the anti-CD151 antibodies according to their calculated binding affinities. Although it has been demonstrated that the QRD motif of CD151 determines the ability of CD151 to form a complex with integrin alpha 3, the differences in the abilities of the antibodies that recognize $CD151^{free}$ and inhibit migration suggest that the actual epitope may be composed of more than these three epitopes or that the epitope maybe inaccessible due to the presence of other amino acids or chemical modifications in and around the epitope that may diminish antibody binding-efficiency. One of the best ways to analyze the differences in antibody binding to CD151 would be to perform antibody docking experiments combined with X-ray crystallography which would allow us to look at the chemical interactions of these antibodies with CD151. Unfortunately, the crystal structure of CD151 has not been determined because transmembrane proteins are extremely difficult to crystalize. Nevertheless, the extracellular domain of CD82 has been crystalized suggesting that the same could be done with CD151 with and without bound antibody. Comparison of physical interactions and structural changes upon antibody binding would

give further insight into the differential activity of these antibodies and how that might relate to the natural activity of CD151.

Expression of CD151^{free} regulates PCa progression

We demonstrated in a retrospective study of metastatic prostate cancer that the detection of CD151^{free} is increased during the progression of metastasis as evidenced by positive staining with the 1A5 antibody. Together with the diminished metastasis in the absence of CD151 this suggest that CD151^{free} is a positive regulator of metastasis. Interestingly, we demonstrate that clustering of CD151^{free} leads to enhanced adhesion via Rap1A activation and subsequently reduces migration. This would suggest that CD151^{free} can certainly act as an inhibitor of metastasis. These seemingly contradictory results, leave us to conclude that it is not yet clear if CD151^{free} is necessary and/or sufficient to promote metastasis. Further evaluation of its specific activity would require the generation of CD151^{free}. Is it possible to dissociate CD151 from α3β1 integrin and determine the contribution of CD151^{free} to migration and metastasis? Chometon et. al reported that the anti-CD151 antibody TS151r is capable of dissociating CD151 from its integrin partner and that this leads to an increase in migration on laminin matrices. Is this a means to generate CD151^{free}? Interestingly, TS151r binds to the same epitope as 1A5 however, unlike 1A5 treatment of cells with this antibody promotes instead of inhibits cell motility. TS151r could be used as a tool to dissociate CD151 from α3β1 and migration analyzed in the presence of 1A5 treatment allowing us to look at the affect on migration mediated by clustering of CD151^{free} It is possible that the effect of TS151r is specific for migration on laminin matrices since it was not tested on other substrates or *in vivo*. Is it possible to disrupt CD151 integrin association in vivo without clustering the tetraspanin? Soluble extracellular domain (EC2) or competing peptides or single-chain antibody might be useful for this purpose. Our laboratory demonstrated that monomeric Fab fragments of 1A5 continue to bind CD151 but fail to inhibit migration. This observation suggests that the detection of CD151^{free} maybe the product of uncoupling CD151 from its integrin

partners. In tissues that express CD151^{free} I demonstrated that they do have reduced levels of integrin α3β1 suggesting that loss of integrin expression correlates with the appearance of CD151^{free}. We demonstrate that the subsequent association with other partners such as ALCAM and the activation of downstream signaling is what controls its contribution to cell migration. This suggests the need for further structure-function investigation of CD151^{free} in order to determine its contribution to migration. This is potentially challenging. because it has been demonstrated that the forced expression of CD151 can promote migration and metastasis of cancer cells (224,225). Nevertheless, further investigations into the protein-protein associations of CD151 will be needed to fully extrapolate its contribution to cancer metastasis and possibly reveal a therapeutic target that can be used to inhibit metastasis. The laboratory has initiated a large-scale cloning project to generate a molecular tool kit that enables my colleagues to rapidly build fluorescent constructs. It may be possible to build a variety of FRET or BRET probes that make it possible to follow the molecular interactions that CD151 engages in as it contributes to cell migration along the metastatic cascade.

Clinically it will be important to determine at what stage during disease progression CD151^{free} become available. To determine this, we will have to perform a more comprehensive analysis of CD151^{free} across the clinical spectrum of prostate cancer and other tumors. A particular emphasis would have to be placed on early stage disease where know evidence of invasive properties are detectable by classic histology. If the detection of CD151^{free} correlates with disease progression for early stage patients, it could serve as a means to determine disease stage prior to the development of visible metastasis or be used as a means to guide early clinical intervention.

