CELL MIGRATION IN CANCER: FROM MECHANISM TO CLINICAL TRANSLATION

Catalina Vasquez

Follow this and additional works at: https://ir.lib.uwo.ca/digitizedtheses

Recommended Citation
Vasquez, Catalina, "CELL MIGRATION IN CANCER: FROM MECHANISM TO CLINICAL TRANSLATION" (2011). Digitized Theses. 3280.
https://ir.lib.uwo.ca/digitizedtheses/3280

This Thesis is brought to you for free and open access by the Digitized Special Collections at Scholarship@Western. It has been accepted for inclusion in Digitized Theses by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.
CELL MIGRATION IN CANCER: FROM MECHANISM TO CLINICAL TRANSLATION

(Thesis format: Integrated Article)

by

Catalina Vasquez

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Catalina Vasquez, 2011
The thesis by

**Catalina Vasquez**

entitled:

**Cell Migration in Cancer: from Mechanism to Clinical Translation**

is accepted in partial fulfillment of the requirements for the degree of
Master of Science

__________________________  ______________________________
Date                               Chair of the Thesis Examination Board
Abstract

Genetic and epigenetic changes that occur in the cell induce oncogenic transformation and increased cell migration resulting in metastasis. We studied molecular mechanisms that involve migratory protein, Rhamm-induced cell transformation by overexpressing it in 10T1/2 cells. We demonstrated that Rhamm isoform $^{163}$, induces cell resistance to anoikis and to growth inhibitory signals through an ERK1,2 dependent mechanism by assessing foci and colony formation in soft agar. We also showed that this isoform of Rhamm exhibits different subcellular localization than Rhamm full lenght (Rhamm$^{FL}$). These differences may account for the oncogenic activity of Rhamm$^{163}$. Additionally, we assessed CD151 immunoreactivity in prostate cancer tissues by using an anti-migratory mAb, 1A5, and found that positive CD151 staining predicts early biochemical failure and metastasis in these patients. These studies increased the understanding of the molecular mechanisms that involve tumour progression and identified a subcellular population of CD151 that associates with poor outcome in prostate cancer.

Keywords: CD151, Full Length RHAMM, RHAMM$^{163}$, cell migration, oncogenic transformation, prognostic factor, prostate cancer, subcellular localization, metastasis
Co-Authorship Statement

CD151 Immunoreactivity Predicts Early Biochemical Failure and Metastasis in Prostate Cancer (PCa)

The chapter under this name is an adaptation of a manuscript prepared for submission for publication to The Lancet Oncology. I reviewed the medical charts and created the patient database, optimized the staining protocol and applied it in the tissue samples, performed data analysis and interpretation, and wrote the report. John D. Lewis, Joseph Chin, and Carlos Martinez participated in the experimental design, data analysis, and in reviewing the report. Andries Zijlstra provided the 1A5 antibody, contributed the microarray data, and was involved in study design. Venu Chalasani and Andrew K. Williams were involved in study design, data analysis and interpretation, and reviewed the manuscript. Susann M. Chan and Jose A. Gomez performed the pathology review and interpretation. I performed the majority of the work in this study.

Rhamm compartmentalization and its functions

The chapter under this name is an adaptation of a manuscript prepared for submission for publication. I designed and expressed the ZsGreen-Rhamm constructs in Rhamm -/- MEFs and 10T1/2 cells. I also performed all immunofluorescence staining, confocal imaging, colocalization analysis, Fluorescence Recovery After Photobleaching (FRAP), western blots, immunoprecipitation experiments and statistical analysis related to this constructs. These experiments are represented in figures 2.4 - 2.8 and supplemental figures 2.1 and 2.2.
Shiwen Zhang and Sara Hamilton performed the colony formation in soft agar and foci formation experiments illustrated in figure 2.1 B, C, and D and figure 2.2. Sara Hamilton performed the nuclear ERK experiments shown in figure 2.9. Pull-down and mass spectrometry analysis of Rhamm was performed by Dr. Turley's lab. Figures 2.1 B, C, D, figure 2.2, and figure 2.9 represent my adaptation from the original ones made by Shiwen Zhang and Sara Hamilton. I performed the majority of the work in this study and wrote this document under the supervision of Dr. Eva Turley.
Acknowledgments

I am heartily grateful to my supervisors Dr. John Lewis and Dr. Eva Turley for having offering me this great opportunity. Thanks to your support, encouragement, and guidance, I was able to become more acknowledgeable not only about the importance of cancer research and the particular subjects of my studies, but also about important lessons in life. Thanks to your entrustment, I have improved as a researcher and as a person.

I would also like to thank to my advisory committee members Dr. Savita Dhanvantari and Dr. Ann Chambers for their support and helpful comments along this journey.

I offer my regards to all the members of the Turley lab, Conny Tolg, Pat Telmer, Jenny Ma, Natalia Akentieva, and very special thanks to Caitlin Ward for her support and invaluable help. I also thank Sara Hamilton, Shiwen Zhang for their contribution to my studies. My special regards are also addressed to the members of the Lewis lab, Hong Leong, Bhavik Manocha, Laura Fung, Balaji Iyengar, Navid Baktash, Amy Robertson, and Chen Lu for their company and patience. Specially, I want to thank Jailal Ablack and Desmond Pink for their insight and advice, Amber Ablack for her patience, lessons and trust in me since I first arrived to the lab. In addition, I would like to give my recognition to Rae-Lynn Nesbitt, Choi-Fong Cho, and Luz Adriana Diaz for their true friendship, infinite patience, supportive words and company throughout this time.

Lastly, I am immensely grateful to my family; my mother and ants who always believed in me even when I had lost the faith, to my father for his proudness towards me, my brothers and sister for their valuable words of support. Finally and most importantly, to Gabriel, my husband, and Santiago, my son, for their transcending patience, encouragement, and love. I could not have finished this endeavour without them.
This thesis is dedicated to my husband, Gabriel Betancur, and to my son, Santiago
Betancur.

Thank you!

Let your mind start a journey thru a strange new world. Leave all thoughts of the world
you knew before. Let your soul take you where you long to be...Close your eyes, let your
spirit start to soar, and you'll live as you've never lived before.

Erich Fromm
## Table of Contents

CERTIFICATE OF EXAMINATION................................................................. ii

Abstract........................................................................................................ iii

Co-Authorship Statement................................................................................ iv

Acknowledgments.......................................................................................... vi

Table of Contents........................................................................................... viii

List of Tables .................................................................................................. xi

List of Figures .................................................................................................. xi

List of Supplemental figures and Appendices .............................................. xiii

List of Abbreviations .....................................................................................xiv

### Chapter 1 Introduction

1.1 Overview.................................................................................................. 1

1.2 Cancer and metastasis as a multistep process ........................................ 2

1.2.1 Oncogenic transformation overview .................................................. 2

1.2.2 Cell migration and cancer ................................................................. 5

1.3 The role of RHAMM in cell migration and cancer progression .............. 10

1.3.1 RHAMM structure ........................................................................... 10

1.3.2 The role of RHAMM in cell migration ............................................... 12

1.3.3 The importance of RHAMM in cancer .............................................. 18

1.4 CD151 in cell migration and cancer progression .................................... 20

1.4.1 CD151 structure .............................................................................. 20

1.4.2 The role of CD151 in cell migration ................................................ 21

1.4.3 The importance of CD151 in cancer ................................................. 26

1.5 Clinical relevance of targeting cell migration associated proteins: RHAMM as a therapeutic target and CD151 as a prognostic factor in PCa .................. 29
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5.1 Therapeutic targeting of the migration-associated protein RHAMM</td>
<td>30</td>
</tr>
<tr>
<td>1.5.2 Use of CD151 as a prognostic biomarker for disease recurrence and</td>
<td>32</td>
</tr>
<tr>
<td>metastasis</td>
<td></td>
</tr>
<tr>
<td>1.6 Final note</td>
<td>33</td>
</tr>
<tr>
<td>1.7 Hypotheses</td>
<td>34</td>
</tr>
<tr>
<td>1.8 References</td>
<td>34</td>
</tr>
<tr>
<td><strong>Chapter 2 Rhamm Compartmentalization and its Functions</strong></td>
<td>49</td>
</tr>
<tr>
<td>2.1 Abstract</td>
<td>50</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>51</td>
</tr>
<tr>
<td>2.3 Methods</td>
<td>55</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>64</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>79</td>
</tr>
<tr>
<td>2.6 Conclusion</td>
<td>84</td>
</tr>
<tr>
<td>2.7 Acknowledgements</td>
<td>85</td>
</tr>
<tr>
<td>2.8 References</td>
<td>85</td>
</tr>
<tr>
<td><strong>Chapter 3 CD151 Immunoreactivity at Diagnosis Predicts Biochemical Failure and Metastasis in Prostate Cancer</strong></td>
<td>92</td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>93</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>94</td>
</tr>
<tr>
<td>3.3 Methods</td>
<td>96</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>99</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>112</td>
</tr>
<tr>
<td>3.6 Acknowledgements</td>
<td>116</td>
</tr>
<tr>
<td>3.7 References</td>
<td>116</td>
</tr>
<tr>
<td><strong>Chapter 4 General discussion and conclusions</strong></td>
<td>121</td>
</tr>
<tr>
<td>4.1 Thesis summary and Significance</td>
<td>121</td>
</tr>
</tbody>
</table>
4.2 Strengths and limitations ................................................................. 126

4.2.1 CD151 immunoreactivity predicts early biochemical failure and metastasis in prostate cancer. ................................................................. 126

4.2.2 Rhamm compartmentalization and its functions ...................... 127

4.3 Future studies ................................................................. 129

4.3.1 CD151 immunoreactivity predicts early biochemical failure and metastasis in prostate cancer. ................................................................. 129

4.3.2 Rhamm compartmentalization and its functions ...................... 130

4.4 References ................................................................. 131

Supplemental information ................................................................. 135

Appendices ................................................................. 138

Curriculum Vitae ................................................................. 144
List of Tables

Chapter 2

Table 2.1 Rhamm binding proteins.................................................................76

Table 3.1 Demographic information of patients that underwent RRP at the London Regional Cancer Program between 1994 and 1998 (n=99).............................................100

Table 3.2 Pathological and clinical outcomes of patients that underwent RRP at the London Regional Cancer Program between 1994 and 1998 (n=99).............................................101

Table 3.3 Demographic information of patients that developed metastasis during follow up at the London Regional Cancer Program between 1994 and 1998 (n=38).....................102

List of Figures

Chapter 1

Figure 1.1 Steps for cell transformation and tumour progression..........................6

Figure 1.2 Steps for cell invasion and metastasis.................................................9

Figure 1.3 Rhamm schematic..............................................................................12

Figure 1.4 Rhamm participation in cell migration pathways...............................19

Figure 1.5 CD151 gene and protein schematic..................................................22

Figure 1.6 CD151 participation in cell migration pathways...............................28

Figure 2.1 RhammA163 transforms growth patterns in 10T1/2 fibroblasts..............66

Figure 2.2 RhammA163 acts upstream of Ras and at the level of ERK to promote growth transformation..........................................................67
Figure 2.3 Proposed mechanisms for RhammΔ163 transforming effects

Figure 2.4 ZsGreen-RhammFL/Δ163 constructs

Figure 2.5 Distribution of the Rhamm constructs in the cell

Figure 2.6 Rhamm isoforms have different motility/dynamics in the cytoplasm of MEFs

Figure 2.7 Rhamm associates with cytoskeletal proteins

Figure 2.8 Rhamm associates with ERK

Figure 2.9 RhammΔ163 influences pERK sustained translocation to different compartments of the cell in Rhamm +/- MEFs and 10T1/2 fibroblasts

Figure 3.1 CD151 is expressed in basal epithelium of prostate tissue

Figure 3.2 mAb 1A5 recognizes a different subpopulation of CD15

Figure 3.3 CD151 staining on RRP samples is prognostic of biochemical recurrence

Figure 3.4 CD151 immunoreactivity associates with earlier onset of metastasis

Figure 3.5 Patients with CD151 positive staining and high PSA have increased risk of developing metastasis

Figure 3.6 Expression analysis of CD151 mRNA in PCa progression
List of Supplemental figures and Appendices

Supplemental figure 2.1 ZsGreen-Rhamm isoforms behave differently in 10T1/2 fibroblasts than in Rhamm -/- cells.................................................................135

Supplemental figure 2.2 ERK activation does not affect the distribution of ZsG-Rhamm^{FL/Δ163} fusion........................................................................136

Supplemental figure 3.1 CD151 expression in adult tissues........................................137

Appendix A: Format for phone informed Consent.................................................138

Appendix B CRIC number.......................................................................................140

Appendix C Ethics approval.....................................................................................141

Appendix D Ethics approval renewal........................................................................142

Appendix E Human tissue use approval.................................................................143
List of Abbreviations

10T1/2: Mouse Fibroblasts
5'UTR: 5' Untranslated Region
A431: Epithelial Carcinoma Cells
ACTA2: Smooth Muscle Actin 2
ACTN1: Actinin 1
Akt: transforming serine/threonine protein kinase
AML: Acute Myeloid Leukemia
Arp2/3: Actin-Related Proteins 2/3
ATCC: American Type Culture Collection
B16F10 cells: Mouse Melanoma
BCa: Breast Cancer Cells
Bcl-2: B-cell lymphoma 2 apoptosis regulator protein
BRCA: Breast Cancer early onset
BSA: Bovine Serum Albumin
Bx: Biopsy
C3 cells: Mouse ras-transformed fibroblasts
Ca++: Calcium
CD151: Tetraspanin 24
CD44: Hyaluronic Acid Receptor
CD63: Tetraspanin-30
CD81: Tetraspanin-28/ Target of the antiproliferative antibody 1
CD9: Tetraspanin-29/ Motility-related protein
Cdc42: Cell division control protein 42
CLL: Chronic Lymphoid Leukemia
CNCC: Cranial Neural Crest Cells
COL5A2: Collagen, Type V, Alpha 2
Cos7: Monkey kidney cells
c-raf (-1): Murine leukemia viral oncogene homolog 1
CXCL12: Chemokine (C-X-C motif) Ligand 12
DC: Dendritic Cell
DMEM: Dulbecco’s Modified Eagle Medium
DRE: Digital Rectal Examination
ECL: Enhanced Chemiluminescence
ECM: Extra Cellular Matrix
EGF: Epithelial Growth Factor
ELISA: Enzyme-Linked ImmunoSorbent Assay
EMT: Epithelial to Mesenchymal Transformation
ER: Estrogen Receptor
ERK1,2: Extracellular Signal-Regulated Protein Kinases 1,2
ESCC: Human Esophageal Squamous Cell Carcinoma Cell Line
EWI-2: Member of the Ig Superfamily
FAK: Focal Adhesion Kinase
FBS: Fetal Bovine Serum
FGF-7: Fibroblast Growth Factor 7
FLNA: Filamin α
FRAP: Fluorescence Recovery After Photobleaching
golgi/ER: golgi/Endoplasmic Reticulum
GPCRs: G-protein-coupled receptor superfamily members
GTPases: enzymes that hydrolyze Guanosin Triphosphate

HA: Hyaluronan/Hyaluronic Acid

HaCaT cells: Human immortalized keratinocytes

HAS: Hyaluronan Synthase

HCF: Human Cementifying Fibroma cells

HEp3: Human Epidermoid Carcinoma cells

HER2: v-erb-b2 Erythroblastic leukemia viral oncogene homolog 2

HIFU: High Intensity Focussed Ultrasound

HLA-A2: Human Leukocyte Antigen subgroup 2

HP: Heparin

HRP/DAB: Horseradish Peroxidase/3,3’-Diaminobenzidine

HUVECs: Human Umbilical Vein Endothelial Cells

IGF: Insulin-Like Growth Factor

IgG: Immunoglobulin G

IHC: Immunohistochemistry

ILs: Interleukins

IP: Immunoprecipitation

J110: Mouse breast cancer cell line

kDa: Kilo-Dalton

Klp2: Kinesin like protein

KO: Knockout

LAMA2: Laminin, Alpha 2

Lck: Lymphocyte-specific protein tyrosine kinase

LEL: Large Extracellular Loop

LLC cells: Lewis Lung Carcinoma Cells
PR: Progesterone Receptor
pRb: Retinoblastoma protein
PSA: Prostate-Specific Antigen
pTNM stage: Pathological Tumour Node Metastasis
PTPμ: Protein Tyrosine Phosphatase
qRT-PCR: Quantitative Real Time Polymerase Chain Reaction
Rac: small GTPase
Rac1: ras-related C3 botulinum toxin substrate 1
Raf: proto-oncogene serine/threonine protein kinase
Ras: for RAT sarcoma oncogene member of the family of small GTPases
REB: Research Ethics Board
Rhamm FL: Receptor for Hyaluronan Mediated Motility Full Length
RHAMM: Receptor for Hyaluronan Mediated Motility
Rho: member of the family of small GTPases, a GDP dissociation inhibitor
ROCK: Rho-associated protein kinase
ROI: Region of Interest
RON: Macrophage stimulating 1 receptor
RRP: Radical Retropubic Prostatectomy
SD: Standard Deviation
SDF1: Stromal Cell-Derived Factor 1
SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEL: Small Extracellular Loop
shRNA: Small Hairpin RNA
siRNA: small interfering RNA
SKOV-3: Human ovarian carcinoma cells
SMCs: Human Smooth Muscle cells

SPNB4: Spectrin β non-erythrocytic 4

SPTA1: Spectrin α erythrocytic 1

Src: viral sarcoma oncogene

TASCs: Tumour Associated Stromal Cells

TBST: Tri-Buffered Saline and Tween 20

TGF-β: Transforming Growth Factor, Beta

TME: Tumour Microenvironment

TNFs: Tumor Necrosis Factor

TPM2: Tropomyosin 2

TPMS2: Tropomyosin 2

T-SCC118: Squamous carcinoma cells

TRUS: Trans Rectal Ultrasound

TUBB4: Tubulin β4

Vav: Guanine nucleotide exchange factor

VEGF: Vasculo-Endothelial Growth Factor

VIM: Vimentin

WASP: Wiskott-Aldrich Syndrome Protein

WAVE/Scar: WASP family Verprolin-homologous protein
Chapter 1 Introduction

1.1 Overview

The tightly regulated steps of cell migration break down during tumor formation from genetic and epigenetic changes that occur in the cell. This deregulation induces cell plasticity, growth and survival, and increases cell migration. Cells begin to ignore inhibitory signals and grow in the absence of optimal substrate during the early steps of transformation [1]. As transformation influences proteins associated with cell migration, cells become more migratory and may eventually give rise to single cells that grow uncontrollably to form a tumour and later on migrate away from it to grow at a distant site to create metastases [1-3].

Normal cell migration is an important function for the formation and differentiation of tissues in the embryo. Later in the adult life, cell migration promotes wound healing, tissue remodelling and immune responses. Pathologic cell migration contributes to processes such as inflammation, autoimmune disease and tumour progression, among others [2, 4-6]. An important regulator of cell migration is the Receptor for Hyaluronan Mediated Motility, RHAMM. Of the various isoforms of RHAMM (Rhamm for the mouse version) only some are associated with aggressive behaviour in cancer cells [7]. Specifically, overexpression of Rhamm^{163} transforms mesenchymal cells [8]. As a last step towards understanding how RHAMM performs oncogenic functions, we compared subcellular compartmentalization and transforming functions of Rhamm^{163} with the full-
length protein. Rhamm$^{\Delta 163}$ had different localization in the cell and significantly higher transformation potential which was dependent on its association with ERK1,2.

Cell migration and cancer metastasis is also regulated by tetraspanin CD151 [9-13]. CD151 is a transmembrane protein linked to poor outcome in many cancers [14-17]. It predominantly localizes to the plasma membrane [18, 19] were it binds integrins in tetraspanin enriched microdomains to influence cell adhesion, migration and proliferation [20, 21]. In this study, we assessed the relationship between CD151 mRNA levels and protein expression in normal prostate epithelium versus metastatic prostate cancer. Patient information collected from multiple databases showed that CD151 levels remain stable during prostate cancer stages [22-25]. However, a CD151 mAb that suppresses cell migration and stromal invasion in human epidermoid carcinoma cells, HEp 3 cells [26], recognized a subpopulation of CD151 associated with poor prognosis in prostate cancer. CD151 immunoreactivity predicted early biochemical recurrence and metastasis.

