LEBANESE AMERICAN UNIVERSITY

The Role of StarD13 in Breast Cancer Cell Proliferation and Motility

By

Samer J. Hanna

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

School of Arts and Sciences

June 2012

LEBANESE AMERICAN UNIVERSITY
School of Arts and Sciences- Byblos Campus

Thesis Proposal Form

Name of Student: Samer Hanna I.D.#: 200904037

Division/Dept: Natural Sciences Department

On 01/06/12: Has presented a thesis proposal entitled:

The Role of StarD13 in Breast Cancer Cell Proliferation and Motility

In the presence of the committee members and Thesis Advisor:

Advisor: Dr. Mirvat El-Sibai Signature: [redacted]

Proposal approved on: 26/09/2011

Comments/Remarks/Conditions to Proposal Approval:

Date: 27/9/11 Acknowledged by: [redacted]
(Dean, School of Arts and Sciences)

cc: Chair
Advisor
Student
File Graduate Studies

LEBANESE AMERICAN UNIVERSITY
School of Natural Sciences- Byblos Campus

Thesis Defense Result Form

Name of student: Samer Hanna I.D.#: 200904037

On: June 1, 2012 has defended a Thesis entitled:

The Role of StarD13 in Breast Cancer Cell Proliferation and Motility

In the presence of the following Committee members:

Advisor : Dr. Mirvat El-Sibai

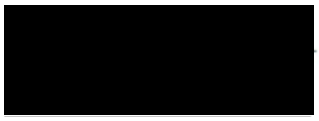
Committee Member: Dr. Sandra Rizk-Jamati

Committee Member: Dr. Roy Khalaf

The student has passed X not passed _____ the Thesis defense in partial fulfillment of the requirements of the degree of MS in Molecular Biology

Comments/Required Changes to Thesis due on: June 15 2012
the student did all the changes recommended by the Committee members

Advisor's report on completion of above Thesis conditions:

Changes Approved by Thesis Advisor: Mirvat El-Sibai Signature: 

Date: 7/6/12 Acknowledged by: 
(Dean, School of Arts and Sciences)

Cc: Registrar
Advisor
File Graduate Studies

Date of the Thesis defense public announcement: 18/05/2012

LEBANESE AMERICAN UNIVERSITY
School of Arts and Sciences- Byblos Campus

Thesis Approval Form

Name of Student :	Samer Hanna		I.D.#: 200904037
Thesis Title	:	**The Role of StarD13 in Breast Cancer Cell Proliferation and Motility**	
Program	:	Masters of Science in Molecular Biology	
Division/Dept	:	Natural Sciences Department	
School	:	**School of Arts and Sciences**	
Approved by	:	[redacted]	[redacted]
Thesis Advisor	:	Dr. Mirvat El-Sibai	
Member	:	Dr. Sandra Rizk-Jamati	
Member	:	Dr. Roy Khalaf	

Date : 7/6/2012

THESIS COPYRIGHT RELEASE FORM

LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants to Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic format and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Samer J. Hanna

Signature: 

Date: June 1, 2012

PLAIGARISM POLICY COMPLIANCE STATEMENT

I certify that I have read and understood LAU's Plagiarism Policy. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me. This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Samer J. Hanna

Signature:

Date: June 1, 2012

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest and most sincere gratitude to Dr. Mirvat El-Sibai, without whom none of this would have happened. I consider myself extremely lucky to have had the opportunity to work with such a talented and candid scientist. Throughout all this time she has shown me continuous support, encouragement, and dedication. I thank Dr. Mirvat for her continuous belief in my potentials and most importantly for being such a wonderful friend.

I would also like to thank Dr. Sandra Rizk-Jamati and Dr. Roy Khalaf for their continuous support and encouragement throughout my thesis work. Also, their participation in my thesis committee was highly appreciated.

I would also like to convey my appreciation to the Dean of Arts and Sciences Dr. Phillippe Frossard, the Chairperson of the Arts and Sciences department Dr. Costantine Daher, the faculty members Dr. Sima Tokajian, Dr. Brigitte Wex and Dr. Ralph Abi-Habib.