<u>The ALCAM/CD151 immobility complex</u>

In Chapter 5 I used a proteomic based approach to identify the protein components of the CD151 TERM complex mediated by clustering of CD151 on the cell surface.

When clustered in the presence of the anti-CD151 antibody 1A5 the tetraspanin localizes to the areas of cell-cell contact and associates with the immunoglobulin protein ALCAM/CD166. I demonstrated further that ALCAM is required for clustered CD151 to regulate cell motility. The physical association of CD151 and ALCAM clearly demonstrated that ALCAM is a protein component of the TERM. Even though, this complex exits on the cell surface it is not known whether this association is direct or indirect. I demonstrated that the complex is relatively stable due to the fact that it can be precipitated under both mild and stringent detergent extraction conditions. In order to determine if the complex formation is through direct interactions with CD151, chemical cross linking experiments could be performed with a cell impermeable cross-linker such as 3′,3′-dithiobis(sulfosuccinimidyl propionate (DTSSP) in the presence of the 1A5 antibody. Although covalent cross-linking is a commonly used technique there is the risk of cross-linking protein complexes with CD151 due to their membrane proximity and not due to their direct physical association with CD151. As I have eluded to previously, protein-protein interactions with tetraspanin proteins involve amino acid residues contained in the hypervariable region of the LEL, therefore it is not difficult to speculate that these same regions are involved in the formation of the CD151/ALCAM complex. In order to determine if the LEL is required for the ability of CD151 to associate with ALCAM CD151it would be necessary to generate CD151 chimeras containing mutant LELs from other tetraspanin proteins. Co-immunoprecipitations can then be performed to determine if the CD151 LEL is required for the formation of the complex with ALCAM. I could also potentially use the CD151GFP human/mouse LEL substitution mutants introduced in Chapter 4 of this thesis. Clustering of CD151 promotes the localization of both CD151 and ALCAM to the areas of cell-cell contact and while we were able to visualize the localization of CD151GFP in live cells in the presence of 1A5 we were unsuccessful in imaging the localization of ALCAM-GFP after 1A5 stimulation. This was primarily due to the fact that, upon transfection, ALCAM-GFP localized to the areas of cell-cell contact and I

was not able to visualize any further localization. Indeed, it is at present not clear wether CD151 brings ALCAM into areas of cell-cell contact or the converse. The data presented in chapter 4 of this thesis demonstrates that the antibody 1A5 binds $CD151^{free}$ which is associated with ALCAM. This raises the possibility that in the absence of α3 CD151 may preferentially associate with ALCAM. Co-immunoprecipitation analysis of alpha3 knock-down cells for the CD151/ALCAM complex could allow us to answer this question.

Traditional scratch assays were used to demonstrate that ALCAM was required for the inhibition of migration mediated by clustering of CD151. Interestingly, when I analyzed the migration of CD151 knockdown cells on ALCAM Fc the inhibition of migration mediated by plating on ALCAM Fc was rescued. These data suggested to us that CD151 and ALCAM are mutually required to control cell motility. The exact molecular and structural relationship between ALCAM and CD151 remains poorly understood. I demonstrated that CD151 is required for ALCAM to activate Rap1A however, the role of CD151 in the regulation of migration is likely to be more complicated than that. The tetraspanin is likely to control the incorporation of ALCAM into macromolecular complexes and even its availability on the cell surface. The ability of ALCAM to connect with tetraspanin enriched microdomains, the cytoskeleton and its availability are also likely to influence the status of post-translation modifications of ALCAM including ubiquitination and shedding (226). These mechanisms could possibly be regulated by the association of ALCAM with CD151. Published work with other tetraspanins have suggested that all of these mechanisms might be available for CD151 to control ALCAM. Some of these regulatory mechanism, including control of post-translational modifications have been recorded for the functional regulation of α3 by CD151 (206). Further structure-function analysis of the ALCAM-CD151 interaction will help guide future experiments that investigate how this relationship can control down stream signaling and its control of cell migration.