1.2 Cancer and metastasis as a multistep process

1.2.1 Oncogenic transformation overview

During the cell cycle a series of events lead to cell replication to maintain tissue homeostasis with normal cell turnover [27]. Normally, there is a delicate balance of growth-inducing signals that promote the cell cycle and proliferation [3, 28]. Oncogenes help to coordinate the cell cycle and may encode growth factors, growth factor receptors, regulators of chromatin, transcription factors, signal transducers and/or regulators of
apoptosis [29]. During cancer progression, oncogenes are often hyper-activated by point mutations, chromosomal rearrangement, or gene amplification, leading to overexpression and deregulation. This can promote survival and/or growth advantages, cell transformation, and tumour formation [29, 30].

Cell transformation and cancer formation is a complex, multistep process [1]. Cancer cells must grow independently of correct growth signals, resist antigrowth or proapoptotic signals, replicate, and induce new blood vessel formation. In order to metastasize, they must additionally migrate and invade through surrounding tissue [1, 3]. Transforming cells produce their own mitogenic factors, overexpress growth factor receptors, and induce growth factor production by neighbouring cells. Some cells even induce export of proteins/receptors that normally function intracellularly. For example, hepatocytes exhibit autocrine production of TGFβ to become resistant to apoptosis and attain EMT properties [31], breast cancers increase Hyaluronan (HA) synthesis [32], and Ras transformed mouse fibroblasts overexpress the putative oncogene, RHAMM [33]. In addition, during oncogenesis and wound repair, cytoplasmic proteins such as RHAMM are transported to the plasma membrane where they couple with CD44 to promote genomic instability and tumour progression [34]. De-regulation or altered expression of transmembrane proteins and extracellular receptors such as tetraspanins also influence cellular transformation. For instance, during colon cancer progression, downregulation of tumour suppressor CD63 takes place [35]. Transforming cells are also capable of inducing constitutive activation of oncogenes such as Ras that, in turn, end up triggering mitogenic pathways, such as the Ras/MEK1/ERK1,2, [36] that results in tumour formation. Conversely, overexpression of
integrins such as α6β4 induces resistance to anoikis in breast tumours [37]. This induces phenotypical changes that lean the cell towards a highly proliferative phenotype.

Second to this self-sufficiency in growth signals is the resistance to anti-growth signals. In general, cells receive cues from the surrounding microenvironment that dictate their entrance into the active cell cycle, thus maintaining tissue homeostasis [27, 28]. These cues induce cell quiescence, halting the cells in the G0 state of the cell cycle until new pro-growth signals are sensed [27, 28]. During tumour progression the anti-growth cues are ignored by the cell by a number of mechanisms that involve loss, downregulation, and/or mutation of tumour suppressor genes [1, 3, 38, 39]. Perhaps the most studied illustration of this could be retinoblastoma protein/gene (pRb) which inhibits cell proliferation by affecting the availability and activity of transcription factors in charge of induction of the cell cycle progression [39-41]. Mutation of the RB1 family induced loss of contact inhibition and sphere formation in Ras transformed mouse fibroblasts [42]. Moreover, injection of these spheres into nude mice caused tumour formation.

Cancer cells must ignore programmed cell death/apoptosis in order to thrive as a malignant tumour. Normal cells that are correctly in tune with environmental cues undergo apoptosis in response to apoptotic signals. Extracellular cues include IGF, IL-3 (sensed by their cognate receptors in the plasma membrane), hypoxia, and cell-cell/cell-matrix adherence signals [1]. Loss of epithelial cell-extracellular matrix interactions affects gene regulation, differentiation, and cell proliferation resulting in cellular imbalance and tissue disorganization that elicits a series of similar intracellular cues [1, 3] that upon detection promote a finely regulated process of orchestrated cell death, anoikis. Oncogene overexpression can provide protection against this [43]. For instance,
overexpression of the anti-apoptotic gene bcl-2 protected fibroblasts and transformed epithelial cells [44]. Resistance to anoikis is crucial during tumour progression in cases such melanoma [45], ovarian [46], oral [47], and prostate cancer [48].

The final, and arguably the deadliest, characteristic that a cancer cell acquires, is its ability to invade surrounding tissue and metastasize to distant sites [1]. During metastasis, a cell must detach from the primary tumour, migrate and invade into surrounding tissue, intravasate into the vasculature, extravasate and ultimately grow as a metastatic nodule in a distant site [49]. Various modifications in gene regulation and, therefore, in protein expression regulate these steps in metastasis. Cancer cells can activate, overexpress, or even export proteins that act as receptors or transmembrane scaffolds that activate cell transformation and increase cell migration. Oncogenes, besides their activity as growth factors, growth factor receptors, regulators of chromatin, transcription factors, signal transducers and/or regulators of apoptosis [29], are important regulators of cell migration. The ability of oncogenes to influence so many stages of metastatic cancer formation makes them likely candidates for intervention and as biomarkers.

1.2.2 Cell migration and cancer

Migration in response to specific stimuli is a common feature among cells. Normally cell migration occurs during embryogenesis and morphogenesis, when cells migrate to generate the three layers of the embryo and to form differentiated tissues. In adults, cell translocation normally allows for tissue remodelling, wound healing and immune
Figure 1.1 Steps for cell transformation and tumour progression

responses. Migratory events are usually tightly regulated. Disrupted and/or pathologic cell migration plays a central role in diseases such as chronic inflammation, vascular disease, autoimmune disorders, osteoporosis, tumour progression and metastasis [2, 4, 5].

Cell migration is often initiated via intricate signalling pathways originating at the cell membrane where many surface receptors, such as integrins or growth factor receptors,
become activated. This, in turn, induces signaling cascades that often act through regulation of the Rho family of small GTPases. A subset of the Ras superfamily, the Rho GTPases include Cdc41, Rac, and RhoA which are important regulators of actin. In response to activation of cell surface receptors, Rho GTPases influence cell migration by regulating actin, tubulins, the phospholipid enzyme phosphoinositide 3-kinase (PI3K), Akt, and vesicular transport [2, 50, 51]. Cell migration can be turned on and off by changes in the expression of adhesion receptors, cytoskeletal-linking proteins, and extracellular matrix (ECM) ligands.

During cell migration, the cell first polarizes and extends protrusions (lamellipodia or filopodia) towards the migration front. After these protrusions form, they attach the cell to the extracellular matrix to provide stability. The cell forms adhesion sites known as focal complexes at its leading edge. Integrins are members of the focal complex and support adhesion to the underlying ECM. Integrins promote maturation of focal complexes into focal adhesions, which adhere strongly and inhibit cell translocation. Adhesion sites at both the front and rear of the cell must assemble and disassemble, respectively in order to allow the cell to form new protrusions at the leading edge and to detach from the rear in a cyclical process influenced by chemotactic and haptotactic stimuli [2].

Cancer cell migration is enhanced by communication between the tumour and its microenvironment (TME). The expression of TME components facilitates tumour cell motility. Tumour associated cells including adipocytes, fibroblasts, stromal cells, immune cells, and endothelial cells, overexpress multiple growth factors (namely EGF, PDGF, and VEGF) and chemokines (such as SDF1 and CXCL12) that act as chemoattractants.
Stromal cells secrete EGFLs, ILs, TNFs, and growth factors such as TGF-β, VEGF, FGF-7, and IGF-2, which bind cancer cell receptors to activate cancer cell migration. These factors also stimulate cancer cell expression of cytokines that attract and stimulate proliferation of tumour associated stromal cells (TASCs). This paracrine positive feedback loop supports a pro-migratory environment for cancer cells.

Cancer cells take advantage of the pro-migratory TME by increasing their expression of ECM receptors that bind components of the modified TME [52]. Integrins, are key regulators of cell-ECM interactions and changes in their expression influences cancer cell fate. In particular, integrins such as αvβ3, α3β1, α6β4 [53, 54] promote tumourigenic and metastatic phenotypes. Other cell surface receptors can be affected. RHAMM, which interacts with HA and forms complexes with transmembrane receptors such as CD44, PDGFR, and RON [55] is not an integrin, but promotes migration of many types of cancer cells. Given this function, it is not surprising that RHAMM protein and mRNA levels are increased in most human cancers and high levels of expression are prognostic of poor outcome in subsets of breast cancer [56, 57], gastric cancer [58], multiple myeloma [59], and colorectal cancer [60, 61].

A significant number of publications have compared gene expression profiles in normal cells with cancer cells [62, 63]. Cancer cells often overexpress pro-migratory genes and therapies that inhibit their motility result in a more compact, less metastatic tumor. Moreover, genes expressed during embryogenesis and early development are turned on during tumourigenesis giving rise to more motile and highly aggressive cells [64].
Epithelial cell markers are downregulated and mesenchymal genes are overexpressed promoting plasticity and motility. These genetic changes contribute to cancer cells that can invade locally and metastasize [62, 65, 66].

Figure 1.2 Steps for cell invasion and metastasis

There is a common theme throughout the above mentioned processes: outside-in signalling determined and activated by proteins present in the cell membrane. Enhanced tumour cell migration results from the integration of multiple factors rather than changes in a few or a single protein. In order to study the mechanisms that influence oncogenicity and metastasis from this perspective, we have chosen to investigate two proteins,
RHAMM and CD151, that act as scaffolds to tether many other proteins into complexes localized to specific areas of the cell and that activate signalling cascades and regulate the motile cycle of polarization, protrusion formation, retraction and displacement. RHAMM, a member of the hyaladherin family of hyaluronan binding proteins, was chosen as an example of a motogenic protein that forms complexes with multiple signalling pathways and that is necessary for cell polarity [67], dynamic nature of leading lamellae and dynamic instability of microtubules. Another example is the transmembrane protein CD151, a member of the tetraspanin family that scaffolds integrins with multiple proteins forming tetraspanin-enriched microdomains in the cell membrane [20, 68]. CD151 associates with specific integrins including α3β1, α6β1, and α6β4, MMPs, and other tetraspanins to induce cell migration and ultimately metastasis. Even though RHAMM and CD151 are very different molecularly and structurally, they both promote migration of cancer cells and their expression is prognostic of poor clinical outcome in a number of human cancers. RHAMM participates in regulation of cell polarity, the turnover of focal adhesions, and the formation of membrane extensions through microtubules and CD151 is involved in cell adhesion, hemidesmosome formation, and retraction of the rear of the cell.

1.3 The role of RHAMM in cell migration and cancer progression

1.3.1 RHAMM structure

RHAMM/HMMR (Receptor for Hyaluronan Mediated Motility), is a Hyaluronan (HA) receptor, which was the first cellular HA binding protein isolated and purified by Dr. Eva
Turley from cultured cardiac fibroblasts [69]. RHAMM binds to HA via a coiled coil carboxyl terminal region that is rich in basic amino acids that possesses a B(X)₇B motif.

RHAMM is a largely coiled coil protein that does not contain a transmembrane domain or classical export signal and is likely exported through an unconventional mechanism that does not involve the golgi/ER. RHAMM is comprised of 18 exons located in chromosome 11 in mice; its mRNA transcript encodes a 95kDa protein. In humans, RHAMM is found on chromosome 5 translated into an 85 kDa protein. Intriguingly, the latter chromosome homes multiple disease associated genes [70]. RHAMM is expressed as multiple isoforms that consist mainly of shorter forms that lack a number of amino acids in the N-terminus of the protein of 60-73 kDa [136]. Exon 4 is responsible for interphase microtubule binding and exon 13 is at least partially responsible for centrosomal localization. Its C-terminus, after aa 600, is also involved in association with mitotic spindles (figure 3). RHAMM also shares high homology with Klp2 in the carboxy-terminal, which is a site of indirect interaction with the dynein/dynactin complex [71]. RHAMM is a ubiquitous protein (for reference see HMMR gene in Gene note and GNF BioGPS databases) found intracellularly as well as extracellularly, and exported in response to cell stress [72, 73]. It occurs mainly in fibroblasts, immune cells, smooth muscle cells, endothelial cells, sperm and keratinocytes [74-79]. Subcellularly, it is distributed at the cell surface, mainly in podosomes and other membrane protrusions, in the cytoplasm of the cells, as well as in the nucleus where it is associated with the mitotic spindle [57, 71, 80-82].
Figure 1.3 Rhamm schematic. Rhamm FL gene encodes a 794 amino acid protein in mice. The translated coiled coil protein possesses two tubulin binding sites: the one in the N-terminus associates with interphase microtubules whereas the C-terminus one has been linked to the centrosomes. One of the most studied isoform lacks the first 163 amino acids in the N-terminus of the protein, but conserves the C-terminal sequence. Bottom: predicted coil distribution.

1.3.2 The role of RHAMM in cell migration

1.3.2.1 RHAMM – HA interactions

RHAMM functions in cell migration are highly dependent on its interactions with HA. Briefly, HA is a glycosaminoglycan composed of repeating units of N-acetyl glucosamine and β-glucuronic acid. The functions of HA within the ECM and cells depend upon its molecular weight, the type of cell, and the HA receptor(s) that target cells express. HA fragments provide signalling functions and are usually present during the ECM remodelling that is associated with morphogenesis, wound healing, or disease. Cancer cells are particularly adept at producing and responding to HA fragments [83-85].
(reviewed in InTech Ward C, Vasquez C et al, in press). RHAMM detects HA oligosaccharides and fragments triggering multiple signalling pathways that result in increased cell motility.

Evanko SP et al. postulated that RHAMM, at least partially, regulates uptake and translocation of HA to the nuclear and perinuclear area, contributing to an HA dependent increase in cell motility, as well as cell cycle regulation, mitosis and RNA splicing [86]. In this study, HA was found nearby microtubules of Human Smooth Muscle cells (SMCs) in vesicle-like organelles. RHAMM was surrounded by these structures in the microtubules of SMCs. Moreover, fluorescein labelled HA bound to microtubules close to the nucleus that were positive for RHAMM staining. HA staining also overlapped with RHAMM in the mitotic spindle during mitosis. Suggesting that intracellular HA may contribute either actively to cell migration by modulating RHAMM function, or passively by balancing biomechanical characteristics that may contribute to the cell stabilization and force balance required during cell locomotion [86].

RHAMM dependent cell migration of arterial smooth muscle cells from rat aorta was demonstrated by Goueffic Y et al, 2006. Stimulation with HA induced increased cell migration as demonstrated by faster closing of gaps in scratch wound assays independently of cell proliferation. RHAMM siRNA experiments further confirmed the role of RHAMM in this process. The same study showed that HA promoted actin reorganization through RHAMM, contributing to stress fibres and lamellipodia formation by stimulating Rac activation in a PI3K dependent mechanism that was independent of RhoA [87].
Analysis of RHAMM mRNA expression in cranial neural crest cells (CNCC) of *Xenopus laevis* embryos (African amphibian) was detectable along with CD44 and HAS1. However, it was also found to be expressed independently of these two genes in “temporal windows” where cell migration was required to continue with its development. Indicating thus that RHAMM performs HA dependent and independent functions in development [88]. Interestingly, CD44 surface display is reduced in mesenchymal cells isolated from RHAMM-/- mice, demonstrating functional interplay between these two HA receptors [89]. Moreover, in CD44-/- mice, RHAMM compensates for the deficit of CD44 by supporting HA engagement on the surface of the cell [90, 91].

### 1.3.2.2 RHAMM – ERK1,2 interactions

ERK1,2 has an extensive effect on cell migration that can be dependent on Ras activation and on integrin signalling but also cooperate with growth factor receptors through a mechanism that does not require Ras-Raf phosphorylation [92]. Not coincidentally, Hatano et al., in 2010 demonstrated that RHAMM not only associated with ERK1,2, but also was required for ERK1,2 activation through a pathway different than Raf-MEK - ERK in HCF (human cementifying fibroma cells) and 10T1/2 mouse fibroblasts [93]. This suggests that RHAMM contributes to pERK1,2 induced cell migration through a different mechanism [94]. Furthermore, the presence of RHAMM 163 was required for ERK activation in response to PDGF in 10T1/2 cells. Levels of RHAMM in the membrane were directly correlated with the degree of ERK phosphorylation [93].
Rhamm/ERK1,2 interactions in cell migration were further demonstrated in our lab by using a Rhamm -/- mouse model created in our laboratory. Gene deletion was performed by homologous recombination of exons 8-16, generating mice with viable offspring; although with reduced fertility [95]. Additional studies showed that Rhamm -/- MEFs (Mouse Embryonic Fibroblasts) respond less to growth factors than corresponding wildtype. Further characterization of Rhamm -/- phenotypes, performed by Tolg et al, 2006 showed that knocking out Rhamm expression in MEFs significantly reduced their ability to close the gap in scratch wound assays and 3D collagen gel assays when compared with Rhamm expressing cells. They also demonstrated that Rhamm not only played a role in ERK activation, but had an additional influence in sustaining ERK1,2 phosphorylation as demonstrated by ELISA, Western Blotting and confocal imaging. Moreover, a MEK inhibitor, U0126, blocked motility in response to HA and serum stimulation of Rhamm -/- cells that were rescued for RhammFL expression but not Rhamm -/- [89]. The same was true when CD44 was blocked indicating that there is interplay between these two Rhamm receptors. Further addition of MEK restored migration of Rhamm -/- cells suggesting that Rhamm-MEK/ERK interactions are important for cell migration. This activation of ERK1,2 by growth factors required CD44/RHAMM interactions.

1.3.2.3 RHAMM and the cytoskeleton

Besides Rhamm/ERK interactions, Rhamm binds to many other proteins in the context of cell migration. It has been shown by immunoprecipitation that during the M phase
Rhamm binds to the dynein/dynactin motor complex previously described as an important player in cell migration that transports cellular cargo by walking along cytoskeletal microtubules towards the minus-end of the microtubule [71]. Moreover, Rhamm shares high homology with Klp2 in the carboxyl-terminus. This Klp2 is a site of indirect interaction with the dynein/dynactin complex. Also, Exon 4 was shown to be responsible for interphase microtubule binding and amino acids 704-707 were suggested to have homology with TPLK consensus cdc2 phosphorylation site. RHAMM is a tubulin binding protein that possesses two microtubule binding sequences, one for interphase microtubules present at exon 4 in full length RHAMM and a mitotic microtubule binding site present at the very C-terminus of the sequence [78, 80].

Turley and others have demonstrated that RHAMM specific antibodies blocked HA dependent migration on different cell lines. Upon binding of RHAMM to HA, a number of signaling events including protein tyrosine phosphorylation of several intracellular proteins take place. For instance, RHAMM was necessary for c-src-induced rescue of cell motility in c-src -/- mouse fibroblasts; suggesting Rhamm as an upstream activator of c-src [96]. Additionally, there is evidence that HA induced a significant increase in locomotion of C3 cells (mouse H-ras transformed fibroblasts) by inducing a rapid but transient phosphotyrosine activity, including FAK, that localized mostly to the forming lamellae in cells stimulated with HA followed by focal adhesion turnover [97]. HA-mediated cell motility was diminished by the use of inhibitors of the kinase activity. Moreover, mutation of the HA binding sites in RHAMM not only reduced protein tyrosine phosphorylation and focal adhesion formation but also reverted ras-induced transformation [8]. These and other studies clearly demonstrate the role of pp125FAK in
addition to the proto-oncogene pp60c-src in the induction of rearrangements in actin filament and cell-matrix adhesion structures that account for cell locomotion in response to HA/RHAMM interactions [97]. In relation to this, Kaverina et al, 2002 postulated that RHAMM contributes to the microtubule instability by inducing their repeated contact with focal adhesions, which promoted microtubule disassembly [90].

The importance of RHAMM in maintaining the integrity of focal adhesions and filopodia by transducing the effects of pericellular HA in human esophageal squamous cell carcinoma cell line (ESCC), OSC1, was also demonstrated recently by Twarock et al. HA was proven to be important in cell morphology, actin cytoskeleton, filopodia, and focal adhesion complexes in that cell line. Total and pFAK, as well as pERK levels decreased after inhibition of HA. The same was true for other ESCC cell lines. This resulted in a significant decrease in cell migration and proliferation. RHAMM knock down by shRNA experiments led to inhibition of filopodia and reduction of FAK, Akt/PKB phosphorylation, and reduction of pERK levels [98].