I thank Dr. Selim Nasser from CMC and Dr. Najla Fakhreddine from Hammoud Hospital for providing us with the tissues.

Finally a special thanks goes to my family and friends without whom none of this would have been possible.

The Role of StarD13 in Breast Cancer Cell Proliferation and Motility

Samer J. Hanna

ABSTRACT

Breast cancer is one of the most commonly diagnosed cancers in women around the world. In general, the more aggressive the tumor, the more rapidly it grows and the more likely it metastasizes. Cell migration, is a complex process, which requires the dynamic regulation of actin cytoskeleton. Members of the Rho subfamily of small GTP-binding proteins (GTPases) play a central role in breast cancer cell motility. The switch between active GTP-bound and inactive GDP-bound state is regulated by Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and Guanine-nucleotide dissociation inhibitors (GDIs). Here we study the role of StarD13, a newly identified Rho-GAP that specifically inhibits the function of RhoA and Cdc42. We aim to investigate its role in breast cancer proliferation and metastasis. The level of expression of this Rho-GAP in tumor tissues of different grades is assayed using immunohistochemistry. Moreover, the role of StarD13 in breast cancer cell lines is studied using two approaches. StarD13 is overexpressed using a StarD13-GFP construct, in the second approach StarD13 is knocked down using a specific siRNA. The effect on the activity of Rho-GTPases is observed using pull down activation assay, which confirmed StarD13 as a negative regulator for Rho and Cdc42 but not for Rac. Our results also showed that StarD13 plays a negative role in cellular proliferation. Moreover to investigate the role of StarD13 in cell motility, StarD13 knock down resulted in an inhibition of cell motility and cells were not able to detach their tail and move forward. Being a Rho-GAP and localizing to focal adhesions, we hypothesize that StarD13 is inhibiting Rho to allow the formation of Rac-dependent focal complexes and the detachment of focal adhesions for the cells to move forward. However, our results show that the knockdown of StarD13 seems to promote breast cancer cell invasion *in vitro*.

Keywords: StarD13, Breast Cancer, Cell proliferation, Cell motility, Focal Adhesions, Cell invasion.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