Requirement for Protein Kinase Cα and Rap1A

The inhibition of cell migration mediated by CD151 and ALCAM is accomplished via signaling through Protein Kinase C Alpha (PKCα). CD151 associates with PKCα and serves an adaptor role between the kinase and integrin α3 (200). It not unreasonable to suggest that ALCAM is a substrate of PKCα. Although it has been demonstrated that ALCAM homotypic adhesion requires PKCα signaling, the lack of a serine or threonine residue in the C-terminal tail of ALCAM challenges the idea that ALCAM is a PKCα substrate (227). Activation of the small GTPase Rap1A in the presence of CD151 clustering and ALCAM mediated adhesion ultimately controls adhesion. The activation of small GTPases like Rap1A is ultimately determined by Guanine Nucleotide Exchange Factors (GEFs) which promote the addition of a phosphate group to the GDP bound small GTPase to promote activation and the initiate of intracellular signaling events. The small GTPase remains activated in the GTP bound state until it is hydrolyzed by the activity of GTPase activating enzymes. There are a number of GEFs that could possibly activate Rap1A to control adhesion and it is likely that PKCα can mediate the activity of one or more regulators of Rap1. In a study performed by Letschka et. al it was determined that another isoform of PKC, PKCθ associates with and phosphorylates the guanine exchange factor RapGEF2 also known as PDZ-GEF2. PDZ-GEF2 activates Rap1A and controls the adhesion of T-cells. Protein Kinase C isoforms are only active when associated with the membrane and while we have determined a role for PKCα in the regulation of motility by CD151 it is not known if CD151 clustering is able to activate PKCα. While we could perform protein kinase activation assays, one of the easiest ways to analyze activation of PKC would be to image the localization of GFP tagged PKCα in live cells in the presence of 1A5 treatment. This experiment will answer two important questions: 1) does 1A5 clustering promote PKCα activation and 2) does activated PKCα localize to similar membrane complexes as CD151? ALCAM is not a PKCα substrate but it is likely that one or more molecular participants in the signaling cascade is. In order to investigate this a targeted screen in which known regulators of Rap1A are knocked down by

RNA could be performed. The knock down that prevents activation of Rap1A by CD151/ALCAM is likely to be a downstream target of PKCα. Alternatively, we could perform radioactive labeling assays in the presence and absence of PKC inhibitors. Comparative analysis of phosphorylated proteins could reveal the proteins targeted by phosphorylation by PKCα in response to CD151 clustering.

Rap1 Activation controls motility

Our data demonstrated that the activation of Rap1A can inhibit motility. Similarly, I demonstrated that the expression of the constitutively active non-hydrolyzable Rap1V12 can similarly inhibit cell migration. Our data conflicts with data generated using other cancer cell types and models that suggest that the activation of Rap1 promotes tumor cell metastasis and invasion . Specifically, Bailey et. al has shown that the activation of Rap1 promotes metastasis of PC3 cells. Mechanistically, they demonstrate that stromal derived factor 1 (SDF-1) is involved in the progression of metastasis which is inhibited when PC3 cells expressing Rap1GAP (GTPase activating protein) are used (228). In another study, McSherry et. al demonstrated that Junctional Adhesion Molecule-A (JAM-A) can promote the migration of MCF-7 breast cancer cells in a Rap1A-dependent fashion (229). These studies imply that the ability of Rap1A to control migration is both stimulus and cell type specific. Most of the current literature focuses on the activation of Rap1A being an positive regulator of migration and metastasis. Our work is one of the first to suggest that the activation of Rap1 could potentially act as an inhibitor of cancer motility and metastasis. It is possible that Rap1 plays opposing roles in cell-matrix adhesion and cell-cell adhesion. While the activation of Rap1 can promote migration by regulating matrix interactions, it may inhibit tumor cell migration by regulating cell-cell adhesion. In Drosophilla, Knox et al. demonstrated that the expression of Rap1 is sufficient to maintain adherens junctions and promote cell-cell adhesion a process that is important for proper wing development. Interestingly, one of the key *in vitro* phenotypes associated with clustering of CD151 is a promotion of platelet cell-to-cell adhesion. In tumor cells I

also demonstrate that clustering of CD151 promotes the localization of CD151 and ALCAM to areas of cell-to-cell contact. The ability to localize GTP-bound Rap1 would allow us to determine if Rap1 is similarly localized with CD151 and ALCAM to similar subcellular locations.