1.3.2.4 RHAMM – integrin interactions

RHAMM induced weak integrin avidity to assist in the process of cell migration. In 1998, Gares SL et al, demonstrated that thymocyte migration and adhesion were regulated at least in part by fibronectin and its receptors α4/5 β1 integrins in a RHAMM mediated mechanism, suggesting a functional association between RHAMM and β1 integrins [99]. Adherence of MN (multi-negative progenitor) thymocytes to fibronectin was blocked by a mAb to α5/β1. RHAMM did not participate in thymocyte adhesion to fibronectin under
conditions of minimal shear. However, it participated and was needed, in conjunction with $\alpha_{4,5}/\beta_1$, for cell motility. The latter were dominant over RHAMM in this process. Thymocytes produced their own HA and it was required for cell migration [100]. Later, Gares and Pilarski showed that $\alpha_{4,5}/\beta_1$ regulated RHAMM expression during differentiation of thymocytes [101].

1.3.3 The importance of RHAMM in cancer

RHAMM is a novel breast cancer susceptibility gene that antagonizes BRCA1 [55, 102] and is prognostic factor for poor outcome [59]. It is overexpressed in 50% of breast cancers, which positions it on the list of the top 10% relevant breast cancer oncogenes, among BRCA1,2, HER2, ER, PR, among others. In a different model, after Ras induced transformation of mouse fibroblasts, there was evidence of a clear increase in a form of Rhamm, an N-terminal truncation that lacks the first 163 aa residues (Rhamm$^{\Delta163}$). This isoform was not only required for Ras transformation but is also transforming in mesenchymal cells [8], strongly suggesting that Rhamm is an oncogene. On the cell surface RHAMM interacts with HA and forms complexes with other transmembrane receptors such as CD44, PDGFR, RON, integrins, etc. [55, 99-101] which results in the activation of several signalling pathways, some of which were discussed above, that influence among others, cancer progression and metastasis.
Figure 1.4 RHAMM participation in cell migration pathways. Once Rhamm binds with HA and/or growth factors, a series of signalling pathways are activated to promote cell migration. Top: schematic of some of the multiple pathways that Rhamm influences on in terms of locomotion. Bottom: Once the cell gets in contact with “clues”, growth factors, cytokines, Ca++, etc., its cell receptors orient towards the signal and the migration steps take place. RHAMM participates in regulation of cell polarity, the turnover of adhesions, and the formation of membrane extensions through microtubules.
RHAMM is elevated in most types of cancer in particular breast [56, 57], glioma [103], endometrial [104], colorectal [60, 61], stomach [58], and prostate cancer [105], as well as in MM [59] and AML [106]. Consistent with these clinical correlations, RHAMM has tumourigenic properties in experimental systems that have been linked to its ability to bind to HA. In BCA cells, RHAMM/CD44/HA complexes sustain phosphorylation/activation of the Ras/MAPK (ERK1,2) signalling pathway, leading to BCA progression and constitutively high rates of motility and invasion [7]. The relationship between RHAMM and ERK1,2 activation has recently been confirmed in BCA samples where concomitant upregulation of phosphorylated ERK1,2 and RHAMM in tumour samples correlates with a high tumour grade (Ward C. in preparation).

1.4 CD151 in cell migration and cancer progression

1.4.1 CD151 structure

CD151 (GP27, SFA-1, PETA-3) is one of 33 members of the mammalian tetraspanin family. The human CD151 gene is located on chromosome 11p15.5 and composed of 9 exons, but the protein is encoded by exons 3 to 9 and is translated into 253 amino acids (Figure 1.5A). It was first identified by Ashman LK et al in 1991 in acute myeloid leukemia cells as a 27kDa platelet surface glycoprotein [107]. There may be alternative splicing of exon 2 in the 5'UTR in humans, varying the length of the mRNA transcript [108]. It is characterized by four transmembrane domains, a large extracellular loop (LEL), small extracellular loop (SEL), and the N and C terminal as two small intracellular tails. The later allocates the YXXΦ motif, relevant for internalization of CD151 and its
binding partners. The LEL is perhaps the most studied sequence of CD151 since it confers it with binding specificity. The reason for this is that it contains the QRD motif between amino acids 194-196, which is responsible for strong and stable binding with the integrin α3β1. The importance of this interaction will be discussed below. Within the LEL there are also two di-sulphide bridges that stabilize the protein folding in this area. CD151 can undergo post-translational modifications such as N-linked glycosylation, producing a larger protein of 32kDa. Moreover, CD151 possess 6 palmitoylation sites which are of pivotal importance for CD151 interaction with other tetraspanin members and other proteins and also stabilization of the molecule by diminishing lysosomal degradation (Figure 1.5B) [20]. It is ubiquitously expressed in humans (for reference see CD151 gene in Gene note and GNF BioGPS databases), but is predominantly found in the plasma membrane of epithelial cells, endothelial cells, smooth muscle cells, and platelets [11, 18-20]. Subcellular localization of CD151 ranges from hemidesmosomes [19], to plasma membrane, endosomes, and endothelial cell junctions [18, 19].

1.4.2 The role of CD151 in cell migration

1.4.2.1 CD151 and integrins

Integrins play an important role in cell migration by activating many signalling pathways that lead to a transformation in cell shape, increase or decrease in cell adhesions to other cells and to the ECM (this is highly dependent on its binding partners and the context of the microenvironment since different isoforms/chains of integrins bind to different substratum i.e. α3/β1 bind to laminin 5 present mostly in the basement membrane), and
ultimately in cell migration. For instance, CD151 modulates MDA-MB-231 cell migration in laminin-332 by regulating glycosylation of α3β1 [109].

![CD151 gene and protein schematic](image)

**Figure 1.5 CD151 gene and protein schematic.** CD151 gene is composed of 9 exons, from which only exons 3-9 get translated (A). The protein encoded spans the plasma membrane four times, possesses 2 intracellular tails, two extracellular loops, 6 palmitoylation sites, and one glycosylation site, (B).

It is perhaps its association with integrins, such as α3β1, α6β1, and α6β4 that makes CD151 such an important mediator in cell migration and cancer progression especially in epithelial cells. It is, therefore, not surprising that a number of researchers had focused their studies on elucidating the CD151-integrin binding motif. Zebian S et al, in 2010 performed mutational analysis of several CD151 domains and found that mutations in the LEL completely abolished association between CD151 and integrins, as well as CD151 with other tetraspanin members. More specifically, alteration of the QRD motif of CD151...
in A431 epithelial carcinoma cells impaired immunoprecipitation with α3β1 under stringent conditions. On the other hand, mutation of CD151 palmitoylation sites inhibited CD151 association with other tetraspanin members [110]. Subsequent experiments showed that the profound negative effect on cell migration as a result CD151 knock-down was not rescued with CD151 mutated on the LEL, but partially rescued by the use of a QRD mutant [110]. CD151 interaction with α3β1, through its QRD sequence has been demonstrated in various cell lines such as MDA-MB-231(BCa cells) [109], Cos7 (monkey kidney cells) and NIH3T3 cells (mouse fibroblasts), among others [111].

To further explore the importance of CD151 in molecular processes, a CD151 -/- mice was generated. Deletion of the CD151 gene resulted in viable offspring with only minor alterations in immune and blood cell counts and platelet aggregation leading to increased bleeding, although no change in integrins α3/β6 expression was noted. Noticeably, however, cell migration in CD151 null keratinocytes was reduced [112]. In addition, reports of CD151 loss in humans and experimental mouse models mention basement membrane related defects, including skin blistering and renal failure as well as defects in wound healing. Both defects strongly associate with defective cell migration [113].

Several scientists have also utilized CD151 knock down approaches on multiple cell lines and animal models to further validate the roles of CD151 in cell migration, invasion, angiogenesis, cancer progression and metastasis. For instance, Winterwood et al., in 2006 silenced CD151 in epidermal carcinoma cells and observed significant reduction in cell migration on laminin-5 that was dependent on interaction of CD151 with α3β1, as well as a decrease in the degree of endogenous laminin-5 deposition by these mutants when compared with wild type cells. In addition, a reduction in migration was observed on collagen I and fibronectin. In a different study, Liu et al 2007 proved that reduced
expression of CD151 lead to diminished internalization of its partners i.e. α3β1 which resulted in reduced tumour cell motility on fibronectin and laminin. Competition assays also demonstrated that CD151 depletion reduced binding affinity of α3β1 to laminin 5 indicating that CD151 not only stabilized this but also regulated integrin trafficking. This clearly implicates that internalization of integrins is required for efficient forward migration [10].

1.4.3 CD151 and tetraspanin enriched microdomains: Outside-in signalling

CD151 has been shown to have a role in various steps of cell migration especially during cell adhesion. One of the most important characteristics of CD151 is the formation of tetraspanin enriched microdomains formed by clusters of tetraspanins that associate with growth factors, integrins, tetraspanins, and other many proteins in the cell membrane and serve as platforms for the initiation of signalling complexes that result in multiple cellular responses [20, 21]. Among these responses are protein trafficking and signalling, platelet aggregation [115], cell adhesion [116], cell fusion, and cell morphology, thereby influencing immune responses, hematopoiesis, fertilization, cell invasion, cell migration [10, 13], and tumour progression and metastasis [9, 11, 12, 117].

To demonstrate the effect that CD151-growth factor interactions have in the context of cell migration, Sedej et al., 2010 compared colonies formed in 3D matrigel by CD151+ and CD151 - MDA-MB-231 cells and found that ~ 80% of the colonies formed by CD151 + cells were less compact and the cells exhibited characteristic elongating protrusions. It was shown that this colony scattered phenotype was dependent on CD151
regulation of integrin mediated phosphorylation of p38 in MDA-MB-231 cells in response to TGFβ1 stimulation. In addition to this role, CD151 also regulated the compartmentalization of TGF β1 receptor [118]. A similar phenotype was observed when HEp3 tumours were treated with a monoclonal antibody specific for CD151. Briefly, after injection of HEp3 cells and treatment with mAb 1A5, treated animals developed more compact tumours composed of cells that were unable to detach. This illustrates the role of CD151 in tumour cell migration and invasion [117]. A different, but related study proved that CD151 silencing on malignant breast cancer cells, i.e. MDA-MB-231 and J110, significantly decreased cell migration that was dependent on CD151-a6 interaction. This was a result of CD151 associated reduction in pFAK and plck. Rac1 integrin dependent activation was also diminished demonstrating the importance of CD151 as an outside-in signaling effector.

There is another example of the role of CD151 as a cell membrane effector of downstream signalling pathways that results in altered cell migration. In disagreement with the multiple studies described elsewhere that show that single cell migration was reduced after silencing of CD151, Johnson et al., in 2009 proposed that CD151 also has an influence in collective cell migration of intact monolayers. Opposite to most studies, they demonstrated that knocking down CD151 expression exerted a significant enhancement in the cell migration rate of these monolayers. This effect was achieved through CD151 influence in mislocalization of E-cadherin, α-catenin, plakoglobin and p120ctn in cell-cell junctions of A431 cells. CD151 participation in the stabilization of actin stress fibers was also noted at the basal surface of CD151 mutants. Noticeably, this pattern was somewhat disorganized, which was a common feature present in β-catenin
interactions as well. Levels of activated RhoA were particularly elevated when CD151 expression was low, explaining at least in part the increase in stress fibers, as well as why cell-cell contacts were threefold more active and therefore had shorter lifespan leading to the increase in intra-monolayer motility [119].

Additionally, loss of expression of CD151 affects integrin binding dynamics. In the absence of CD151, α3β1 no longer associated with the member of the Ig superfamily, EWI-2, nor it did with the tetraspanins CD81 and CD9, which is indicative of the ability of CD151 to form TEMs. Moreover, CD151 silencing induced formation of lateral membrane protrusions and changes in directionality. Reduction of α3β1 internalization was also observed, suggesting that CD151 dependent integrin trafficking to the cytoplasm was necessary for proper cell motility. Interestingly, α6 dependent cell adhesion and spreading was also disturbed in mutants [120]. The results obtained by this group were not surprising since Yáñez-Mó M in 1998 demonstrated that CD151 associated with CD9, CD81 and α3β1 to modulate HUVECs (Human Umbilical Vein Endothelial Cells) migration and invasion in Collagen I gels [13, 121]. Furthermore, integrin-tetraspanin interaction, more specifically α3β1-CD151 regulated cell adhesion complexes involved in cadherin mediated adhesion through gene regulation of protein tyrosine phosphatases (PTPμ) [122].

1.4.4 The importance of CD151 in cancer

After summarizing the effect of CD151 on cell migration, it is not surprising that CD151 has been associated with tumourigenicity. In fact, CD151 is frequently up-regulated in
cancer [20] and its expression is often associated with poor outcome in several cancers. In hepatic cancer, for instance, increased mRNA levels and protein expression associated with larger tumours, poor differentiation and metastasis. Two and five year survival rates were higher for patients with low CD151 expression [123]. In addition, Ke AW et al, in 2009 showed that high CD151 expression as detected by qrtPCR and IHC associated with higher metastatic potential. Patients with elevated CD151 expression had multiple larger tumours with vascular invasion and had significantly lower overall survival when compared with patients with low CD151 expression [14]. Similarly, in lung cancer, CD151 positivity, evaluated by rtPCR and IHC, influenced overall five year survival rate in adenocarcinoma patients [16]. In the case of clear cell renal carcinoma there was significant association between high CD151 staining and pTNM stage, nuclear grade, tumour size, age, lung and bone metastasis, in addition to shorter cancer-specific survival and progression-free survival [17]. Lastly, but not less important, Susuki S et al, 2011 demonstrated that CD151 positive expression in esophageal squamous cell carcinoma associated with tumour depth, metastasis, increased proliferation and lower five-year survival rates [15].

Additionally, Takeda et al, 2011 demonstrated the importance of CD151 in cancer metastasis by using CD151 knockout (KO) mice. Briefly, they injected either MLECs (mouse lung endothelial cells), B16F10 cells (mouse melanoma) or LLC cells (Lewis Lung Carcinoma cells) into CD151 KO mice C57BL/6J and noticed that mice lacking CD151 had fewer lung metastasis in an experimental metastasis assay [124].
Figure 1.6 Participation of CD151 in cell migration pathways. Upon CD151 binding with integrins and other proteins to form the TEMs, a series of signalling pathways get activated to promote cell migration. Top: schematic of some of the multiple pathways that CD151 influences on in terms of locomotion. Bottom: Once the cell gets in contact with "clues", growth factors, cytokines, Ca++, etc., its cell receptors orientate towards the signal and the migration steps take place. CD151 is involved in cell adhesion, hemidesmosome formation, and retraction of the rear of the cell.
This reduction was not due to an effect on cell growth, microvascular density, apoptosis or differences in leukocyte interaction, but to a reduction in cell adherence of B16F10 cells to MLECs in KO mice, reduced cell spreading and disorganized laminin ECM. After VEGF stimulation, Src, and Akt activation was reduced in KO mice. Additionally, transendothelial migration of B16F10s through CD151-null endothelial cells was reduced due to a decrease in tumour cell induced endothelial retraction (permeability). These results indicate that CD151 also plays a role in endothelial response to VEGF.

Lastly, we have demonstrated the importance of CD151 by showing that CD151-specific monoclonal antibody 1A5 (mAb 1A5) prevented metastasis in mouse and chicken embryo models by inhibiting tumour cell intravasation without affecting primary tumour growth, tumour cell arrest, extravasation, or growth at the secondary site [117]. In vivo, this activity, unique to mAb 1A5, results from a strong inhibition of migration due to enhanced tumour cell-matrix interactions. Other CD151-specific antibodies promoted tumour cell migration [125, 126].

1.5. Clinical relevance of targeting cell migration associated proteins: RHAMM as a therapeutic target and CD151 as a prognostic factor in PCa

Most therapies are addressed to kill the primary tumour, but if clinicians want to avoid dissemination of the tumour, they need to treat with aggressive approaches, causing innumerable negative side effects on the patients. It is, therefore, necessary to implement new, different ways of treatment for these patients. More and more researchers have been focussing their studies on finding ways to stop the occurrence of early events of
metastasis such as cell migration. Targeting this event could potentially not only reduce the risk of dissemination of cancer cells from the primary tumour to a distant site, but also help to prevent local invasion and further dissemination after metastasis has been identified.

In theory, approaches that address any step in the metastatic cascade should be a good therapeutic target. However, in our lab, we strongly believe that inhibiting the very first aspects of metastasis are the best targets since their abrogation translates into a greater chance of reduction of dissemination. Targeting cell migration can stop local invasion and metastasis by limiting the disease and therefore reducing mortality and morbidity since clinicians may no longer need to provide aggressive treatments to patients at higher risk. This endeavour is not easy though. It is necessary to find therapeutics that only disrupt pathologic cell migration without affecting normal physiological function.

The main challenge at the moment in designing any anti migration therapy is the time to start it and its duration. Unfortunately, there are not very many biomarkers for metastasis. If one could predict which patients are at higher risk of developing metastasis for each type of cancer, the design of a treatment scheme should be easier and perhaps more successful.

1.5.1 Therapeutic targeting of the migration-associated protein RHAMM

As discussed above, RHAMM plays a major role in cell migration and tumour progression. Moreover, RHAMM upregulation occurs under very specific conditions,
namely, embryogenesis, wound healing, and cancer. This is very advantageous in terms of therapeutic targeting. Since it is not expressed in many tissues during normal conditions, one would expect that treatment options targeting RHAMM would be highly specific and would not cause major adverse side effects. It is based on this hypothesis that a number of scientists have intended to cure different types of malignancies with RHAMM based therapies.

Currently, there is a vaccine based on a RHAMM peptide that has been used in a number of clinical trials. Specifically, RHAMM R3 peptide has been assessed recently in Phase I/II clinical trials for treatment of AML, MM, and CLL [127-129]. This treatment resulted in blast reduction in the bone marrow and leukemic blast lysis and avoided the need for blood transfusions for one patient. Also, immunological response, marked by an increase in T cell frequency, was observed in 70% of AML, MM, and MDS patients in an initial study [129]. In agreement with the above mentioned advantages, treatment with RHAMM peptide has yielded very low toxicity because it is not expressed in healthy bone marrow tissue. Additionally, vaccination with DC pre-stimulated against the previously mentioned peptide has also undergone Phase I/II clinical trials for treatment of CLL. Clinical response was correlated with an increase in CD8+ T cell proliferation and in some cases a decrease in Treg population. Interestingly, in B-CLL patients with clinical response to vaccination with stimulated DC cells, the CD8+ cytotoxic T cell and IL-12 anti-tumour response was increased, whereas the Treg cell population was decreased [130]. Subsequently, Greiner et al., in 2010 demonstrated that there was no dose-dependent effect of this vaccine, indicating that RHAMM is an effective therapeutic target even at low levels [131].
This treatment strategy has not yet been used for epithelial tumours, however, as RHAMM is a prognostic marker for many of them and is overexpressed in many cases which currently do not have a specific targeted therapeutic option (e.g. basal subtype of BCa) and given the magnitude of the response, along with such low toxicity, it is an approach which merits further consideration.

1.5.2 Use of CD151 as a prognostic biomarker for disease recurrence and metastasis

As mentioned above CD151 is a tetraspanin frequently up-regulated in cancer and has been able to predict the outcome of multiple cancers. It is therefore not surprising that a number of scientists have proposed the use of mAbs to CD151 and other tetraspanins for the treatment of tumours. Multiple antibodies to CD151 have been designed and used throughout the years in vitro and also in vivo in animal models [132]. These recognize different epitopes in different tissues in vitro and some are specific for CD151 bound to other proteins i.e. integrins and have been able to either inhibit or enhance not only cell migration, but also metastasis in animal models [117, 133, 134]. Many of these antibodies target sequences present in the LEL of CD151 disrupting with this its interactions with integrins and resulting in reduced cell migration (SFA1.2B4) [134]. This was accompanied by altered/enhanced adhesion by gluing the rear of the cell to the primary tumour (1A5)[117], increasing homotypic cell attachment which enhances cell-cell junctions (11B1.G4) [135], and/or decreasing in invasion and angiogenesis (50-6) [133]. Most likely, the effect observed with these antibodies is due to an effect on ligand binding that ultimately alters downstream effectors such as reduction in activation of RhoA, Raf,
PI3K, Akt, as well as stabilization of E-cadherin and inhibition of β-catenin, among other implications.