One significant distinction between the inhibition of cell motility and metastasis seen in our studies vs. the promotion of motility seen in other studies is the nature and duration of Rap1 signaling. Most studies investigate dynamic activation of Rap1 in response to a soluble ligand, activation which is normally short in duration. In contrast, the activation that we have investigated in response to cell-surface adhesion promotes persistent (chronic) activation of Rap1. The activation of Rap1 mediated both by CD151 clustering and ALCAM-ALCAM adhesion is robust and maintained for more than 48 hours post-stimulation. Is it possible that the perpetual activation of Rap1 inhibits migration while cycling of Rap1 from the GDP bound to the GTP bound state is able to promote migration? Our *in vitro* and *in vivo* data associated with the expression of the Rap1V12 suggests that this is indeed the case. Further support for this possibility comes from work done by Freeman et. al who demonstrated that the expression of Rap1V12 is able to inhibit the migration of B16 by altering focal adhesion turnover. They further demonstrated that focal adhesion turnover and migration is dependent on 1) Rap1 activation and 2). Rap1 cycling between the GDP bound and GTP bound states. Stabilization of focal adhesions is also seen in response to CD151 clustering. *In vivo* Freeman et. al. also show that the activation of Rap1 is able to inhibit the metastasis of B16 cells to the lungs in murine experimental metastasis assays (230). Further analysis of Rap1 activation in scenarios of cell-cell contact might reveal its contribution to cell migration. It is highly likely that the transient activation of Rap1 in response to soluble ligands is functionally and spatially distinct from the activation in response to adhesive interactions. To visualize Rap1 activation in live cells treated with 1A5 or plated on ALCAM-Fc, FRET analysis could be performed in HEp3 cells expressing pRaichu-Rap1, a commonly used

biosensor of Rap1 activation (231). A comparison of Rap1 activation in response to cytokines vs tetraspanin-mediated adhesions could provide some guidance on future investigations of this signaling mechanism. Furthermore, detailed investigation of Rap1 in the migration of non-transformed cells is warranted to determine how the GTPase contributes to migration prior to oncogenesis. There maybe distinct changes that occur as a function of oncogenic transformation and the mode of tumor cell migration (ie. epithelial vs mesenchymal) might determine the ultimate contribution of Rap1 during metastasis.

Subcellular localization of the components of the immobility complex

I have presented data demonstrating that the tetraspanin CD151 controls the motility and metastasis of tumor cells through the formation of a cell surface complex containing a number of very diverse proteins. The order of events as presented here suggests that clustering of CD151 forms a complex with ALCAM on the cell surface. The formation of this complex ultimately activates small GTPase in a PKCα-dependent manner. All of the proteins that we have found to be involved in this mechanism are organized on the cell surface and their activity is likely to be controlled at least in part by their spacial orientation. This is at leas in part supported by the fact that migration in 3D and *in vivo* is inhibited much more by anti-CD151 antibodies than it is *in vitro*. Visualization all of the proteins involved in this mechanism will be important for determining how the spatial orientation of the cell and its cell-surface interactions will influence cell migration. Our fluorescent tool kit currently under construction may help facilitate new experimental imaging of these molecules in 3D and *in vivo*. Many of the discoveries presented in this thesis were made in the chick chorioallantoic membrane (232). Imaging the spatial distribution of CD151, ALCAM, Rap1 and other participants may greatly clarify how the organization of these complexes can control tumor cell motility and their subsequent metastasis.

The integrated model of motility regulation by $CD151^{free}$

Chapter 4 of this thesis demonstrated that there are two pools of CD151 expressed on the tumor cell surface integrin associated ($CD151^{ITGA}$) and integrin free CD151 ($CD151^{free}$). Additionally, I demonstrated that the ability of CD151 to control motility is independent of α3β1 integrin and that $CD151^{free}$ is potentially a metastasis promoter. In tumor tissues I also demonstrated that the presence of $CD151^{free}$ is concomitant with the loss of α3 expression. The identity of the protein associating with $CD151^{free}$ was answered in chapter 5 where I demonstrated that $CD151^{free}$ associates with ALCAM/CD166 ($CD151^{ALCAM}$) and that this complex promotes migration. The clustering of $CD151^{ALCAM}$ by 1A5 inhibits motility in a PKCα and Rap1A dependent manner. Collectively the data generated in these chapters allows me to generate the integrated model of motility regulation by CD151 (Fig.37).

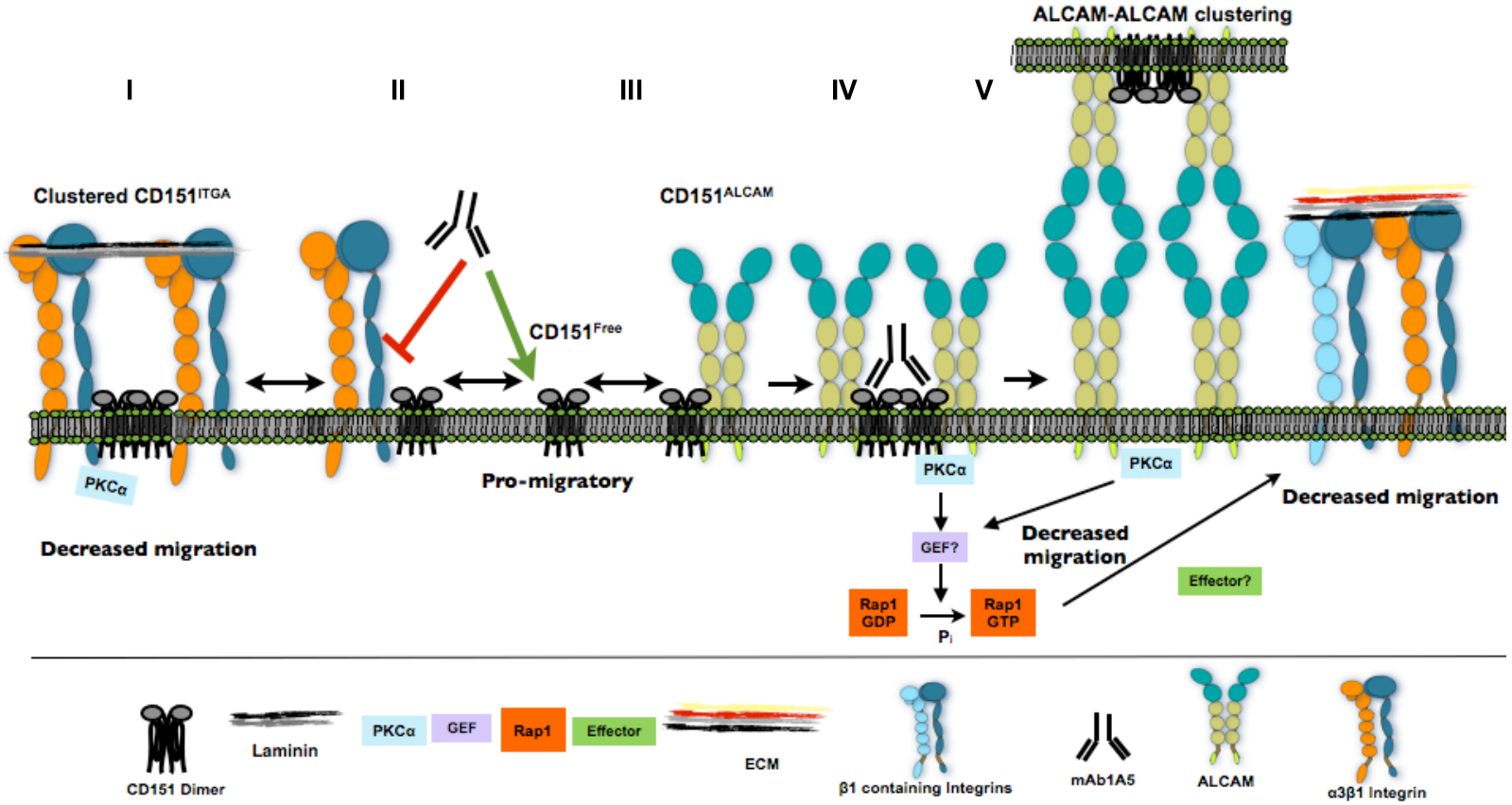

Fig. 37.. Integrated model of motility regulation by CD151. I). Clustering of CD151^{ITGA} inhibits tumor cell migration on laminin matrices by promoting stable adhesion. II).1A5 binds to integrin free CD151 CD151^{Free} which in tumor tissues arises due to the loss of α3β1 expression. III). In the absence of α3β1 CD151^{Free} associates with ALCAM/CD166 and functions to promote migration. IV). Clustering of CD151^{ALCAM} using the 1A5 antibody inhibits motility and migration through PKCα to activate the small GTPase Rap1A. Activation of Rap1A promotes cell-matrix adhesion and inhibits migration presumably through integrin activation and ECM binding. V. ALCAM-ALCAM mediated adhesion can control motility in a PKC, Rap1 dependent manner.