Even though CD151 has demonstrated to be a good prospective therapeutic target in the cancer field, the fact that it is a ubiquitous protein (for reference see CD151 gene in Gene note and GNF BioGPS databases) and that the use of these antibodies in humans influence for instance platelet aggregation, has made researchers step back on this approach. Remaining, though, is its ability to predict the clinical outcome of patients with cancer. We strongly believe that the identification of CD151 immunoreactivity in the initial biopsy tissue can help physicians to determine what patients could benefit from more aggressive treatment modalities increasing with this their survival.

1.6 Final note

The intent of this thesis is, by no means, to find an association or interactions between these two proteins, but to demonstrate that protein members of different families can have similar downstream effects i.e. on cell migration. Additionally, my goal is to show how targeting of proteins involved in cell migration can be of great use in the clinic. Although CD151 to date cannot be targeted for therapy because of the above mentioned pitfalls, it indeed can help to predict clinical outcome of several types of cancer. On the other hand, RHAMM has been proven to be useful in the clinic as a treatment for blood malignancies; although the molecular mechanisms that make RHAMM an oncogenic protein are still subject of study.
Here I describe the utility of CD151 as a prognostic factor in prostate cancer and contribute to a better understanding of the Rhamm molecule by showing how one Rhamm isoform induces cell transformation through an ERK1,2 related mechanism and localizes at different subcellular compartments when compared with the full length form.

1.7 Hypotheses

1. CD151 as detected by mAb 1A5 is a molecular prognostic factor for prostate cancer disease progression and metastasis.

2. Rhamm FL and Rhamm Δ163 isoforms have different functions and subcellular localization in MEFs and 10T1/2s.

1.8 References


Palmer T, Zijlstra A. CD151 UCSD Molecular Pages. 2011(doi:10.1038/mp.a004123.01).


Chapter 2
Rhamm compartmentalization and its functions

C. Vasquez\textsuperscript{1}, S. Hamilton\textsuperscript{2}, S. Zhang\textsuperscript{2}, J.D Lewis\textsuperscript{1} and E.A. Turley\textsuperscript{2}

\textsuperscript{1}Department of Medical Biophysics, Schulich School of Medicine & Dentistry, The University of Western Ontario, 339 Windermere Road London, ON, Canada N6A 5A5

\textsuperscript{2}Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, 339 Windermere Road London, ON, Canada N6A 5A5

Keywords: RHAMM, cell transformation, subcellular localization, MEK1/ERK1,2, nuclear translocation

Address correspondence to:
Eva A. Turley
London Regional Cancer Program
790 Commissioners Road East
London, Ontario, Canada
N6A 4L6
Phone: 519 685 8600 x53677
Email: eva.turley@lhsc.on.ca
2.1 Abstract

The transforming potential of many oncoproteins can be "activated" by alternative gene splicing and post-translational modifications. These modifications may expose cryptic active sites, remove inhibitory sequences or influence the localization/function of oncoproteins. RHAMM is an oncoprotein, tumor marker, and novel breast cancer susceptibility gene that belongs to a family of hyaluronan binding proteins defined as hyaladherins. It is displayed both inside the cell, where it decorates the mitotic spindle, and outside the cell where it binds to receptors such as CD44 and growth factor receptors. RHAMM is subject to alternative splicing and post-translational modification and both of these processes are thought to affect its subcellular compartmentalization. Furthermore, some of these modifications have been associated with aggressive tumour behaviour.

Here we demonstrate that RHAMMFL can be "activated" into a transforming form by the removal of its N-terminal 163 amino acids, as demonstrated by foci formation and anchorage independent growth. These oncogenic effects require MEK1/ERK1,2 activity. To assess if this change in function of activated RHAMM is associated with altered subcellular compartmentalization, we transiently expressed ZsGreen -RhammFL-FLAG and Δ163 in RHAMM -/- MEFs. Both forms were observed at the cell surface and on mitotic spindles as assessed by confocal microscopy. However, in interphase cells, intracellular RHAMMFL appears primarily fibrillar and associates with microtubules. On the cell surface, RHAMMFL is present as an amorphous coat on the end of cell processes. In contrast, intracellular, oncogenic RHAMMΔ163 is diffuse and is present in several compartments in interphase cells, including the nucleus and vesicular structures. RHAMMΔ163 also localizes to podosomes, which are structures required for cell motility.
and invasion. Mass spectrometric analysis of RHAMM$^{FL}$ and $^{A163}$-interacting proteins show that both associate with components of the cytoskeleton. RHAMM$^{FL}$ binds more robustly to TPM2, tubulin, vimentin, actin and myosin light chains, consistent with its observed association with the fibrillar cytoskeleton. Furthermore, FRAP analysis suggests that RHAMM$^{FL}$ is less mobile than RHAMM$^{A163}$. Since the oncogenic functions of RHAMM$^{A163}$ require ERK1,2 activity, the co-association of these MAPK with both RHAMM forms was compared using confocal and immunoprecipitation assays.

In RHAMM$^{-/-}$ MEF and 10T1/2 cells, both RHAMM forms associate with total and phospho-ERK1,2, but the interaction is greater with RHAMM$^{A163}$ than RHAMM$^{FL}$. Furthermore, RHAMM is required for transformation by H-Ras and mutant active MEK1. In a reciprocal relationship, RHAMM requires both Ras and MEK1 expression for oncogenic activity. These results are consistent with previous experimental data showing that RHAMM acts at two locations on a RAS-ERK1,2 pathway: as a cell surface co-receptor for HA-CD44 interactions, which activate ERK1,2 at the cell membrane, and as part of a protein complex with MEK1 and ERK1,2. RHAMM$^{A163}$ expression promotes nuclear accumulation of p-ERK1,2. These results suggest that intracellular RHAMM may act as a scaffold for translocating pMEK1, pERK1,2 to the cell nucleus and that this property is required for its transformation activity.

2.2 Introduction

It is becoming apparent that many proteins are multifunctional and that this is in part affected by their subcellular localization. Specialized targeting sequences that guide and
dock proteins to specific intracellular and extracellular sites are modulated by alternative splicing and post-translational modifications. One well-studied example of this mechanism is the signal peptide, which targets proteins to the Golgi-ER and leads to their export into the extracellular compartment. Until recently, this paradigm was thought to be the only means of secretion but this has recently been challenged [1, 2] by evidence that a growing group of proteins lacking classical export signal sequences are found on the surface of the cell. Examples include epimorphin/syntaxin 2 [2, 3], bFGF1,2 [1] and RHAMM/HMMR [4]. These proteins are not exported through golgi/ER but by independent routes in response to cell stress such as tissue injury and neoplastic transformation. The unconventional export mechanisms involve exocytosis, membrane blebbing, transport through the endolysosomal pathway, export via channel proteins and export complexes that “flip” the protein across the membrane [4]. Importantly, proteins that are unconventionally exported to the cell surface often perform distinct functions in each compartment. For example, epimorphin is involved in mammary gland morphogenesis when expressed outside the cell and a vesicle protein (also called syntaxin 2) involved in endocytosis when inside the cell [2-4]. Similarly, the precise intracellular compartmentalization of proteins also affects their function. For example, whether or not a protein is oncogenic can depend upon its ability to enter specific subcellular compartments. Src requires access to focal adhesions in order to transform cells [5] while p-ERK must gain access to the cell nucleus to exert its oncogenic effects [6, 7]. In fact, more than 60% of ERK1,2 targets are located in the nucleus, indicating that ERK translocation to the nucleus of the cell is required for its function [8].
Based on this, it is clear that products of single genes can have opposing functions, even during oncogenesis. For example, CD44, a hyaluronan receptor that is expressed as multiple isoforms [9] resulting from alternative mRNA splicing can both promote tumor progression, as is the case of CD44 v6, in colorectal cancer [10] or act as a tumor suppressor, CD44-s [11]. Furthermore, CD44-s, the best characterized isoform, has been identified as a tumour suppressor in prostate cancer [12] and its loss predicts poor outcome in this disease [13, 14]. These differentially expressed isoforms vary in their subcellular distribution, association with the matrix, function and their contribution to tumour progression [15].

RHAMM is typically expressed after injury, but is also upregulated in many inflammatory diseases including arthritis, diabetes and cancer. It belongs to the hyaladherin family of hyaluronan binding proteins and is a multifunctional protein that is present in both the intracellular and extracellular compartments. A wide variety of cell types express RHAMM; including fibroblasts, immune cells, smooth muscle cells, endothelial cells, sperm and keratinocytes in culture [16-21]. Intracellular RHAMM decorates interphase microtubules, centrosomes and mitotic spindles, but it is also diffusely localized throughout the cytoplasm [22-26]. It also localizes to podosomes, filopodia and other membrane protrusions [25, 27, 28]. RHAMM is exported to the cell surface after wounding (in vivo and in vitro) or at subconfluent levels after stimulation with growth factors [28, 29]. On the cell surface, RHAMM interacts with HA and complexes with transmembrane receptors such as CD44 [30], PDGFR [31], RON [32], and integrins [33]. It promotes the activation of signalling cascades through these receptors. For example, RHAMM/CD44 interactions promote maximal activation of
ERK1,2. RHAMM likely acts as a co-receptor in this activation since it does not have a membrane spanning sequence [34].

RHAMM is expressed as both alternatively spliced and post-translationally modified proteins [22, 35, 36]. Isoforms that are shorter than the full length protein are transiently expressed following injury [37, 38] and constitutively expressed upon neoplastic transformation [39]. One of these, RHAMM\(^{A163}\) is highly expressed in H-Ras transformed fibroblasts and its HA binding ability is required for H-Ras to sustain fibroblast transformation [39]. There is also clinical evidence to support an oncogenic role for RHAMM. RHAMM is elevated in most types of cancer and its over-expression has been associated with poor outcome and onset of metastasis in glioma [40], breast [24, 41], endometrial [42], colorectal [43, 44], stomach [45], prostate cancer [46], multiple myeloma [35] and AML [47]. However, the oncogenic effects of RHAMM isoforms other than RHAMM\(^{A163}\) have not been reported. Several studies suggest that RHAMM isoforms differ in their distribution. For example, cell surface protein biotinylation experiments suggest that only a 70 kDa protein, the size of RHAMM\(^{A163}\), is exported to the cell surface [38]. The alternatively spliced RHAMM\(^{Δexon4}\) exhibits reduced association with interphase microtubules compared to the full length protein and selectively traffics to the cell nucleus [22, 26].

Here, we assessed the oncogenicity of truncated RHAMM proteins that are highly expressed in aggressive human breast cancer cell lines [34]. We related the differences in transformation ability to the distinct subcellular localization of these RHAMM isoforms. To assess this, we utilized the 10T1/2 model of cellular transformation and assayed foci formation and growth in soft agar. We show that only RHAMM\(^{A163}\) is transforming in...
this model. To unambiguously identify the subcellular distribution of each RHAMM isoform, we transiently expressed recombinant ZsGreen-RHAMM fusion proteins in RHAMM -/- MEFs. We show that RHAMM FL, which is not transforming, is associated with microtubules while RHAMM A163 is significantly more mobile within the cell and is present in many subcellular compartments including the cell nucleus.

2.3 Methods

Cell culture

Rhamm knockout (-/-) Mouse Embryonic Fibroblasts (MEFs) were isolated as described by Tolg et al., 2003 and 2006 [48, 49] and grown as monolayers in low glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Multicell) supplemented with 10% FBS (Multicell) and 10mM HEPES (Multicell) at pH 7.2 at 37°C in a humidified 5% CO₂ atmosphere. C3H/10T1/2, mouse fibroblasts were purchased from ATCC (Manassas, VA) and maintained under the same conditions as MEFs.

Antibodies

The following antibodies were used in this study: α tubulin rat mAb (Abcam), β tubulin mouse mAb (SIGMA), FLAG Tag M2 mouse mAb (SIGMA), FLAG M2 agarose beads from mouse (SIGMA) (a kind gift from Dr. Joe Mymryk’s lab), ERK1 and MEK1 (Santa Cruz Biotechnology, Inc.), ERK1,2 rabbit polyclonal #9102 (a kind gift from Dr. Dimattia’s lab (Cell Signaling), phosphorylated ERK1,2 rabbit polyclonal #4370 (Cell
Signaling), ZsGreen rabbit polyclonal (Clontech), Rhamm mouse mAb 6B7B7 (Promab), Rhamm polyclonal antibody against the sequence QLRQQDEDFR (aa 543-553), non-immune IgG (Chemicom) was used as negative control, and species specific IgG + Alexa Fluor secondary antibodies (Invitrogen).

**Recombinant Rhamm protein constructs**

ZsGreen was amplified using the following primers (SIGMA) 5' EcoRI-ZsGreen (AAAAAAA GAATTC ATG GCCCAGTCCAAGCACGG) and 3' 10 first N-terminal base pairs of Rhamm FL/A 163 - ZsGreen C-terminus (FL: TAGGAAAGGA GGGCAAGGCGGAGCCGG and A163: TTAGAGCTCT GGGCAAGGCGGAGCCGG) by using the Phusion high fidelity PCR kit (NEB) and following the manufacturer recommended conditions. Rhamm FL and Rhamm A163 were amplified by PCR using 5' 10 last C-terminal base pairs of ZsGreen and the first 16-17 base pairs of the N-terminus of Rhamm (FL: CGCCTTGCCC TCCTTTCTAAGGCAGCC and A163: CGCCTTGCCC AGAGCTCTAAGCCTTG), and 3' last 17 C-terminal base pairs of Rhamm-FLAG tag-XhoI (AAAAAAA CTCGAG TCA CTTGTCATCGTGCTCCTTGTAGTC GCAGCAGTTTGGTGTTGC). Afterwards, the product of the ZsGreen reaction was PCRed with the product of either Rhamm FL or Rhamm A163 to allow for annealing of the two sequences. After purification (Bio Basic Inc. Gel extraction and PCR product purification kits) and enzymatic digestion with EcoRI and XhoI (NEB), the construct was ligated into pCDNA 3.1+ plasmid under the control of the CMV promoter, sequenced, and transiently expressed into i) Rhamm -/- MEFs, ii) Rhamm FL rescued Rhamm -/-
MEFs, iii) Rhamm^{Δ163} rescued Rhamm-/- MEFs, iv) 10T1/2s, and v) Rhamm-/- transfected with mutant active MEK. A plasmid containing only the ZsGreen sequence was used as negative control.

Untagged Rhamm^{FL/Δ163} cDNAs were inserted into the pHApr-1-neo expression vector under the control of a β-Actin promoter as previously described by Zhang et al., 1998. The mutant active MEK and dominant negative MEK1 constructs were kind gifts from Dr. Natalie Ahn. Mutant active Ras (H-Ras) and dominant negative H-Ras cDNA (DN-RasN17) were kindly provided by Dr. Channing J. Der.

A dominant negative Rhamm^{Δ163} with an N-terminal Leucine zipper mutation was created previously in our lab by altering the Hyaluronan Binding Domain (HABD) that overlaps with ERK D-docking motif. The following substitutions were made, K748N, R749N, and K750W.

Rhamm expression in MEFs and 10T1/2 cells

ZsGreen-Rhamm^{FL/Δ163} and ZsGreen only constructs were expressed into the cells by transient transfection using jetPRIME (Polyplus transfection reagent). Immortalized Rhamm-/- MEFs or 10T1/2s were grown in monolayers to a confluency of 80-90% in DMEM supplemented with 10% FBS for 24 hours. The plasmids were then incubated with the transfection reagent as recommended by the manufacturer and added to the cells in a ratio of 1μg of DNA : 2μL of transfection reagent, brought back to incubation for 12 hours, and treated according to the experiment needed.
10T1/2s were transfected with untagged Rhamm\(^{FL}\), Rhamm\(^{\lambda 163}\), Rhamm\(^{\lambda 163-\text{HABD}}\), H-Ras, dominant negative H-Ras, or mutant active MEK 1 using Lipofectamine Plus (Invitrogen) as described previously [31] stably expressing cells were established by selecting with 1-5mg/mL G418 (SIGMA).

Rhamm \(-/-\) MEFs were infected using a retroviral system for expression of untagged Rhamm\(^{FL/\lambda 163}\), as described by Tolg et al., 2003 [49] and stable transfectants were selected with 1-5mg/mL G418 (SIGMA selected using 1-5mg/mL G418 (SIGMA).

**Western Blot analysis**

12 hours after transfection, Rhamm \(-/-\) ZsGreen-Rhamm\(^{FL/\lambda 163}\) cells were rinsed twice with PBS, detached from the dish with a cell scraper, centrifuged at 1400 rpm for 4 minutes and then lysed with NP40 lysis buffer (1% NP-40, 50mM Tris-HCl, pH 8.0, 150 mM NaCl) containing protease inhibitor cocktail. Protein was collected and concentration was measured by the Bradford protein assay (BioRad). 30\(\mu\)g of protein was loaded onto 8% gels, separated using SDS-PAGE, and transferred to a PVDF membrane (GE Healthcare). Then, the membrane was rinsed with TBST (10mM Tris base pH 7.4, 150mM NaCl, and 0.1% Tween-20) and blocked for an hour at room temperature in 5% skim milk in TBST. Subsequently, the membranes were incubated with rabbit pAb to ZsGreen (1:1000) or mouse mAb to FLAG tag (1:5000) in 5% skim milk in TBST overnight at 4°C. After several washes with TBST, horseradish peroxidase conjugated secondary antibody (1:10,000 in 5% skim milk in TBST) incubation was performed for one hour at room temperature followed by several washes with TBST. The presence of
protein was evidenced by using the enhanced chemiluminescence (ECL) detection kit (GE Healthcare).

Cell lysates from 10T1/2 cells were isolated with RIPA buffer (25mM Tris-HCl pH 7.2, 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate, 0.15M NaCl, 1mM EDTA, and 50mM HEPES pH7.3) containing protease and phosphatase inhibitor cocktails on ice. Lysates were then incubated on ice for 20 minutes and then were centrifuged for 20 minutes at 13,000 x g at 4°C. Protein concentration was determined using the DC protein assay (Bio-Rad). Nuclear extracts were prepared following the manufacturer’s instructions (Nuclear Extract kit, Active Motif). 10-50μg of protein were electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad). Nonspecific binding was blocked with 3% skim milk in TBST at 4°C. Primary and secondary antibodies were incubated in 1% skim milk in TBST for 1 hour at room temperature. Signal was developed with the ECL detection system (Amersham).

**Foci formation and colony formation in soft agar**

After 10T1/2 cells were stably transfected with H-Ras, dominant negative H-Ras, Rhamm<sup>FL</sup>, Rhamm<sup>Δ163</sup>, mutant Rhamm<sup>Δ163-HABD</sup>, or empty vector using Lipofectamine Plus as described above, foci formation was assessed as follows. Post-transfected cells were grown in DMEM supplemented with 10% FBS during 10-15 days at 37°C with 5% CO<sub>2</sub>. Then, cells were fixed with 4% formaldehyde for 10 minutes at room temperature and stained with methylene blue as described by Zhang et al., in 1998 [31]. The number
and size of foci were analysed and then normalized according to transfection efficiency measured by western blot. All experiments were performed in triplicate.

Colony formation in soft agar was measured by suspending $1 \times 10^5$ transfected 10T1/2 cells in 0.3% agarose in 4mL of DMEM supplemented with 10% FBS. The cells were plated onto a 60mm petri dishes pre-coated with 0.8% agarose in DMEM 10% FBS. The cells were then incubated for 15-20 days at 37°C with 5% CO₂. The number of colonies was quantified and normalized according to transfection efficiency measured by western blot. All experiments were performed in triplicate.

**Immunofluorescence staining and co-localization analysis**

After transfection, the cells were seeded onto glass coverslips at a confluency of 50-60% and allowed to attach for 7-8 hours. The cells were then washed and fixed with 4% formaldehyde solution in PBS for 10 minutes at room temperature. After 2-3 washes with PBS, the cells were incubated one hour at room temperature with PBS- 1% BSA with or without 0.1% Triton X-100 (BioShop) depending on the need for intact membranes. For pERK staining, TBS was used instead of PBS. Subsequently, the cells were incubated with primary antibodies (Anti α tubulin, β tubulin, FLAG, pERK 1:1000) overnight at 4°C followed by several washes with PBS/TBS and incubation with species specific Alexa Fluor conjugated secondary antibodies (1:2000) (Invitrogen) for one hour at room temperature. Non-immune IgG was used as negative control. After rinsing, the coverslips were then mounted using Prolong Gold DAPI antifade reagent (Invitrogen) and imaged.
Confocal images were taken using an oil 63x/60x objective in either a spinning-disk confocal microscope, using a specialized instrument (Quorum Technologies, Guelph, ON, Canada) comprised of an upright Zeiss Axio Examiner Z1, LUDL filter wheels and large format motorized stage, a Yokogawa spinning disk head and a Hamamatsu 9100-12 Image EM CCD camera, controlled by Volocity (Improvision, UK) or an Olympus Fluo View TM FV1000 coupled to the IX81 Motorized Inverted laser scanning confocal microscope LX2 series. For co-localization analysis, images were acquired at 60-63x magnification with oil objectives and 24 regions of interest were selected in a total of 6 cells per group and Pearson's correlation was performed by the software Volocity after establishing background threshold.