Concluding Remarks

The data presented within this dissertation highlights the role of the tetraspanin CD151 as a molecular integrator of migration and metastasis. I demonstrated that clustering of CD151 with the migration and metastasis inhibitory antibody 1A5 functions independently of the integrin α3β1 to control migration. I also show that anti-CD151 antibodies that bind to the integrin-binding motif are able to inhibit migration by clustering integrin free CD151 ($CD151^{free}$). The most exciting aspect of this work is that I was able to apply a basic molecular mechanism of migration to an enhanced understanding of the progression of metastatic prostate cancer. Mechanistically, clustering of CD151 promotes both cell-matrix and cell-cell adhesion to control migration. This data supports the idea that the primary roles of tetraspanins are to function as scaffolding proteins that are able to regulate cellular processes by promoting the association of diverse signaling proteins and processes. Clinically we demonstrated that $CD151^{free}$ is present in advanced cancers,and can independently predict recurrence and metastasis free survival. This data demonstrates that the dissociation from the integrins is clinically relevant. Collectively, the work presented herein highlights a number of key findings: 1) the tetraspanin CD151 functions as a master integrator of cell migration and metastasis 2) clustering of CD151 is sufficient to control both cell-cell and cell-matrix adhesion 3) there are potentially two pools of CD151 integrin associated ($CD151^{ITGA}$) and non-integrin associated ($CD151^{free}$) and 4) the expression of $CD151^{free}$ has clinical significance to the progression of metastatic cancer. In addition to improving our understanding of tetraspanins and associated microdomains I elucidated a large part of the mechanism by which the anti-CD151antibody 1A5 functions to control migration and metastasis. Understanding the function of CD151 could ultimately aid in the creation of therapeutics targeting cell motility in the treatment of metastatic disease.

Both CD151 clustering and ALCAM-mediated adhesion trigger Rap1 activation and are sufficient to promote tumor cell immobility *in vitro* and *in vivo*. Ultimately, I demonstrated that CD151^{free} forms a complex with ALCAM and clustering through tetraspanin antibodies or ALCAM binding both lead to down stream activation of Rap1A Rap1 activation has been suggested to promote metastasis however, our data suggests the opposite. I demonstrated that perpetual activation of Rap1 is able to inhibit motility by promoting adhesion. In addition to the clarity of molecular signaling and the clinical relevance of both CD151^{free} and ALCAM, this work could be useful in the design of therapies designed to target motility in metastasis. This work demonstrates that persistent and stable adhesions mediated by Rap1 signaling inhibit invasion and metastasis. This implies that cells which lose such stable adhesions are prone to become metastatic. While this has been demonstrated for E-cadherin, this thesis suggests that ALCAM maintains a positive feed-forward loop that maintains stable adhesions. This loop can be disrupted by temporarily or permanently disabling ALCAM. The laboratory is actively investigating the regulation of ALCAM. Interestingly, the loss of ALCAM inhibits metastasis suggesting that it indeed contributes to dissemination. This role is supported clinically by the findings that ALCAM expression not only persists but is frequently upregulated in tumor tissues. Conversely, at the protein level ALCAM is subjected to shedding by ADAM17 and ubiquitination by a, currently unknown, E3 ligase. ALCAM shedding elevates greatly during cancer progression and this shedding corresponds with poor patient outcome(17). Our current interpretation of these observations is that dynamic regulation of ALCAM-ALCAM interactions allows tumor cells to engage adhesive properties via Rap1 activation when needed. Disabling these same interactions can disable the feed-forward loop, reduce adhesion and promote dissemination. Future therapeutic targeting of this process could be successful by simply limiting the turnover of ALCAM or limiting the inactivation of Rap1. There are likely to be several approaches that could accomplish this. While targeting CD151 is likely to have many off-target effects in normal homeostasis, targeting AL-

CAM shedding or Rap1 (in)activation may provide a means to limit metastatic tumor cell migration more specifically.

Ultimately this work suggests that metastasis is facilitated by the dynamic regulation of migration not a mere activation of motility. Therefore, in order to truly make inroads into the treatment of metastatic disease, it will be necessary to develop a deeper understanding of motility and accomplish greater control over the mechanisms that drive tumor cell migration.

APPENDIX

A. RAP1 ACTIVATION IS POTENTIALLY A GLOBAL TETRASPANIN MECHANISM

In chapter 5 of this thesis we determined the identity of the components of the immobility complex mediated by the clustering of CD151 on the cell surface. These data demonstrated that ALCAM is component of the TERM complex and is functionally required for the clustering of CD151 to inhibit cell migration. This inhibition of motility was dependent on the downstream activation of the small GTPase Rap1A in a PKCα dependent fashion. Like CD151, ALCAM is involved in the regulation of cell motility. Interestingly, when we stimulated ALCAM-ALCAM homotypic interactions by plating tumor cells on ALCAM Fc, it also leads to the activation of Rap1 in a PKCα dependent manner. Independent of CD151 clustering and ALCAM mediated adhesion, perpetual Rap1 activation inhibits cell motility *in vitro* and *in vivo*. We focused on the role of CD151 in the regulation of motility; however, as mentioned earlier CD151 is not the sole cell motility and cancer associated tetraspanin. CD9, CD63, CD82, and CD81 are associated with motility and monoclonal antibodies to these tetraspanins have been used as tools to cluster the tetraspanin. We first verified the expression level of these tetraspanins on the cell surface of HEp3 cells by flow cytometry and demonstrated that they are all expressed albeit at varying levels. In order to determine if the clustering of these tetraspanins regulates migration we performed transwell migration assays in the presence of these antibodies. Cell migration was inhibited significantly in the presence of antibodies to all of the tetraspanins except CD63. We then analyzed Rap1 activation levels in the presence of all of these antibodies including MAb 1A5 (anti-CD151) and demonstrated

that Rap1 is activated in the presence of the migration inhibitory antibodies. This data clearly suggests that the activation of Rap1 to control migration mediated by tetraspanin clustering is potentially a global mechanism (Fig. A1). This data motivates a number of key questions; 1). is PKCα signaling involved in the activation of Rap1 and the inhibition of motility 2). are some of the same partner proteins associated with the regulation of by CD151 involved and 3). if these tetraspanins do not associate specifically with ALCAM do TERM complexes generated by the clustering of these molecules contain CD151 and by association ALCAM? Interestingly, in a recent paper by Gilsanz et al it was demonstrated that CD9 forms a complex with ALCAM in T-cells and that this interaction is important for regulating activation, proliferation and adhesion of T-cells (233)

Rap1 activation is the common mechanism of CD151 clustering and ALCAM mediated adhesion. The demonstration that the activation of Rap1 is sufficient to control migration and metastasis by Freeman et. al strongly supports the data presented in this thesis. The decrease in focal adhesion turnover, combined with an an increase in paxillin localization in Rap1V12 expressing cells further supports the role of adhesions in the regulation of migration and subsequent metastasis(230). Interestingly, CD151 clustering also leads to increased focal adhesion formation as evidenced by an increase in paxillin staining in 1A5 treated cells (Fig. A2). It would interesting to determine if the clustering of CD151 is able to control migration through the regulation of focal adhesion turnover(24).