**Fluorescence recovery after photobleaching and confocal imaging**

After transfection, cells were plated in chambered coverglass plates (Lab-Tek) with phenol red free DMEM (Multicell) 10% FBS, 0.2% HEPES and allowed to attach for 7-8 hours. FRAP was performed with the Olympus inverted laser scanning confocal microscope LX2 series enclosed in a temperature controlled chamber. Briefly, a region of interest (ROI) of 45 μm² was delineated and fluorescence of ZsGreen-Rhamm Fl/Δ163 was measured before and after photobleaching. Four images were taken before photobleaching and the ROI were then exposed to a circular tornado scan with a 488nm laser at 100% power. 120 images at intervals of 2.5 seconds were acquired with a 60x oil objective after exposure was performed. A total of 6 cells were analysed per group and initial fluorescence intensity was normalized to 1. Measurement of fluorescence intensity
before and after photobleaching was performed at 120 timepoints. Then, fluorescence recovery was plotted against timepoints and non-linear curve fitting was carried out by the software FV 10-ASW 1.6.

Immunoprecipitation assays

12 hours after transfection of Rhamm -/- MEFs with ZsGreen-RhammFL/Δ63, the cells were serum starved for 12 hours and then stimulated with 10% FBS for 10-15 minutes. Immediately after the cells were removed from the plate with a cell scraper, centrifuged at 4°C, the pellet was rinsed three times with PBS and lysed with F buffer (Described by Michael Cole's laboratory: 10mM TRIS pH7.5, 50mM NaCl, 30mM Sodium Pyrophosphate, 50mM Sodium Fluoride, 5μM ZnCl2, and 0.5% Triton X-100; (a kind gift from Dr. Joe Mymryk's lab) with protease inhibitor cocktail and 1mM Orthovanadate for five minutes on ice. The lysates were then centrifuged at 13500rpm for 10 minutes at 4°C. Subsequently, the protein solution was incubated with mouse anti-FLAG M2 agarose (SIGMA) (another kind gift from Dr. Joe Mymryk's lab) overnight at 4°C. After centrifuging the pellet was rinsed 5 times with F buffer and resuspended in SDS-DTT loading dye. SDS-PAGE and protein transfer was performed as described above and probed with ERK1/2 and pERK1/2 rabbit polyclonal antibodies. Cell lysates/input were also tested following the same procedure.
Mass Spectrometry analysis of proteins

Recombinant GST-RHAMM^FL and RHAMM^A163 were prepared as described previously [50] and linked to Glutathione-linked beads. Cell monolayers were grown to culture confluence and solubilised in RIPA buffer. A cell scraper was used to remove all of the cellular debris and extracellular material from the culture dish. The solubilised and scraped material was mixed using a vortex then the pellet was prepared by centrifugation. The supernatant was discarded and the pellet was solubilised using a modified RIPA buffer and vortexing. The pellet contained primarily cytoskeleton proteins as judged by Mass Spec analysis. Recombinant RHAMM-beads were mixed with the solubilised cell pellet overnight at 4°C, beads were washed and then proteins that were bound to RHAMM were released using 10mM glutathione. Released proteins were separated by SDS-PAGE (7-14% gradient gels) and lightly stained with silver to identify protein bands. Bands that were clearly separated from one another were cut out and analysed by MALDI-TOFF (Proteomics Research Center, University of Toronto).

Statistical analysis

Differences between multiple groups were measured using One-way ANOVA and Tukey’s comparison post-test. Statistical differences between two groups were assessed using two-tailed Student’s t-test method. P-values <0.05 were considered statistically significant.
2.4 Results

**Rhamm^{A163} induces growth transformation in 10T1/2 fibroblasts**

Our first aim was to test if overexpression of Rhamm^{A163} confers the cells with the ability to proliferate independently of both inhibitory signals, such as the absence of a solid substratum, and regardless of growth contact inhibition signals. For this, the transforming potential of the truncated Rhamm protein forms shown in Figure 2.1A was assessed in two separate assays: i. foci formation and ii. growth in soft agar. H-Ras transformed 10T1/2 fibroblasts were used as positive controls for these assays. Rhamm^{A163} was found to be transforming in 10T1/2 cells. As shown in Figure 2.1B, both H-Ras and Rhamm^{A163} transfected 10T1/2 cells formed foci after 10-15 days of incubation, but empty vector transfected and Rhamm^{FL} transfected 10T1/2 fibroblasts did not. The ability of these transfectants to form colonies in soft agar, which is a measure of resistance to anoikis, was also measured (Fig. 2.1D). Both H-Ras and Rhamm^{A163} formed abundant colonies in this assay. Rhamm^{FL}, formed fewer colonies than either Rhamm^{A163} or H-Ras expressing cells but more than empty vector transfected cells. These results suggest that expression of either Rhamm isoform provides some protection against anoikis.

**Rhamm^{A163} acts at the level of Ras and MEK1 to promote growth transformation in 10T1/2 cells**

We and others have previously shown that Rhamm acts through the Ras pathway to control cell migration and transformation [51]. Since Ras influences multiple pathways,
we next determined through which RAS effectors RhammA\textsuperscript{A163} mediates signalling. Since Rhamm/HA or growth factor interactions can activate ERK1,2, we assessed if RhammA\textsuperscript{A163}/ERK1,2 interactions were required for this process.

For this purpose a mutant inactive version of RhammA\textsuperscript{A163} was created by site directed mutagenesis in the overlapping HA and ERK binding sites of RhammA\textsuperscript{A163} (illustrated in figure 2.1A). We first co-transfected 10T1/2 fibroblasts with a plasmid containing H-Ras and with the mutant inactive RhammA\textsuperscript{A163 H\textsubscript{A}B\textsubscript{D}\textsuperscript{+}} sequence; an empty vector was used as a negative control. Foci formation was reduced in about 50% in the cells transfected with the RhammA\textsuperscript{A163 H\textsubscript{A}B\textsubscript{D}\textsuperscript{+}} cDNA when compared with those transfected with H-Ras + empty vector (Figure 2.2A) indicating that RhammA\textsuperscript{A163} is acting upstream of Ras. In a different but related experiment, RhammA\textsuperscript{A163} and mutant inactive H-Ras cDNA \textsuperscript{(DN-Ras\textsuperscript{N17})} were transfected in 10T1/2 cells and the ability of the cells to form foci was assessed. The effect of RhammA\textsuperscript{A163} on contact inhibition of cell growth was reduced by approximately 60% after co-expression of the Ras mutant indicating a role of RhammA\textsuperscript{A163} upstream of Ras in this process (Figure 2.2 B).

To dissect, in more detail, the downstream effects of RhammA\textsuperscript{A163}, we developed a model in which we transfected simultaneously RhammA\textsuperscript{A163} with a dominant negative MEK1 cDNA and observed that inhibition of MEK/ERK1,2 activation had a statistically significant negative effect on RhammA\textsuperscript{A163} dependent foci formation (Figure 2.2 B). Moreover, co-transfection of 10T1/2 cells with a constitutively active MEK1 and mutant RhammA\textsuperscript{A163 H\textsubscript{A}B\textsubscript{D}\textsuperscript{-}} reduced foci formation by more than 50% (Figure 2.2C). These results indicate that Rhamm/ERK1,2 interaction is required for the transforming effect of MEK1 (Figure 2.3).
**Figure 2.1** Rhamm\(^{\Delta 163}\) transforms growth patterns in 10T1/2 fibroblasts. Colony growth in soft agar and foci formation was assessed in H-ras transformed 10T1/2 fibroblasts overexpressing Rhamm\(^{FL}\) and Rhamm\(^{\Delta 163}\). A) Schematic of Rhamm isoforms. Rhamm\(^{FL}\) possesses two tubulin binding sites (leucine zippers represented as red squares), two HA binding sites at the C-terminal sequence (illustrated in purple squares) and a D-domain ERK binding site on residues 748-756 of the protein (yellow square). Rhamm\(^{\Delta 163}\) isoform lacks the N-terminal tubulin binding site and Rhamm\(^{\Delta 163} \text{ HABD}^\dagger\) was mutated by site directed mutagenesis in the D-domain and HA binding site as indicated. B) Rhamm\(^{\Delta 163}\) promotes foci formation when overexpressed in 10T1/2s. Quantification of foci formation is represented in C. D) Representative image of colony formation in soft agar of 10T1/2 after transfection with either Rhamm\(^{FL}\) or Rhamm\(^{\Delta 163}\). H-Ras transformed fibroblasts were used as positive control, both experiments were performed in triplicate, and the results were normalized to the expression levels as assessed by western blot analysis after transient transfection.
Figure 2.2 Rhamm\textsuperscript{A163} acts upstream of Ras and at the level of ERK to promote growth transformation. Mutant Rhamm\textsuperscript{A163 HABD}, inactive Ras (DN-RasN17), and double negative MEK1 (DN MEK1) were transfected in 10T1/2 fibroblasts and their effect in foci formation was assessed. 10T1/2 cells were transfected with: A) H-Ras and Rhamm\textsuperscript{A163 HABD}, B) Rhamm \textsuperscript{A163} and either inactive Ras or inactive MEK1, or C) Mutant active MEK1 and Rhamm\textsuperscript{A163 HABD} and foci formation was measured. The relative number of foci was arbitrarily set to 100 and the data was then normalized to the positive control in each experiment. The experiments were performed in triplicate. * p < 0.05
Figure 2.3 Proposed mechanisms for Rhamm$^{Δ163}$ transforming effects. Rhamm upstream of Ras and at the level of ERK1,2 to induce cell transformation. Additionally, Rhamm$^{Δ163}$ induces pERK translocation to the nucleus of the cell and mediates its sustained expression there. This may influence Rhamm associated oncogenicity.
Subcellular localization of ZsGreen-Rhamm isoforms in MEFs

To assess if the transforming effect of Rhamm^{Δ163} is associated with altered subcellular compartmentalization, and to observe the subcellular localization of different Rhamm isoforms, we prepared ZsGreen-FLAG-Rhamm^{FL} and Rhamm^{Δ163} constructs (Figure 2.4A and B) then transiently expressed these in Rhamm^{-/-} MEFs. Figure 2.4C demonstrates similar transfection efficiency for both isoforms and the ability of the FLAG and ZsGreen antibodies to detect the expressed proteins in Rhamm^{-/-} MEFs. Confocal analysis suggested that both forms localize to mitotic spindles during mitosis (Figure 2.5A). In addition, staining using a ZsGreen antibody in cells that were not previously permeabilized suggests the presence of both isoforms of Rhamm at the cell surface (Figure 2.5B). However, during interphase, intracellular Rhamm^{FL} appears primarily fibrillar, whereas on the cell surface Rhamm^{FL} is present as an amorphous coat on the end of cell processes. In contrast, the intracellular pool of Rhamm^{Δ163} is diffuse throughout the cytoplasm and localizes to several compartments in interphase cells, including the nucleus and vesicles (Figure 2.5A). Additionally, Rhamm^{Δ163} seems organized within podosome-like structures, which are structures required for cell motility and invasion. The present results indicate not only that Rhamm isoforms have different subcellular distribution, but also that having attached a fluorescent protein on the N-terminus and a FLAG tag on the C-terminus of Rhamm does not affect the previously reported localization of Rhamm.
Figure 2.4 ZsGreen-Rhamm^{FL/Δ163} constructs. ZsGreen-Rhamm^{FL/Δ163} sequences were amplified and fused by PCR and inserted into pcDNA 3.1+. A FLAG tag was also inserted at the C-terminus of Rhamm (A and B). C) Western blots using ZsGreen and FLAG antibodies to assess expression of ZsG-Rhamm in MEFs after transfection demonstrate comparable transfection efficiency for both constructs.
**Rhamm^{Δ163} motile/dynamic within the cell**

To determine if the different subcellular distribution of Rhamm isoforms are related to the motility of these proteins within the cell, we performed fluorescence recovery after photobleaching (FRAP) analysis of our constructs in transiently transfected MEFs. Rhamm surface diffusion velocity reflects the state of the labelled protein in that unbound protein diffuses faster than bound protein. We found that ZsGreen-Rhamm^{FL} exhibited a significantly lower recovery rate than its counterpart, ZsGreen-Rhamm^{Δ163} (p<0.0009) (Figure 2.6). This suggests that a pool of Rhamm^{FL} is stably associated with rigid cellular structures, which would reduce its ability to diffuse through the cell cytoplasm.

**Rhamm associates with cytoskeletal proteins**

Rhamm has been previously described as a tubulin/actin binding protein [20, 22, 26, 34, 48, 52]. Based on Rhamm^{FL} distribution and motility in the cytoplasm, we hypothesized that this isoform has higher binding affinity for cytoskeletal proteins. To test this, we transfected Rhamm^{FL} or Rhamm^{Δ163} into 10T1/2 cells, performed a pull-down and analysed the proteins that attached to Rhamm using matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectroscopy (MS). In general, Rhamm^{FL} seems to preferentially bind to cytoskeletal proteins when compared to Rhamm^{Δ163} (Figure 2.7 A). These results suggest that both isoforms of Rhamm bind to actin and tubulin, and to other actin scaffold proteins such as Filamin α (FLNA), Spectrin α erythrocytic 1 (SPTA1), and Spectrin β non-erythrocytic 4 (SPNB4).
Figure 2.5 Distribution of the Rhamm constructs in the cell. Confocal images of MEFs transfected with either ZsGreen-Rhamm\(^{FL}\) or Rhamm\(^{\Delta163}\) illustrate differences in the distribution of Rhamm isoforms in the cytoplasm (A). Rhamm\(^{FL}\) is expressed in fibre-like structures whereas Rhamm\(^{\Delta163}\) is diffuse in the cytoplasm. Both isoforms translocate to the nuclei of MEFs during mitosis where they decorate the mitotic spindle. B) Immunofluorescence staining of MEFs transfected with ZsGreen-Rhamm using a ZsGreen antibody (red) detects the presence of ZsGreen-Rhamm fusion in the membrane of the cells. The cells were stained in the absence of membrane permeabilization or detergent. Scale bars, 20\(\mu\)m.
However, Rhamm\textsuperscript{FL} seemed to associate to a higher degree with smooth muscle actin 2 (ACTA2), Vimentin (VIM), Tubulin\(\beta\)4 (TUBB4), Tropomyosin 2 (TPMS2), and Myosin (My 1,4,6, and 6B) than Rhamm\textsuperscript{Δ163} (Table 2.1).

Furthermore, co-localization analysis performed on MEFs demonstrated that ZsGreen-Rhamm\textsuperscript{FL} more strongly co-localized with \(\alpha\) and \(\beta\) tubulin than ZsGreen-Rhamm\textsuperscript{Δ163} (p<0.0001) (Figure 2.7 B). ZsGreen alone was used as negative control and did not show co-localization with tubulin.

**Rhamm immunoprecipitates with ERK1/2 and co-localizes with pERK1/2 in MEFs**

Since the oncogenic functions of RHAMM\textsuperscript{Δ163} require ERK1,2 activity, the co-association of both Rhamm forms with ERK1,2 was compared using confocal and immunoprecipitation assays. After transient transfection of our constructs into Rhamm\textsuperscript{-/-} MEFs and staining with pERK1,2 antibody, Pearson's correlation of co-localization of our constructs with pERK demonstrated that there is a modest degree of co-distribution between both isoforms and activated ERK1,2. The range of co-localization was slight although significantly more pronounced in the cells transfected with ZsGreen-Rhamm\textsuperscript{Δ163} (p<0.009) when compared with ZsGreen-Rhamm\textsuperscript{FL}. No association was found between pERK and MEFs transfected with ZsGreen (Figure 2.8 A). Additionally, we assessed for interactions between the above mentioned proteins by immunoprecipitation assays and observed that both isoforms of Rhamm in these constructs associated with ERK 1,2. The same effect was not noticed when cells containing the plasmid with ZsGreen alone were immunoprecipitated (Figure 2.8 B).
Figure 2.6 Rhamm isoforms have different motility/dynamics in the cytoplasm of MEFs. Fluorescence Recovery After Photobleaching (FRAP) analysis was performed in Rhamm -/- MEFs transiently transfected with ZsGreen-RhammFL/A163 constructs. ZsGreen-RhammA163 was more mobile than ZsGreen-RhammFL. A total of 6 cells were analysed per group. Mean normalized fluorescence recovery was plotted against timepoints. Each line in the graph represents the mean recovery rate of one group (±SEM). Scale bar, 20µm.
**Rhamm$^{163}$ induces ERK1,2 trafficking and phosphorylation**

We have demonstrated thus far that Rhamm/ERK1,2 interactions are required for growth transformation, and previous studies have shown that ERK1,2 translocation to the nucleus of the cell is required for cell transformation [6, 7]. Thus, we hypothesized that Rhamm$^{163}$ plays a role in this process. To investigate this further, we looked for differences in ERK levels between Rhamm -/- MEFs and Rhamm -/- transfected with Rhamm$^{163}$. The levels of pERK1,2 in the nuclei and in the membrane of our MEFs were higher after serum stimulation. These high levels were also sustained in the cells expressing Rhamm$^{163}$ over a period of 24 hours as detected by immunofluorescence staining (Figure 2.9 A). Additionally, Western blot analysis of pERK1,2 after nuclear extraction in 10T1/2 cells demonstrated that the over-expression of the N-terminal truncated Rhamm$^{163}$ induced the translocation of pERK1,2 to the nucleus which persisted for at least 60 minutes post-stimulation with FBS (Figure 2.9 B). In addition, when a nuclear export signal (NES) was added to the MEK1 sequence to reduce the levels of active MEK/ERK in the nuclei of LR21 cells (Clonal population of 10T1/2 mouse fibroblasts transfected with Rhamm$^{163}$), a significant reduction in tumour formation after injection in NOD SCID mice was observed (P< 0.0005) (data not shown), suggesting that the role played by Rhamm$^{163}$ in cellular transformation is at least partially due to its ability to activate and induce enhanced and retained nuclear trafficking of ERK1,2. Moreover, the presence of pERK1,2 in the membrane of the cell (Figure 2.4B) indicates that there is an interplay between differentially localized Rhamm$^{163}$. 
<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>Rhamm^FL^</th>
<th>Rhamm^Δ163^</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLNA</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SPTA1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SPNB4</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ACTN1</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ACTA2</td>
<td>+++++</td>
<td>++</td>
</tr>
<tr>
<td>VIM</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>TUBB4</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>TPM2</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>My 1,4,6,6B</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2.1 Rhamm binding proteins.** After transfection of 10T1/2 fibroblasts with Rhamm^FL/Δ163^, pull-down with recombinant Rhamm was performed and mass spectrometry-MALDI TOF analysis was performed comparing protein interactions between the two proteins.
**Figure 2.7 Rhamm associates with cytoskeletal proteins.** Confocal imaging and Pearson's correlation of Rhamm KO MEFs transfected with ZsG-Rhamm FL Δ163 fusion and stained using mAbs to α and β tubulin (red). For analysis, four regions of interest were delineated per cell on a total of 6 cells per group. One-way ANOVA with Tukey's post test were used to assess statistical significance. Scale bars, 20μm.

**Figure 2.8 Rhamm associates with ERK.** A) Confocal imaging and Pearson's correlation of Rhamm KO MEFs transfected with ZsG-Rhamm FL Δ163 fusion and stained using pAb to pERK evidenced that ZsGreen-Rhamm Δ163 fusion co-localizes more with pERK (Red). For analysis, four regions of interest were delineated per cell on a total of 6 cells per group. B) Immunoprecipitation was performed by using agarose beads coated with FLAG mAb to pull down ZsGreen-RHAMM FL Δ163. Then, associations were detected by probing with rabbit pAb to ERK1,2. Both fusion proteins co-immunoprecipitated with ERK1,2. Scale bars, 20μm.
Figure 2.9 Rhamm^{Δ163} influences pERK sustained translocation to different compartments of the cell in Rhamm -/- MEFs and 10T1/2 fibroblasts. Activation of ERK and its trafficking was determined after transfection of Rhamm^{Δ163} in either Rhamm -/- MEFs or 10T1/2 cells. A) Confocal images of Rhamm -/- MEFs and Rhamm -/- MEFs transfected with Rhamm^{Δ163}. The cells were imaged before and after stimulation with 10%FBS at different timepoints as indicated in the figure. pERK was present in the nuclei, cytoplasm and membrane of the MEFs. However, greater fluorescence was evidenced in the cells that expressed Rhamm^{Δ163}. This activation was also more stable over time in these cells. B) The presence of pERK1,2 was obvious in the nuclei of 10T1,2 transfected with Rhamm^{Δ163} after serum stimulation and nuclear extraction at different timepoints when compared with its counterpart, parental 10T1/2. The images are representative of experiments performed in triplicate. Scale bars, 20 μm.
2.5 Discussion

Tumour progression and metastasis are complex and intricate processes that involve a series of events that begin with mutations that confer the ability of a cell to ignore growth and migration inhibitory signals. This allows them to proliferate while ignoring contact inhibitory signals and accumulate additional mutations that may eventually confer a migratory phenotype that results in cell invasion and migration to a distant site. A growing number of proteins have been implicated in these processes. Our study focussed on one protein, Rhamm\textsuperscript{A163}, whose expression promotes loss of contact inhibition and results in tumourigenic transformation in mesenchymal cells, as well as spontaneous metastasis in two mouse models of mesenchymal tumors [39].

With this study we were able to identify several properties that contribute to the oncogenicity of Rhamm\textsuperscript{A163}. First, we demonstrated that Rhamm\textsuperscript{FL} is not transforming but that it can be activated by the truncation of the N-terminal 163 amino acids. Rhamm\textsuperscript{A163} promotes loss of contact inhibition of growth and resistance to anchorage-dependent programmed cell death as determined by foci and colony formation in soft agar (Figure 2.1).

The loss of growth restraint is a key step in tumourigenesis and may be the mechanism by which Rhamm influences Ras mediated transformation [39]. Our results indicate that Ras transformation pathways are affected by the oncogenic activities of Rhamm. This is consistent with previous studies where high levels of Rhamm\textsuperscript{A163} were found in H-Ras transformed fibroblasts [39]. To expand on this, we demonstrated that it is Rhamm\textsuperscript{A163} that is implicated in cell transformation, and that its subcellular distribution is critical for this function. By using mutant inactive H-Ras we were able to reduce the transforming
ability of Rhamm$^{Δ163}$, indicating that this isoform acts upstream of Ras in 10T1/2 cells (Figure 2.2 B). Similarly, after using a mutant/inactive Rhamm$^{Δ163 \text{ HA}_{BD}}$ construct, inhibition of foci formation was observed, indicating that Rhamm is required downstream of Ras to meet its Ras-associated oncogenic functions (Figure 2.2 A). These results strongly suggest that cytoplasmic Rhamm$^{Δ163}$ influences the transforming capability by acting at multiple levels to promote cell growth advantage. This confirms our hypothesis that these two Rhamm isoforms have distinct functions that are dependent upon their subcellular distribution.

While this study confirms a role for Rhamm upstream of Ras, evidence is also presented implicating Rhamm downstream of Ras through association with MEK/ERK. ERK1,2 MAP kinases are involved in cell migration and proliferation in response to growth factors and ECM receptor activation [53]. There is an immense number of ERK/MAPK substrates, necessitating the presence of regulatory mechanisms that confer ERK1,2 with substrate and functional specificity [54]. Spatial resolution appears to be one of these regulatory mechanisms. For instance, nuclear accumulation of ERK takes place after mitogenic stimulation; moreover, pERK presence in the nucleus is required for ERK induced transformation [6-8]. It is clear that Rhamm plays a role in the regulation and stabilization of ERK1,2 activation [34, 48, 52, 55], however we are the first to show not only that Rhamm$^{Δ163}$/ERK1,2 interaction is required for Rhamm-dependent growth transformation (Figure 2.2 B,C), but also that Rhamm$^{Δ163}$ is responsible for the stable translocation of pERK into the nucleus (Figure 2.9). The importance of Rhamm-mediated trafficking of pERK to the nucleus in cellular transformation and cell migration in 10T1/2 cells is a subject of current study.
In accordance with previous studies, we demonstrated that the full length isoform of Rhamm preferentially binds to cytoskeletal proteins (Figure 2.7 A), which is at least partially responsible for its localization in the cytoplasm (Figure 3.7 B). Rhamm$^{FL}$ possesses two tubulin binding sites, located at the N-Terminus of the sequence between amino acids 129-149 (Rhamm$^{A163}$ lacks this binding site) and the other at amino acids 727-741 at the C-terminus (Figure 2.1 D). Site directed mutagenesis using GFP-RHAMM fusions [22, 26] and co-localization analysis of ZsGreen-Rhamm constructs indicate that the N-Terminal tubulin binding site is required for its fibrillar distribution. This enhanced association for tubulin and cytoskeletal proteins might serve as a reservoir for Rhamm whereby cleavage of its N-Terminus would make it available by releasing then the active $^{A163}$ form. Further studies are required to confirm this working model.

We were also able to demonstrate that our ZsGreen-Rhamm fusions displayed equivalent localization to that previously reported for wild type Rhamm. Briefly, we observed both isoforms of Rhamm in the cytoplasm of the cell, as well as in the nucleus decorating the mitotic spindle during cell division and at the cell surface in podosomes [22, 26-28]. This confirmed that the fusion of ZsGreen at the N-terminus and FLAG at the C-terminus of Rhamm does not affect its trafficking and subcellular localization (Figure 2.5 A).

The activation of the oncogenic properties of Rhamm by truncation is analogous to that of other proteins. This is the case of proteins like c-raf (-1) and Vav that get activated by removal of a portion of the full length protein [56, 57]. Gene rearrangement and recombination of the original sequence gives rise to the active form of c-raf (-1), suggesting that the truncation of the regulatory portion leads to c-raf (-1) activation. Furthermore, the Vav family of Rho GEFs is composed of three isoforms. Similar to
Rhamm, Vav 1 is transforming when over-expressed in fibroblasts and an N-terminal extension of the protein inhibits Vav interactions with its downstream GTPase substrates. The mechanism by which Rhamm<sup>Δ163</sup>, but not Rhamm<sup>FL</sup> is transforming is yet to be fully elucidated.

Fluorescence recovery after photobleaching (FRAP) analysis is an established method to assess intracellular protein motility dynamics [58-60]. Here we investigated whether different isoforms of ZsGreen tagged Rhamm differed significantly in their fluorescence recovery time after photobleaching. Indeed, it was evident that ZsGreen-Rhamm<sup>Δ163</sup> was more dynamic in the cytoplasm of Rhamm<sup>-/-</sup> MEFs as they presented higher fluorescence recovery when compared with Rhamm<sup>FL</sup>. In general, the fluorescence recovery index represents the repopulation of the bleached areas with the fluorescent protein present in the remaining, unbleached area of the cell. The surface diffusion velocity reflects the state of the labelled protein, with the key assumption being that unbound protein diffuses faster than bound protein. Therefore, these results can be explained by the stable association of Rhamm<sup>FL</sup> with rigid components of the cell, such as cytoskeletal tubulin, actin or even Dynein/dynactin-microtubules. Such associations have been shown previously with Rhamm [20, 22, 26]. It is possible that the second tubulin binding site in the Rhamm N-terminus makes it more likely to bind to either those cytoskeletal proteins or to other scaffolds, such as Filamin α (FLNA), Spectrin α erythrocytic 1 (SPTA1), Spectrin β non-erythrocytic 4 (SPNB4), or Actinin1 (ACTN1) that attach it to the cytoskeleton. Based on its increased mobility, it is likely that a substantial portion of Rhamm<sup>Δ163</sup> is free in the cytoplasm. This population of Rhamm<sup>Δ163</sup> may arise as a result of cleavage of Rhamm<sup>FL</sup>. This may help to explain its enhanced
oncogenic activity, which could be due to an increased ability to traffic to subcellular compartments that Rhamm$^{FL}$ does not have access to. Interestingly, when we transfected 10T1/2 cells with our ZsGreen-Rhamm constructs, the distribution of Rhamm$^{FL}$ was far less fibrillar than in Rhamm $^{-/-}$ MEFs (Supplemental figure 2.1A). 10T1/2 cells have higher expression of Rhamm$^{FL}$ [20] and it might be that endogenous Rhamm in these cells are binding with higher affinity to cytoskeletal proteins in the cells inhibiting interactions of ZsGreen-Rhamm$^{FL}$. In support of this hypothesis, FRAP experiments performed in this cell line demonstrated that Rhamm$^{FL}$ was almost as dynamic as Rhamm$^{A^{163}}$ (Supplemental figure 2.1B). Despite the fact that the two cell lines used for these experiments are mouse fibroblasts, they come from different genetic backgrounds. Our results lead us to think that ZsGreen-Rhamm$^{FL}$ is less likely to bind to tubulin (Supplemental figure 2.1C) and more available to bind to ERK in the cytoplasm of 10T1/2s (Supplemental figure 2.1D). This theory, although in its very early stages, agrees with our previous one that suggests that Rhamm$^{A^{163}}$ is free in the cytoplasm to bind to ERK and to target it to the nucleus of the cell, however differs as to the role of Rhamm$^{FL}$, whereby “free” ZsGreen-Rhamm$^{FL}$ co-localizes more with pERK in 10T1/2 cells than the tubulin bound ZsGreen-Rhamm$^{FL}$ in Rhamm $^{-/-}$ MEFs. We also speculated that dimerization of endogenous Rhamm ZsGreen-Rhamm$^{FL}$ was not responsible for this change in distribution because transfection of ZsGreen-Rhamm$^{FL}$ in Rhamm $^{-/-}$ that were stably expressing either Rhamm$^{FL}$ or Rhamm$^{A^{163}}$ did not influence the fibre-like appearance of Rhamm$^{FL}$ in this cell line to the extent of transfection into 10T1/2 cells (Supplemental figure 2.1B).
It is important to note that our MALDI-TOF MS analysis of Rhamm^{FL/Δ163} binding partners has several limitations. Cytoskeletal proteins are much more abundant than other intracellular proteins, and this bias in our assay may have obscured other potential RHAMM protein partners not only in the cytoplasm, but also in the nucleus and membrane of the cell. While further studies are required for us to come to a better understanding of the molecular interactions that make Rhamm^{Δ163} an oncoprotein, the present study provides some meaningful insight in the transformation process. In summary, we demonstrated that the expression of Rhamm^{Δ163} in mouse fibroblasts induced loss of contact inhibition of growth and resistance to anchorage-dependent programmed cell death. These phenomena can be explained by the presence of a feedback mechanism in which Rhamm^{Δ163} acts at the level of Ras and MEK/ERK, promoting activation of these substrates and inducing a cascade of events that result in cell transformation. In addition to Rhamm^{Δ163} interactions with ERK1,2, in the cytoplasm, this transforming isoform also promotes translocation of pERK1,2 to the nucleus and stabilizes it there for extended periods of time.

2.6 Conclusion

A naturally occurring Rhamm isoform with a 163 amino acid N-terminal truncation, in contrast to the full length protein, has oncogenic activity in mouse fibroblasts. This activity may be due in part to observed differences in cellular localization, a differential interaction with Ras and MEK/ERK in the cytoplasm, and by an increased ability to recruit pERK1,2 to the nucleus.
2.7 Acknowledgements

This study was supported by NSERC grant # R3170A09 to JDL and EAT. We thank Cornelia Tolg, Pat Telmer, Caitlin Ward, and Jailal Ablack for their valuable technical support and editorial input.

2.8 References


Chapter 3

CD151 Immunoreactivity Predicts Early Biochemical Failure and Metastasis in PCa

C. H. Martínez\textsuperscript{1,4*}, C. Vasquez\textsuperscript{1*}, S.M. Chan\textsuperscript{2}, V. Chalasani\textsuperscript{1,5}, J.A. Gomez-Lemus\textsuperscript{2}, A.K. Williams\textsuperscript{1}, J. Chin\textsuperscript{1}, A. Zijlstra\textsuperscript{3}, and J.D. Lewis\textsuperscript{1}

\textsuperscript{1} Translational PCa Research Group, London Regional Cancer Program, 790 Commissioners Rd. E, London, Ontario, Canada N6A 4L6
\textsuperscript{2} Department of Pathology, Schulich School of Medicine & Dentistry, The University of Western Ontario, 339 Windermere Road London, ON, Canada N6A 5A5
\textsuperscript{3} Vanderbilt University Department of Pathology, 1161 21st Ave. S., C-2104A MCN, Nashville, TN 37232-2561
\textsuperscript{4} Present address: Hospital Pablo Tobon Uribe. Calle 78B No. 69-240, Oficina 148 Medellin, Antioquia, Colombia
\textsuperscript{5} Present address: University of Sydney, 603/20 Bungan Street, Mona Vale, NSW, 2103, Australia

* These authors have contributed equally to this study and are listed in alphabetical order.

Keywords: CD151, PCa, Monoclonal Antibody 1A5, Metastasis, Biochemical failure, Biomarker.

Address correspondence to:
John D. Lewis
London Regional Cancer Program
790 Commissioners Road East
London, Ontario, Canada
N6A 4L6
Phone: 519 685 8600 x57194
Email: john.lewis@lhsc.on.ca

The corresponding author has had access to all data in the study and had the final responsibility for the decision to submit for publication.

Competing Interests: None
3.1 Abstract

Metastasis is the deadliest aspect of prostate cancer (PCa), and influences its prognosis and treatment. Current diagnostic methods fail to identify patients with occult metastases, highlighting the need for more effective prognostic indicators of disease progression. Through immunostaining of prostate tissues we evaluated the prognostic value of the metastasis blocking, anti-CD151 antibody 1A5 (mAb 1A5), in detecting PCa disease progression and metastasis. CD151 immunoreactivity was assessed by immunohistochemistry using mAb 1A5 in PCa tissue from 99 non-metastatic patients that underwent radical retropubic prostatectomy (RRP) and 38 biopsy samples from patients that did not undergo RRP but exhibited metastasis during their follow-up. To gain additional insight into the predictive value of mAb 1A5 compared to global CD151 expression, CD151 mRNA levels in PCa were evaluated using publically available microarrays. Immunoreactivity as assessed by mAb 1A5 was higher in malignant tissue than in benign tissue adjacent (p=0.01) or distant from the tumor (p<0.01). CD151 staining in the prostate luminal epithelium correlated positively with time to biochemical recurrence (p=0.023) in the RRP cohort. CD151 immunoreactivity in diagnostic biopsy samples was prognostic for the occurrence of overall metastasis (p<0.01), bone metastasis (p=0.01), and hormone resistance (p<0.01). Microarray analysis of PCa revealed no significant change in CD151 expression across clinical stage nor did it correlate with metastasis, disease relapse, or survival. Multivariate analysis indicates that CD151 immunoreactivity with mAb 1A5 is an independent prognostic marker that indicates significant risk of early metastasis. These results indicate that mAb 1A5 staining of CD151 correlates with biochemical recurrence and metastasis of PCa. The absence of a
similar correlation with CD151 mRNA suggests that epitope-specific CD151 immunoreactivity may serve as a biomarker to help identify PCa patients with a high risk for rapid progression.

3.2 Introduction

Prostate cancer (PCa) is the most common malignancy in North American men, accounting for 10% of all cancer-related deaths in 2010 [1]. With the ubiquitous use of prostate specific antigen (PSA) testing (normal <4ng/mL) and PCa screening programs, an increasing number of low grade early PCa are being diagnosed. Due to the low overall risk of these cancers developing into clinically-relevant disease, many patients opt for active surveillance rather than radiation or radical retropubic prostatectomy (RRP). In a significant number of these cases, disease progression can accelerate and pose an elevated risk that may have been avoided with earlier intervention [2]. The vast majority of cancer-related mortalities are due to metastatic disease [3].

Current diagnostic tools such as PSA, digital rectal examination (DRE), biopsy, Gleason score, TNM stage (Tumour, lymph Node invasion, and distant Metastasis), Partin’s nomogram and tables [4], and D’Amico’s risk stratification scheme [5] accurately predict PCa-specific mortality [6]. However, these tools do not accurately identify those cases that harbour occult metastatic disease [7-9]. PSA in particular is a valuable tool for disease diagnosis and the evaluation of treatment response, but it lacks the sensitivity and specificity necessary to predict metastasis [10-12]. The inability of PSA to determine disease progression is highlighted by the discrepancy between the lifetime risk of PCa
diagnosis (16%) and death from PCa (3-4%).[13-15] Although there is a well-established 
association between biochemical recurrence and PCa-specific death,[16, 17] it remains 
difficult to identify patients likely to experience biochemical recurrence by pathology or 
other currently available diagnostic techniques.[17]

CD151 (GP27, SFA-1, PETA-3) is one of 33 members in the mammalian Tetraspanin 
family and is characterized by four transmembrane domains. It is ubiquitously expressed, 
but is predominantly found in the plasma membrane of epithelial cells, endothelial cells, 
smooth muscle cells, and platelets.[18-21] Several related functions have been attributed 
to CD151, including platelet aggregation,[22] cell adhesion,[23] cell migration,[24, 25] 
tumour cell invasion and metastasis.[21, 26, 27] Its loss in humans and experimental 
mouse models leads to basement membrane failure, including skin blistering and renal 
failure.[28, 29] CD151 is frequently up-regulated in cancer.[21] Its expression is 
associated with poor outcome in several cancers, including esophageal cancer,[30] 
hepatic cancer,[31, 32] lung cancer,[33] and clear cell renal carcinoma[34]. While 
previous studies have shown that CD151 expression in PCa is a better prognostic 
indicator than Gleason grading,[35] a role for CD151 expression in the prediction of 
metastasis has not been explored.

We have shown previously that CD151-specific monoclonal antibody 1A5 (mAb 1A5) 
prevents metastasis by inhibiting tumour cell intravasation without affecting primary 
tumour growth, tumour cell arrest, extravasation, or growth at the secondary site.[27] In 
vivo, the unique ability of mAb 1A5 to strongly inhibit migration is due to enhanced 
tumour cell-matrix interactions. Because other CD151-specific antibodies have been 
described to promote tumour cell migration,[36, 37] we surmised that mAb 1A5 might
recognize an epitope of CD151 that is particularly relevant for PCa cell migration and metastasis. To test this, CD151 immunoreactivity was assessed by immunohistochemistry using mAb 1A5 in two cohorts, 99 cases from patients that underwent radical retropubic prostatectomy (RRP) and 38 biopsies from patients who exhibited metastasis during their follow-up but who did not undergo RRP.

Here, we show that CD151 immunoreactivity as determined using mAb 1A5 is a prognostic indicator of PCa disease progression and metastasis in RRP and diagnostic prostate biopsy specimens.

3.3 Methods

Patients and samples

A total of 137 patients with PCa were included in this retrospective study with a mean follow up of 12.1 years. The first cohort of 99 cases had undergone RRP between 1994-1998 at the London Health Sciences Centre with pathological stage pT2 - pT3 PCa (Tables 3.1 and 3.2). A second cohort of 38 cases was comprised of diagnostic biopsy specimens from patients at the same center who did not undergo RRP and developed metastatic disease detected during their follow up (Table 3.3). Pathological staging was re-evaluated according to the TNM staging regarding presence and size of prostate tumour, Lymph node metastasis and distant metastasis (AJCC 2002). Patient characteristics and disease-related outcomes were obtained from a review of the medical records (Tables 3.1-3.3). This study obtained approval from an independent local Ethics
Committee (UWO REB #15084E) (Appendices B-D) and obtained the informed consent from all patients for the research use of their tissues (Appendix A).