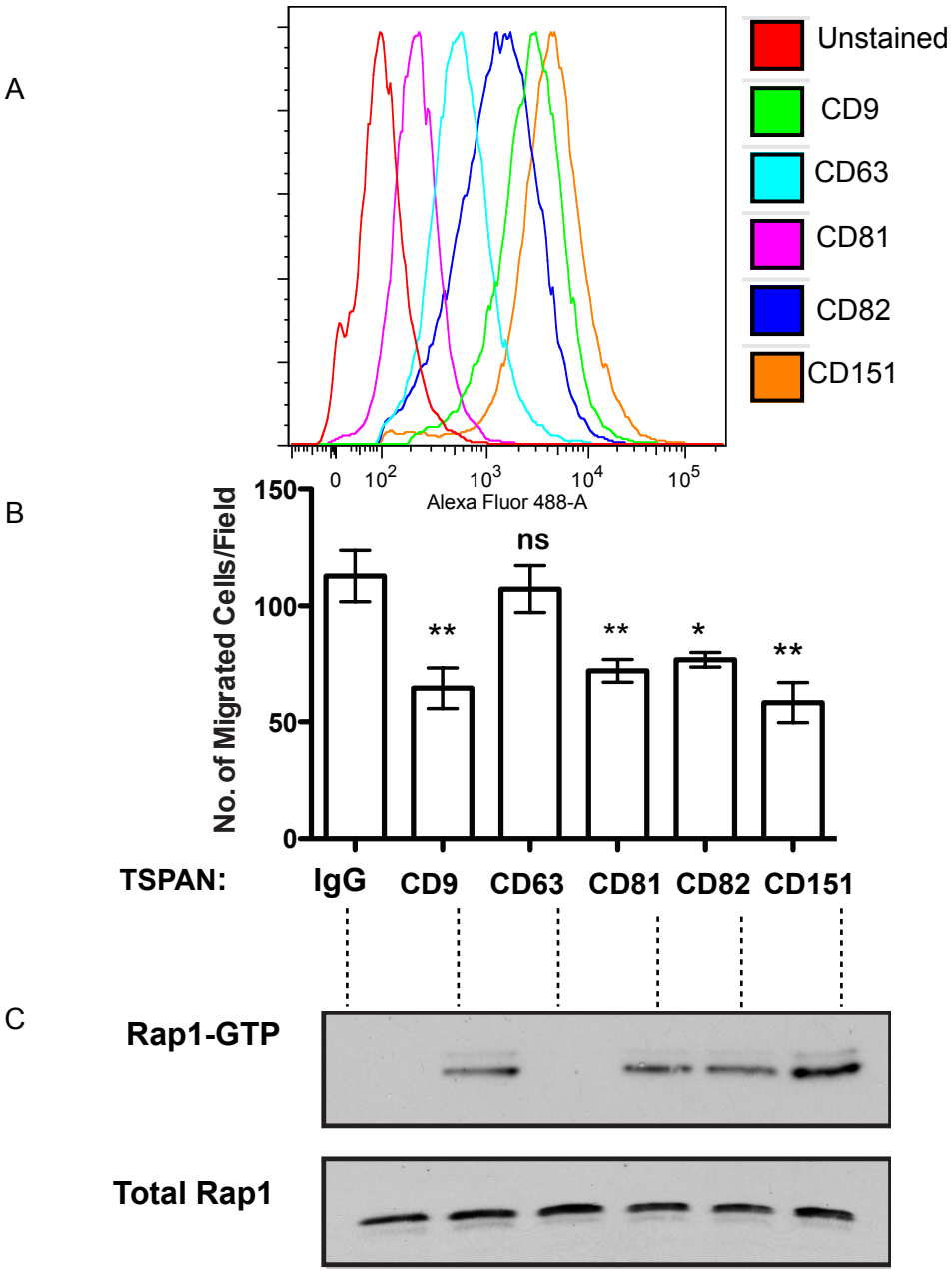

Fig. A1. Rap1 activation is a conserved mechanism of Tspans that inhibit motility. A). Flow cytometric histograms of the indicated tetraspanins on the surface of HEp3 cells. B). Transwell migration in the presence of the indicated antibodies. C). Rap1 activation was analyzed in the presence of the indicated antibodies. Data is representative of 3 independent experiments. Migration assays in B performed in triplicate and data represented as SEM * ($p>0.05$) ** ($p<0.01$) *** ($p>0.001$)

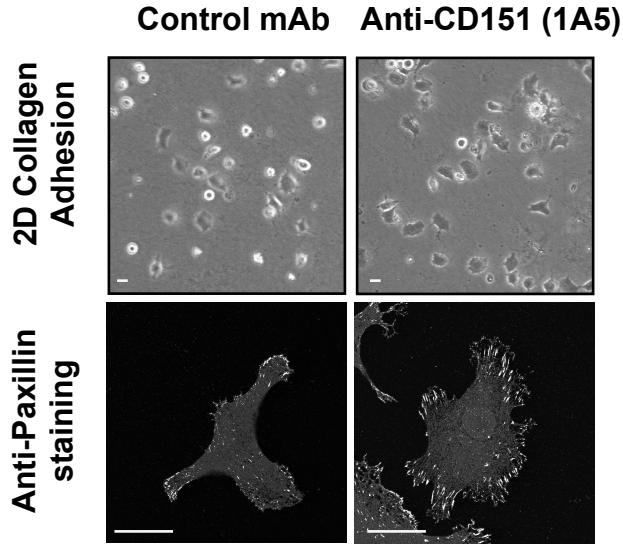

Fig. A2. 1A5 treatment promotes focal adhesion formation. (A). Treatment of HEp3 cells with 1A5 promotes an increase in paxillin expression compared to untreated cells (reproduced from (24) Data is representative of 3 independent experiments with $\geq$ 5 cells imaged per experiment.

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