**Immunohistochemistry**

The mouse monoclonal antibody 1A5 specific for CD151 was generated using a subtractive immunization approach as previously described [38, 39]. 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, and incubated with pre-warmed pepsin (digest-all™ 3, Invitrogen) for 3 minutes. After washing with PBS-Triton-X, the specimens were stained using the UltraVision Detection System (Thermo-Scientific). CD151 mAb 1A5 was diluted 1:800 in 1% BSA in PBS, and CD151 mAb 11G5α (abcam) was diluted 1:100 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 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 and benign tissue adjacent and distant from areas of tumour. Biochemical recurrence after RRP was defined as two consecutive PSA measurements greater than 0.2ng/mL. Hormone resistance was defined as the progression of metastatic lesions and/or an increase in testosterone levels over original castration levels and/or significant PSA increase in at least two consecutive measurements while the patient was receiving hormone therapy. Bone metastasis was defined as presence of metastatic lesions on a bone scan.

**Statistical analysis**

All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC). The primary endpoint for the cohort of patients who had RRP was biochemical free survival; the primary endpoint for the biopsy cohort was metastasis free survival. Correlations between 1A5 immunoreactivity and biochemical free survival in malignant areas compared to benign areas were analyzed by McNemar’s chi-square test. Kaplan–Meier plots were used to assess the univariate and multivariate analysis of biochemical free survival and metastasis and groups comparison was made using the log-rank test. The Cox proportional hazard model was applied for multivariate analysis. In all statistical analyses, a two-sided P < 0.05 was considered statistically significant.

Gene expression profiling studies analyzed for the PCa data were obtained from the NCBI GEO. These included GDS2545 (n=171)[40], GDS1439 (n=19)[41], GSE3933 (n=112),[42], and GSE6099 (n=102)[43]. 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 publically available expression dataset GDS3113 (n=3/tissue)[44] to demonstrate relative levels of CD151 expression in prostate compared to other tissues.

Role of the funding source

The study sponsor (London Health Sciences Centre) and sources of funding (Canadian Cancer Society Research Institute and the US National Institutes of Health) had no role in the design of the study, collection, analysis, or interpretation of the data, or in writing the report, or the decision to publish the results. Interpretation of the data and the final content of this report were approved by all authors. The corresponding author had full access to the data and takes full responsibility in submitting the report for publication.

3.4 Results

Patients

For the RRP cohort, the patient characteristics, pathological and clinical outcomes are summarized in tables 3.1 and 3.2. The mean age of these patients at the time of diagnosis with PCa was 62.4 years ±5.7 SD, initial PSA median was 9.7ng/mL ±5.2 SD and median follow-up was 12.1 years ±1.6 SD. The clinical tumour stages (TNM staging) were as follows: 36.4% were classified as T1, 55.5% as T2, 2% as T3 and 6.1% as unknown. The pathological T stages were distributed as: 60.6% in pT2, 35.4% in pT3, and 4% in pT4. Gleason 8 or higher was recorded in 27.3% of the patients while 72.7% of patients had a score of 7 or less. Positive margins were noted in 23.2% of cases and 12% had positive
seminal vesicles at the time of the RRP, 35.4% developed biochemical failure, 4.2% metastatic disease and 1% hormone resistance.

<table>
<thead>
<tr>
<th>Variable</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.4 ± 5.7 SD</td>
</tr>
<tr>
<td>PSA (ng/mL)</td>
<td>9.7 ± 5.2 SD</td>
</tr>
<tr>
<td>Follow up (years)</td>
<td>12.1 ± 1.6 SD</td>
</tr>
<tr>
<td>Clinical Stage (%)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>36 (36.4%)</td>
</tr>
<tr>
<td>T2</td>
<td>55 (55.5%)</td>
</tr>
<tr>
<td>T3</td>
<td>2 (2.0%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (6.1%)</td>
</tr>
</tbody>
</table>

Table 3.1 Demographic and clinical staging information of patients that underwent RRP at the London Regional Cancer Program between 1994 and 1998 (n=99)
<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathological stage (%)</td>
<td>pT2 60 (60.6%)</td>
</tr>
<tr>
<td></td>
<td>pT3 35 (35.4%)</td>
</tr>
<tr>
<td></td>
<td>pT4 4 (4%)</td>
</tr>
<tr>
<td>Gleason (%)</td>
<td>≤6 26 (26.2%)</td>
</tr>
<tr>
<td></td>
<td>7 38 (38.3%)</td>
</tr>
<tr>
<td></td>
<td>≥8 27 (27.3%)</td>
</tr>
<tr>
<td></td>
<td>Unknown 8 (8.0%)</td>
</tr>
<tr>
<td>Margins (%)</td>
<td>Negative 76 (77.6%)</td>
</tr>
<tr>
<td></td>
<td>Positive 23 (23.2%)</td>
</tr>
<tr>
<td>Seminal vesicles (%)</td>
<td>Negative 87 (87.9%)</td>
</tr>
<tr>
<td></td>
<td>Positive 12 (12.1%)</td>
</tr>
<tr>
<td></td>
<td>Positive Nodes 0</td>
</tr>
<tr>
<td>Biochemical failure</td>
<td>35 (35.4%)</td>
</tr>
<tr>
<td>Hormone resistance</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>Solid organ metastasis</td>
<td>2 (2.1%)</td>
</tr>
</tbody>
</table>

Table 3.2 Pathological and clinical outcomes of patients that underwent RRP at the London Regional Cancer Program between 1994 and 1998 (n=99)
Table 3.3 summarizes the demographic information from the biopsy cohort of patients. Median age was 66.7 ± 9.76 SD years. From this group of patients, 15.7% were at clinical stage T1, 50% at T2, 26.3% at T3, and 2.6% at T4 at the time of diagnosis. Gleason 7 or lower was reported in 50% of the patients. Bone metastasis was present in 89.4% of patients with metastatic disease. 81.5% developed hormone resistance during their treatment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.7 ± 9.76 SD</td>
</tr>
<tr>
<td>Clinical Stage (%)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>6 (15.7%)</td>
</tr>
<tr>
<td>T2</td>
<td>19 (50%)</td>
</tr>
<tr>
<td>T3</td>
<td>10 (26.3%)</td>
</tr>
<tr>
<td>T4</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (5.2%)</td>
</tr>
<tr>
<td>Gleason</td>
<td></td>
</tr>
<tr>
<td>≤7</td>
<td>19 (50%)</td>
</tr>
<tr>
<td>≥8</td>
<td>19 (50%)</td>
</tr>
<tr>
<td>Hormone resistance</td>
<td>31 (81.5%)</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>34 (89.4%)</td>
</tr>
</tbody>
</table>

Table 3.3 Demographic and clinical staging information of patients that developed metastasis during follow up at the London Regional Cancer Program between 1994 and 1998 (n=38)
CD151 is expressed in basal epithelium of prostate tissue.

The mAb 1A5 was generated previously using a subtractive immunization approach that was designed to generate a library of function-blocking antibodies for metastasis. We first wanted to determine if mAb 1A5 could detect CD151 in paraffin embedded prostate tissue. To determine if mAb 1A5 staining is predictive of disease progression we compared immunostaining of mAb 1A5 with the commercially available mAb 11G5a. As expected, some immunoreactivity for both antibodies was observed in normal prostate epithelium and prostate adenocarcinoma (figure 3.1). However, mAb 1A5 staining was significantly higher in the tumor area (figure 3.1A) when compared with benign tissue adjacent to the tumor (p=0.01) (figure 3.1B) and benign tissue distant from the tumor (p<0.01) (figure 3.1C). In contrast to the immunoreactivity observed with mAb 1A5, mAb 11G5a detected CD151 expression in the membrane and cytoplasm of both benign prostate epithelium and tumor (figure 3.2, right). Moreover, mAb 11G5a signal was evident in fibroblasts (red arrow) and immune cells which are known to express CD151 but which are not stained by mAb 1A5 (not shown). These divergent staining patterns suggest that mAb 1A5 detects a distinct pool of CD151 that is present at elevated levels in the tumor.

CD151 staining on RRP samples is prognostic of biochemical recurrence

We then wanted to confirm if by staining patient samples with mAb 1A5 we could detect more aggressive subpopulations of cells and could predict patient prognosis. In order to
Figure 3.1 CD151 is expressed in basal epithelium of prostate tissue. CD151 immunoreactivity was detected by immunohistochemistry (IHC) using mAb 1A5 on formalin fixed, paraffin embedded primary tumors (RRP) stained following the HRRP/DAB method. CD151 staining was higher in tumor area (A) (20x) than in benign tissue close to the tumor (B) (p=0.01) and benign tissue distant from the tumor (C) (p<0.01) Scale bars left, 50 μm, right, 8 μm.
Figure 3.2. mAb 1A5 recognizes a different subpopulation of CD151. CD151 Immunoreactivity was detected by immunohistochemistry (IHC) using mAb 1A5 (A, left) Bar 50μm) and mAb 11G5a (A, right), on formalin fixed, paraffin embedded primary tumors (RRP) stained following the HRRP/DAB method. CD151 as detected by mAb 1A5 was expressed mostly in basal epithelium of prostate tumor tissue whereas mAb 11G5a detected different subpopulations of CD151. CD151 is present throughout the membrane and cytoplasm of prostate epithelium (black square), fibroblasts (red arrow). Figures 2B left are representative images of samples from patients that did not develop biochemical recurrence that were stained with mAb 1A5. Patient tissues on figures on the right were stained with mAb 11G5a.
do that we generated Kaplan-Meier curves by correlating Biochemical free survival data with CD151 immunoreactivity in epithelium of prostate tissue after RRP. Log-rank analysis of the biochemical free survival data of patients with PCa demonstrated a strong correlation between biochemical recurrence and positive mAb 1A5 staining of CD151 (p=0.023) (figure 3.3).

On the other hand, CD151 staining using mAb 1A5 in benign tissue adjacent to the tumor and benign tissue distant from the tumor failed to demonstrate association with biochemical recurrence. Moreover, 85% of the patients that had PSA recurrence were CD151 positive in the tumor area. In contrast, staining with 11G5a was not predictive of survival nor did it correlate with biochemical recurrence. This is represented in figure 3.2B for two patients who did not developed biochemical failure. Tumor tissue was stained with mAb 1A5 (left) with no significant detection of signal. However, serial tissue sections from the same patients stained with mAb 11G5a (right) showed strong CD151 immunoreactivity. Based on these data we can conclude that staining with mAb 1A5 may be useful in detecting those patients at risk of biochemical recurrence.

**CD151 immunoreactivity associates with earlier onset of metastasis and adds predictive value for hormone resistance**

After we found significant predictive value of mAb 1A5 in RRP specimens, we attempted to determine if this staining would be predictive of progression to metastasis. Biopsies were obtained from patients whom, at the time of PCa diagnosis did not undergo RRP,
and eventually developed metastasis identified at follow-up. In those biopsies, CD151 immunoreactivity positively correlated with overall metastasis ($p<0.01$), bone metastasis ($p=0.01$), and hormone resistance ($p<0.01$) (Figure 3.4). In fact, 76.3% of the patients with elevated CD151 staining on the diagnostic biopsies developed hormone resistance; 41.3% within the first three years of treatment.

![Log-rank p=0.023](image)

**Figure 3.3** CD151 staining on RRP samples is prognostic of biochemical recurrence. Kaplan-Meier curves were generated using the Biochemical free survival data against CD151 immunoreactivity in epithelium of prostate tissue after RRP. Log-rank analysis of the biochemical free survival data of patients with PCa showed correlation between PSA increase after RRP and positive staining for CD151 in tumor area ($p=0.023$).
Figure 3.4 CD151 immunoreactivity associates with earlier onset of metastasis. Kaplan-Meier survival curve performed with data from patients that developed metastasis during their follow-up at the London Regional Cancer Program between 1994 and 1998. Log-rank analysis of the survival curve shows association between CD151 immunoreactivity on diagnostic biopsy samples and earlier onset of metastasis (p=0.001).
Stepwise Cox proportional hazards regression was used to obtain a multivariate model. PSA (p<0.001) and CD151 immunoreactivity (p=0.009) entered the model. CD151 positive staining was still significant after adjusting for PSA levels showing that patients with high PSA (p<0.01) and CD151 staining (p<0.01) have the highest risk of developing metastatic disease. CD151 immunoreactivity demonstrated additional risk of earlier metastasis as an independent variable improving the model. For display purposes PSA was categorized as 20ng/mL or higher and lower than 20ng/mL, and was associated CD151 positive or negative immunoreactivity as detected by mAb 1A5 and the time to metastasis using the Kaplan-Meier technique (figure 3.5).

PCa expression microarray data was evaluated to confirm that the detection of CD151 by mAb 1A5 was due to the increased epitope availability and not due to increased gene expression. In comparison to other normal tissues, expression of CD151 in prostate tissue is greater than in testis, liver, and brain, but lower than in lung, breast, and heart (Supplemental figure 3.1). Three distinct datasets assessing CD151 expression in normal prostate and PCa were evaluated. Within these samples CD151 expression remained unchanged (figure 3.6 A-C). Expression analysis in epithelial cells by laser capture microdissection confirmed that the epithelial cells, in its progression to cancer cells did not significantly alter CD151 expression (figure 3.6D).
Figure 3.5 Patients with CD151 positive staining and high PSA have increased risk of developing metastasis. Kaplan Meier curves of CD151 expression and PSA in patients that developed metastatic disease during follow up at the London Regional Cancer Program between 1994 and 1998. Stepwise Cox proportional hazards regression was used to obtain a multivariable model. PSA (p<0.001) and CD151 immunoreactivity (p=0.009) entered the model.
Figure 3.6 Expression analysis of CD151 mRNA in PCa progression. Public microarray datasets were obtained through Gene Expression Omnibus (GEO) at The National Center for Biotechnology Information (NCBI). Normalized expression data obtained for CD151 was plotted for normal prostate, PCa, and metastatic prostate in panel A-C (GDS2545 (n=171) [40], GDS1439 (n=19) [41], and GSE3933 (n=112) [42]. An analysis of individual cell populations obtained by laser capture microdissection (panel D, GSE6099, n=102) [43] was used to confirm that the epithelial derived cancer did not alter expression of CD151 during progression.
3.5 Discussion

In this study we demonstrate that detection of a specific pool of CD151 in PCa tissue from RRP specimens is associated with earlier biochemical failure of patients. These early failures might represent localized disease at the time of RRP or a subset of patients that harbored unrecognized micrometastatic disease. CD151 expression, as detected by monoclonal antibody 1A5, may predict patients with elevated risk for failure and potentially could avoid the associated morbidity of RRP in patients without significant chance of cure. These early failures have been shown to respond poorly to local salvage with external beam radiation therapy presumably because early PSA failure is the result of occult micrometastatic disease. This emphasizes the need for a more accurate diagnostic tool to avoid overtreatment and to detect those who have an increased risk of metastatic disease and death. Especially because over treating patients is problematic. A recent European screening randomized trial confirmed that 48 patients need to be treated in order to save one life. The associated morbidity of treating men with low risk PCa creates significant undue stress on patients and is also a significant burden economically. Strategies like active surveillance have helped to reduce this morbidity, but accurate markers which can define high risk patients in the early stages of disease are lacking. Effectively tailoring patient treatment requires more accurate markers to identify those patients with an increased risk of developing biochemical failure, metastatic disease, hormone resistance, and death.

The findings from our study demonstrate that mAb 1A5 detects a pool of CD151 that is elevated in tumors, highlighting the ability of CD151 staining to predict early disease
recurrence. Therefore, CD151 detection in prostate adenocarcinoma tissue identifies patients at higher risk of biochemical failure, which may benefit from further treatments such as chemotherapy for presumed micrometastatic disease or pelvic and nodal radiation. This data can be made available both at the time of TRUS biopsy and immediately after surgery with the pathology report, allowing earlier intervention and avoiding unnecessary delays in higher risk patients.

In patients with metastatic disease detected during their follow-up, CD151 staining was independently associated with shorter metastasis free survival, and progression to bone metastasis and hormone independence, adding accuracy to the multivariate model. Whilst we demonstrated a clear biological rationale for the association of CD151 positivity with the development of metastasis, the link with castrate resistant disease is likely complex. We theorize that the ability of PCa to metastasize results in an increase in tumor mass allowing for subsequent alterations within the malignant cells androgen receptor culminating in a clonal population of androgen independent cells. Regardless of the mechanism, these data consistently suggest that CD151 immunoreactivity early in the disease is associated with worse prognosis. To further validate the functional predictive value of the 1A5 antibody, a significantly larger database should be assessed.

This, to the best of our knowledge, is the first study to use the 1A5 mAb to measure immunoreactivity of CD151 in human prostate tissue but it is consistent with the findings of others. Ang et al. showed association between CD151 staining and PCa progression.[35] They found a positive correlation between CD151 expression and cancer dedifferentiation, a hallmark indicator of disease progression, and patient death. Gleason
score association with recurrence was not strong enough to reach statistical significance in our cohort. This could result from antibody differences used to detect the levels of CD151. Previous CD151 studies used different antibodies that recognize intracellular epitopes of CD151. 1A5 mAb was produced by subtractive immunization [38] against an extracellular epitope of CD151 that was necessary for cancer cell metastasis in animal models.[27] It was shown that mAb 1A5 blocked CD151 induced cell migration and reduced spontaneous metastasis by more than 80%, demonstrating the importance of CD151 in metastasis. mAb 1A5 might specifically recognize a pool of CD151 that is relevant for clinical metastasis.

CD151 expression has been associated with metastasis and progression in other types of cancer. In aggressive mammary ductal carcinoma in situ, CD151 expression correlates with markers of progressive disease, including elevated Erk and c-Akt activation and increased cell proliferation [49]. Changes in CD151 expression are associated with poor prognosis of colon cancer patients [50]. In one of the most invasive forms of human cancer, pancreatic adenocarcinoma, there is an increased association of CD151 with integrin α6β4 that is thought to contribute to its aggressive nature [51]. The association of CD151 with integrins may play an important role in the dissemination of cancer cells. A major mechanism by which CD151 supports cancer progression is through integrin regulation, and CD151 has been found to regulate integrins containing either α3 [52] or α6 [53] subunits. Blocking CD151 function inhibits the metastasis of human epidermoid carcinoma and fibrosarcoma without influencing growth of the primary tumor cells [27]. Since CD151 is generally associated with the migratory phenotypes of many cancers, the
association of CD151 with aggressive forms of prostate cancer reported here is likely to have functional significance to disease progression.

This study is limited by the retrospective collection of the demographic and clinical data. Overall survival analysis was limited, despite a follow-up of 12.1 years ±1.6 SD, because there were only 2 deaths in the group of patients who underwent RRP. Another limitation is the number of patients in the group of biopsy specimens which can limit the interpretation of the results, however because of the significant findings we have decided to include them in this manuscript.

Further investigation is required into the role of CD151 in predicting metastatic variants of PCa. We have demonstrated the ability to reliably perform immunohistochemical analysis on prostate biopsy specimens that have been embedded in paraffin for significant periods of time. This suggests that not only can CD151 be used as tool to add to the initial treatment decision but could also be useful in assessing patients suitability to undergo potentially morbid salvage treatments.

In conclusion, the CD151 staining with mAb 1A5 is associated with earlier biochemical failure, metastasis and hormone resistance. In conjunction with PSA measurement, detection of CD151 adds significant prognostic value to predict biochemical failure and the outcome of patients with PCa and might help to stratify and treat patients accordingly.
3.6 Acknowledgements

This study was supported by CCSRI Grant #700537 to JDL and NIH/NCI grant #CA120711-01A1 and CA120711-01A1 to AZ. We gratefully recognise the patients who participated in this study, and all experiments were performed in accordance with the regulations and guidelines of the Research Ethics Board at the London Health Sciences Centre. We thank Amber Ablack and Carl Postenka for their valuable technical support, Kristin Kain for editorial input, and Larry Stitt for the statistical analysis.

3.7 References


Palmer T, Zijlstra A. CD151 UCSD Molecular Pages. 2011(doi:10.1038/mp.a004123.01).


Chapter 4

General discussion and conclusions

4.1 Thesis summary and Significance

Tumour cell migration has an enormous influence on cancer progression since it accounts for tumour dissemination to cause not only local recurrence, but also distant metastasis [1-4]. It is, therefore, not surprising that many researchers are focusing their efforts on dissecting in detail the mechanisms by which a cell gains the ability to productively move from a primary tumour to a distant site, the implications that this has for tumour metastasis, and, lastly, the therapeutic pathways that can be followed to prevent this phenomenon from occurring. In order to find any therapeutic agent to inhibit the cell’s ability to detach from the primary tumour and migrate towards a distant site, molecular microdissection of the mechanisms involved in this process is required.

Multiple attempts to develop potential therapeutic agents to reduce tumour cell migration have been made. In fact there are currently a number of clinical trials evaluating reagents that target cell motility [5-8]. However, due to the complex mechanisms involved and the high degree of specificity that they must exhibit in order to avoid off target effects this endeavour is very challenging. While some groups focus on developing new anti-migratory drugs as therapeutic reagents, other laboratories have concentrated on finding other applications, for instance, using signatures of motogenic proteins to predict the outcome of cancer patients [9-11]. Over the long term, the development of a deeper
knowledge of the molecular mechanisms of cancer cell migration will potentially translate into the clinics in the future.

Of particular interest for us are two proteins that have been demonstrated to have an effect on cell migration. The first is CD151 for which a mouse monoclonal antibody was proven to significantly inhibit cell migration away from the primary tumour and invasion of surrounding stroma. This inhibition resulted in significant reduction of spontaneous metastasis after injecting HEp3 cells in both a chicken embryo model and in SCID mice, along with experimental metastasis in a chicken embryo model [12]. Unfortunately, since CD151 is a ubiquitous protein and CD151 is expressed in platelets and endothelial cells, this antibody induced platelet aggregation in human blood making it unsuitable for cancer therapy (data not shown). Nevertheless, it is a prognostic biomarker for biochemical recurrence and metastasis in prostate cancer.

The second protein, Rhamm, is currently being used as a therapeutic target for AML and MM and has completed phase II clinical trials with minimal patient toxicity and favourable clinical results [13-15]. Under normal physiological conditions, RHAMM is not expressed in most human cells. However, during cancer progression, it is highly overexpressed and this expression is often linked with poor clinical outcome. This, added to the restricted expression of RHAMM during wound healing and tumour formation and progression, makes it a more specific therapeutic target for tumour treatment inducing less adverse side effects. However, to date, the molecular mechanisms by which one isoform of this protein, Rhamm<sup>A163</sup>, influences transformation and oncogenicity have not been fully established.
The aim of this study was to demonstrate the avenues by which seemingly different cell migration inducing proteins can be studied in order to help to achieve a deeper understanding of the transformation process and to predict the clinical outcome of cancer patients and therefore treat them accordingly, diminishing not only adverse effects, but also the onset of metastasis. We integrated the use of an anti-metastatic/anti-migratory monoclonal antibody to CD151, 1A5 [16], to predict disease recurrence and metastasis free survival in patients with prostate cancer. Additionally, we investigated the effect that two different isoforms of an oncoprotein with a significant effect on cell migration, Rhamm^{FL/A163}, have on subcellular localization and transforming events in mouse fibroblast cell lines.

In the third chapter of this thesis we demonstrate the ability of the mouse monoclonal antibody 1A5 to identify a subpopulation of CD151 present mostly in patients who developed biochemical recurrence after having radical retropubic prostatectomy (Figure 3.2) and in patients that developed metastasis during their follow up (Figure 3.3). Database scavenging for gene expression proved that CD151 mRNA and protein expression are not significantly different at any stage of prostate cancer progression starting from normal prostate to prostate cancer and metastasis (Figure 3.6). This indicates that in prostate cancer, as opposed to other cancers such as hepatocellular carcinoma [17], non-small cell lung carcinoma [18], and clear cell renal cell carcinoma [19], CD151 is not overexpressed [20-23] but there is rather a potentially mutated subgroup of this protein that confers cells with a more aggressive phenotype. Based on the fact that this antibody had an effect on the more migratory cells in vivo by inhibiting their ability to detach from the primary tumour, invade through surrounding tissue, and
migrate to a secondary site to cause metastasis [12], we hypothesized that mAb 1A5 was able to detect that subpopulation of CD151 that actually conferred the cells with migratory/metastatic abilities. After performing immunohistochemistry on paraffin embedded prostate cancer specimens, we demonstrated that the antibody detects CD151 in a subset of patients that later on developed biochemical recurrence in the RRP cohort and metastasis in the biopsy cohort (Figure 3.3). These findings are of pivotal importance since we were able to detect which patients are at higher risk of recurrence or metastasis. We suggest that CD151 immunoreactivity be assessed with this antibody in diagnostic biopsies to help clinicians to assess which patients are at higher risk of recurrence which will dictate the treatment options for the patient. The epitope targeted by this antibody, its dynamics of recognition, and the mechanisms by which this subgroup of CD151 associates with metastasis and biochemical recurrence are subjects of study by our group.

In a separate study described in chapter 2, we demonstrated that a naturally occurring isoform of Rhamm that lacks the first 163 N-Terminal amino acids is transforming, while RhammFL is not, indicating that Rhamm may be activated to its oncogenic potential by truncation of key inhibitory amino acids. Microdissection of the initial steps of the transforming mechanism of RhammA163 revealed that this isoform confers the cells with growth transformation potential (Figure 2.1). Here, we demonstrate that RhammA163 owes its transforming abilities to at least two mechanisms in 10T1/2 fibroblasts. The first mechanism is the ability to induce loss of contact inhibition of growth as detected by foci formation experiments and resistance to anchorage-dependent programmed cell death (anoikis) (Figure 2.1).
It is now well known that the process of transformation from normal to tumourigenic cells involves a cascade of events that are triggered by a combination of oncogene overexpression and inhibition or loss of tumour suppression [24, 25]). This cascade starts with simple mutations and progresses to the point where cell cycle regulation is no longer existent [26]. Hanahan et al, 2001 postulated six players in the cascade of events required for tumour progression. These are, “Self sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis” [25]. In this study, we show that Rhamm$^{\Delta 163}$ makes 10T1/2 fibroblasts insensitive to contact-dependent growth-inhibitory signals and able to evade anchorage dependent programmed cell death. This is not surprising since it has been postulated that Rhamm expression induces the loss or inactivation of tumour suppressor genes such as BRCA1 [27, 28], and p53 [29] resulting in the cell’s proliferation advantage. In addition, several scientists have documented the role of Rhamm in cell migration and in cell cycle regulation, demonstrating role of this protein in multiple steps of the tumourigenic transformation.

The second mechanism by which Rhamm induces transformation is associated with its subcellular compartmentalization. Here, we demonstrated that Rhamm$^{FL}$ is bound to cytoskeletal proteins whereas Rhamm$^{\Delta 163}$ has a much wider distribution and mobility within the cytoplasm (Figure 3.5). We postulated that similarly to c-raf(-1) and Vav [30, 31], Rhamm$^{FL}$ undergoes either transcriptional or post-translational modifications that give rise to a shorter, transforming form, Rhamm$^{\Delta 163}$. It may be that the full-length form needs to detach from these cytoskeletal proteins be able to traffic to key sites such as the nucleus.
4.2 Strengths and limitations

I was fortunate to have the opportunity to work in two labs that are located at the London Regional Cancer Program. This makes it possible for us to come into contact with patients and clinical oncologists, as well as, attendance to oncology grand rounds which help immensely to have a broader knowledge of the clinical implications and possibilities of our current research.

4.2.1 CD151 immunoreactivity predicts early biochemical failure and metastasis in prostate cancer.

This is the first study that has used the mAb 1A5 to assess immunoreactivity of CD151 in human prostate cancer. We strongly believe that this antibody is an asset due to its intrinsic properties. Contrary to many other antibodies [32], 1A5 was created by an unusual technique named subtractive immunization that allows for the production of antibodies specific to antigens present in highly metastatic cells and disregarding epitopes shared by metastatic and non-metastatic cells [16]. This confers the antibody with the ability to recognize a subpopulation of cells that are more aggressive, making it possible for us to predict which patients have more aggressive cancers. Moreover, studies performed by our group and others indicate that 1A5 recognizes specifically CD151 regardless of binding state or associated proteins [16, 33]. Other commercially available antibodies recognize CD151 only when it is in a binding complex with different integrins, which, although informative, may obscure the results and requires further interpretation [32].
In addition, the patients included in this study had an average of 12.1 years of follow-up. This is important in the context of prostate cancer since it is a very slow growing disease and the length of the surveillance on these patients is pivotal in detecting late disease recurrence and onset of metastasis. Comparison of our study with others positions us among the retrospective studies with the longest follow up [17-19, 34-37].

This study is limited by its retrospective nature. Furthermore, even though we initially aimed to compare the onset of metastasis in the cohort of patients that underwent RRP, only four patients in this group developed metastasis forcing us to include a second cohort of patients who developed metastasis during their follow-up and did not undergo RRP.

Our goal was to have a significantly higher number of patients included in our database, however, a number of patients did not give their informed consent (Appendix A). Other subjects were lost because at the time of sectioning the tissue samples for our study, the portion of tissue with tumour was very small and had been used almost completely for either diagnosis or staging. Finally, another group of patients were diagnosed at different hospitals making it very difficult for us to have access to the tissues. Due to the above mentioned reasons we were forced to exclude a significant percentage of subjects from this study.

4.2.2 Rhamm compartmentalization and its functions

This is a novel study because it not only is the first to compare the full length version of Rhamm with an isoform that has been proven to be transforming and induce metastasis [38], but also because we are the only group that possesses a Rhamm -/- cell line that allowed us to identify individual characteristics of each isoform of Rhamm in terms of
function and subcellular localization. Despite the fact that this is a small study, we were able to demonstrate, at least partially, the mechanisms that make Rhamm\textsuperscript{163} oncogenic. This study, along with others published by our group, link Rhamm with many of the steps required for tumour progression. Namely, we have demonstrated that Rhamm contributes to loss of contact inhibition of growth, resistance to cell anchorage dependent programmed cell death, enhanced cell migration and invasion [38-42], and regulation of cell division by controlling mitotic spindle stability [43-45]. It is evident that this protein is an important player in the oncogenic process.

Because Rhamm is a coiled coil protein [46], the generation of specific antibodies can be difficult. At present, we are working on developing new monoclonal antibodies specific for Rhamm. A delay in the production of such reagents significantly slowed the data acquisition for my project. To overcome this problem, I developed this model in which I fused ZsGreen-Rhamm-FLAG tag for visualization purposes. Even though the system met our goal, making stably transfected ZsGreen-Rhamm-FLAG expressing cells was impossible since they lost their fluorescence overtime, making it very difficult for us to control which cells were still expressing our construct. Furthermore, in accordance with previous reports, our ZsGreen-Rhamm construct induced a blockage in the cell cycle making it impossible for us to perform cell proliferation studies. We hypothesize that the expression of Rhamm under the CMV promoter, which is very efficient and drives high protein production levels in the cell, negatively impacts cell cycle progression. Both overexpression and underexpression of Rhamm have previously been shown by our laboratory to induce genomic instability and apoptosis in the former case. Our lab is
currently developing a system under the control of a β actin promoter that has proven to be less toxic for our cells.

4.3 Future studies
The results obtained from these two studies open many possibilities for us to pursue in the future.

4.3.1 CD151 immunoreactivity predicts early biochemical failure and metastasis in prostate cancer.
Arrangements are being made to assess the immunoreactivity of CD151 in a significantly higher number prostate cancer tissue from both non-metastatic and metastatic patients. If the results of this new study are similar to those found here, we plan to take advantage of our strategic position at the London Regional Cancer Program to propose a prospective project in which CD151 immunoreactivity is assessed at the time of diagnosis to help physicians plan treatment options and then measure disease recurrence, onset of metastasis and reduction in morbidity and adverse effects of unnecessary therapeutic approaches. We are currently in the process of obtaining tissue samples from patients that underwent high intensity focussed ultrasound (HIFU) and cryotherapy as treatment for local radio-recurrent prostate cancer and that exhibited cancer recurrence after 6-8 months of treatment. CD151 immunoreactivity will be assessed and the results will be analysed to determine if there is any correlation between local recurrence and expression of the protein in this cohort.
Additionally, we are planning on exploiting the potential of our 1A5 antibody to assess the presence and significance of CD151 expressing tumour microparticles in patients with prostate cancer.

4.3.2 Rhamm compartmentalization and its functions

The results obtained from the ZsGreen-Rhamm constructs open the road for many opportunities. The first step that our group is taking is the creation of these constructs under the control of the β actin promoter in order to create stable cell lines expressing the construct. Also, interaction between the two isoforms can be evidenced in part by fusing one of them to a different fluorescent protein such as m-cherry or td-tomato. We also plan to use the stable cell lines to observe the dynamics of these isoforms in vivo in a wound healing environment using the chicken embryo model. The same model can also be used to assess if RhammA163 has an effect on tumour growth arrest after injection, intra/extravasation, proliferation and metastasis in an experimental metastasis assay.

In addition, experiments using a nuclear export signal (NES) to constitutively direct pERK out of the nuclei of the cell are currently being performed. The aim is to further confirm that RhammA163 not only favours pERK translocation to this compartment, but also induces its retention there promoting cell transformation.

Additionally ongoing are scratch wound assays to compare the migratory capacity that the two isoforms confer to our Rhamm-/- MEFs.
Results of these experiments will help to further elucidate the molecular mechanisms by which Rhamm$^{163}$ exerts its oncogenic functions.

4.4 References


Supplemental information

Supplemental figure 2.1 ZsGreen-Rhamm isoforms behave differently in 10T1/2 fibroblasts than in Rhamm -/- cells. A) Confocal images of 10T1/2 cells transfected with ZsGreen RhammFL or ZsGreen-RhammA163. B) Fluorescence Recovery After Photobleaching (FRAP) analysis was performed in Rhamm -/- MEFs transfected with ZsGreen-RhammFL/A163 fusion. C) Pearsons correlation of co-localization of Rhamm -/- MEFs transfected with ZsG-RhammFL/A163 fusion and stained using mAbs to α and β tubulin and pERK (D). For analysis, four regions of interest were delineated per cell on a total of 6 cells per group.
Supplemental figure 2.2 ERK activation does not affect the distribution of ZsG-Rhamm\textsuperscript{FL/Δ163} fusion. A) Confocal imaging was performed after transfection of ZsG-Rhamm\textsuperscript{FL/Δ163} fusion on Rhamm KO MEFs that were previously stably transfected with constitutively active MEK. B) Rhamm basal expression does not affect the distribution of ZsG-Rhamm\textsuperscript{FL/Δ163} fusion. Confocal imaging was performed after transfection of ZsG-Rhamm\textsuperscript{FL/Δ163} fusion on Rhamm KO MEFs that were previously stably transfected with either isoform of Rhamm. Scale bars, 20 μm.
Supplemental figure 3.1 CD151 expression in adult tissues. CD151 expression levels were extracted from publically available expression dataset GDS3113 (n=3/tissue, Dezso ref) to demonstrate relative levels of CD151 expression in prostate compared to other tissues.
Appendices

CD151 immunoreactivity predicts biochemical failure and metastasis in prostate cancer

Appendix A: Format for phone informed Consent

Local Principal Investigator:  Dr. J. Chin MD, FRCSC

London Health Sciences Centre

PURPOSE

You have been invited to participate in a research study because you have previously been diagnosed with prostate cancer and have had a biopsy to confirm this. You may also have had a radical prostatectomy (total removal of your prostate) or a trans-urethral prostatectomy.

The purpose of this study is to examine your previously removed tissue from one of the methods described above and check it for the presence of a protein marker called CD 151.

Previous studies have shown that this protein may be a superior prognostic indicator for overall survival. Developing this kind of markers we will use the specimens also for future but presently unidentified markers. This may help us to understand the behavior of the prostate tumors.

PROCEDURES

If you agree to participate in the study please indicate so to the person that is calling you.

By agreeing, you are permitting us to access your medical records for this research purposes.
BENEFITS

There will be no benefit to you from participating in this trial. The information collected from your participation may assist us in treating patients with prostate cancer in the future.

RISKS

There are no known risks associated with this study.

CONFIDENTIALITY

All information collected in this study will be kept strictly confidential. A study number will code your research records.

COMPENSATION (MEDICAL/FINANCIAL) TO BE PROVIDED

There is no payment to you for your participation in the study. By giving consent you are in no way waiving your legal rights or releasing the investigator from his legal and professional responsibilities.

OTHER INFORMATION

If you have any concerns regarding this study you can contact the Principal Investigator, Dr. J. Chin at [Contact Information] or the Urology Research Office at [Contact Information]. For questions concerning your rights as a research subject you may contact Dr. David Hill, Scientific Director, Lawson Health Research Institute at [Contact Information].
LAWSON HEALTH RESEARCH INSTITUTE

CLINICAL RESEARCH IMPACT COMMITTEE

RESEARCH OFFICE REVIEW NO.: R-08-227

PROJECT TITLE: CD151 as a prognostic factor in prostate cancer

PRINCIPAL INVESTIGATOR: Dr. J Chin

DATE OF REVIEW BY CRIC: October 21, 2008

Health Sciences REB#: 15084E

Please be advised that the above project was reviewed by the Clinical Research Impact Committee and the project:

   Was Approved

PLEASE INFORM THE APPROPRIATE NURSING UNITS, LABORATORIES, ETC. BEFORE STARTING THIS PROTOCOL. THE RESEARCH OFFICE NUMBER MUST BE USED WHEN COMMUNICATING WITH THESE AREAS.

Dr. David Hill
V.P. Research
Lawson Health Research Institute

All future correspondence concerning this study should include the Research Office Review Number and should be directed to Sherry Paiva, Room C210, Nurses Residence, South Street Campus.

cc: Administration
Appendix C Ethics approval

Office of Research Ethics
The University of Western Ontario
Room 4180 Support Services Building, London, ON Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 851-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethrcs

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. J.L. Chin
Review Number: 15084E
Review Date: April 23, 2008
Protocol Title: CD151 as a prognostic factor in prostate cancer
Department and Institution: Urology, London Health Sciences Centre
Sponsor:
Ethics Approval Date: September 18, 2008
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent (V2 Sept 9, 2008)
Documents Received for information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada ICH Good Clinical Practice Practices: Consolidated Guidelines, and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REBs as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information-consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participants and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information

☐ Janice Sutherland (sutherland@uwo.ca)
☐ Elizabeth Wambolt (ewambolt@uwo.ca)
☐ Grace Kelly (grace.kelly@uwo.ca)
☐ Denise Grafton (digrants@uwo.ca)

This is an official document. Please retain the original in your files.
Appendix D Ethics approval renewal

Office of Research Ethics
The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone (519) 661-3395 Fax (519) 661-3485 Email: ethics@uwo.ca
Website: www.uwo.ca/researchethics.

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. J. L. Chin
Review Number: 15044E
Revision Number: 1
Review Date: August 18, 2009
Revision Date: Expedited
Protocol Title: CD151 as a prognostic factor in prostate cancer
Department and Institution: Urology, London Health Sciences Centre.
Sponsor:
Ethics Approval Date: August 18, 2009
Expired Date: August 31, 2010
Documents Reviewed and Approved: Revised Study End Date
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and the Health Canada/ICCH Good Clinical Practice Practices: Consolidated Guidelines and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REBs as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above, assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time, you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB. The protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g., change of monitor, telephone number). Expedited review of minor changes in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
Dr. J. Chin
Urology Department, C3-120
London Health Sciences Centre
800 Commissioners Road E.
London, ON N6A 4G5

Dear Dr. Chin:

The Tissue and Archives committee has approved your application for tissue for biomedical research, pending receipt of a copy of your University of Western Ontario Research Ethics Board Approval, under the title:

TA # 462 - CD151 as a prognostic factor in prostate cancer.

Your request for tissue has been given the Tissue Application # 462. Please refer to this number in any future correspondence.

The Research Assistant has been advised that this protocol has been approved with a 3 section limit per patient case where there is adequate tissue available and will aid you hereafter in obtaining the requested materials.

Relevant technical charges will be billed directly to your office. Please see the enclosed cost estimate. For funded research, payment is expected in full at your earliest convenience. Please make certain that the appropriate funds have been reserved for this.

It is London Health Sciences Centre policy not to release original pathology material (slides or blocks).

It is the responsibility of each investigator to maintain the new standards of privacy legislation.

Any publications should include acknowledgment of the Department of Pathology, London Health Sciences Centre.

Yours sincerely,

M. Moussa, MD, FRCPC
Co-Chair, Tissue and Archive Committee

cc. Dr. J. Gomez-Lemus, Pathologist
Dr. Carlos H. Martinez
Jeanette Botz, Research Assistant
Cathie Crukey, Manager Pathology Laboratories

Telephone: (519) 663-3375 • Fax: (519) 663-2930 • karen.mackie@lhsc.on.ca

A Joint Venture of London Health Sciences Centre and St. Joseph's Health Care London