DCIS: Ductal carcinoma in situ

LCIS: Lobular carcinoma in situ

IBC: Inflammatory breast cancer

BRCA1: Breast cancer gene 1

BRCA2: Breast cancer gene 2

ATM: Ataxia telangienctasia mutated

TP53: Tumor protein 53

ARLTS1: ADP-ribosylation factor-like tumor suppressor gene 1

ER: Estrogen receptor

PR: Progesterone receptor

HER2: Human epidermal growth receptor 2

VEGF: Vascular endothelial growth factor

EGFR: Epidermal growth factor receptor

GPCR: G-protein-coupled receptor

NF-κB: Nuclear factor κB

IKK: Inhibitor of NF-κB kinase

TRAIL: Tumor necrosis factor-related apoptosis inducing ligand

VEGFR: Vascular endothelial growth factor receptor

G1: Gap phase 1

G2: Gap phase 2

S: Synthesis phase

M: Mitosis phase

G0: Gap 0

CDK: Cyclin-dependent kinase

PI3-K: Phosphatidylinositol 3-kinase

RTK: Receptor tyrosine kinase

PIP3: phosphatidylinositol (3,4,5)-triphosphate

PIP2: Phosphatidyl 4,5-bisphosphate

PH: Pleckstrin homology

PDK1: Phosphoinositide-dependent kinase-1

mTOR: Mammalian target of rapamycin

Akt: Acutely transforming retrovirus

Bcl-2: B-cell lymphoma 2

Bad: Bcl-2 associated death promoter

Bax: Bcl-2 associated x protein

PARP: Poly-ADP-ribose polymerase

Ras: Rat sarcoma

MAPK: Mitogen-activated protein kinase

mdm2: Mouse double minute 2

pRb: Retinoblastoma protein

GTPase: Guanosine triphosphatase

ECM: Extracellular matrix

MMP: Matrix metalloproteinases

TIMP: Tissue inhibitor of metalloproteinase

Arp2/3: Actin-related protein 2/3

N-WASP: Neural wiskott-aldrich syndrome protein

MT1-MMP: Membrane-type 1 matrix metalloproteinase

LIMK: LIM kinase

TESK: TES kinase

WASP: Wiskott-aldrich syndrome protein

SCAR: Suppressor of cyclic AMP

WAVE: WASP-family verprolin-homologous

VCA: Verprolin homology

EMT: Epithelial to mesenchymal transition

MET: Mesenchymal to epithelial transition

Rho: Ras homologous member

Rac: Ras-related C3 botulinum toxin substrate

Cdc42: Cell division cycle 42

GDP: Guanosine diphosphate

GTP: Guanosine triphosphate

GEF: Guanine Nucleotide Exchange Factor

GAP: GTPase Activating Protein

GDI: Guanine Nucleotide Dissociation Inhibitor

DH: Dbl homology

SH3: Src homology 3

LPA: Lysophosphatidic acid

PDGF: Platelet-derived growth factor

EGF: Epidermal growth factor

Sos: Son of sevenless

Src: Schmidt-Ruppin A-2

Syk: Spleen tyrosine kinase

FAK: Focal adhesion kinase

p130Cas: Crk-associated substrate protein 130

DOCK180: Dedicator of cytokinesis 180

PKL: Paxillin kinase linker

PIX: p21 kinase-interacting exchange factor

RhoA: Ras homologous member A

ROCK: Rho-associated coiled coil-containing protein kinase

MLC: Myosin light chain

MBS: Myosin binding subunit

mDia: Mammalian homolog of diaphanous

ERM: Ezrin-radixin-moesin

PAK: p21 activating kinase

CRIB: Cdc42/Rac interactive binding

MLCK: Myosin Light Chain Kinase

ADF: Actin depolymerization factor

PIP5-Kinase: Phosphatidylinositol-4-phosphate 5-kinase

GIT: G protein-coupled receptor kinase-interacting

C3T: C3 transferase

ATF-2: Activating transcription factor 2

FTI: Farsenalytrasferase inhibitor

DLC2: Deleted in liver cancer 2

StarD13: steriodogenic acute regulatory protein-related lipid transfer domain-containing protein 13

START: STAR-related lipid transfer

DLC1: Deleted in liver cancer 1

FAT: Focal adhesion targeting

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

siRNA: Small interfering Ribonucleic Acid

GFP: Green Fluorescent Protein

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PVDF: Polyvinylidene fluoride

PBS: Phosphate Buffered Saline

ECL: Enhanced Chemiluminescence

RT-PCR: Reverse Transcription Polymerase Chain Reaction

WST-1: Water-soluble tetrazolium salt

BSA: Bovine Serum Albumin

GST: Glutathione S-transferase

RBD: Rhotekin binding domain

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ROI: Region of interest

Chapter I

LITERATURE REVIEW

1.1. Breast Cancer

1.1.1. Introduction

Breast cancer is one of the most commonly diagnosed cancers in women around the world. Ductal and lobular carcinomas are the two most frequent types of breast cancer. They can be either noninvasive referred to as in situ carcinoma, or invasive infiltrating carcinoma (Jiang, Enomoto, & Takahashi, 2009). The most important factor in predicting patient outcome is the stage of the disease. In general, the more aggressive the tumor, the more rapidly it grows and the more likely it metastasizes. However, there are many small breast cancers with a highly aggressive behavior that remain undertreated because there is no marker capable of identifying them (Kleer, et al., 2002).

1.1.2. Classification of Breast Cancer

According to the US National Cancer Institute, breast cancer can be classified into five progressive stages. Stage 0 is referred to as carcinoma in situ, which can be either ductal carcinoma in situ (DCIS) or Lobular carcinoma in situ (LCIS). DCIS may become invasive in later stages of the tumor and spread to other tissues (Bombonati & Sgroi, 2011; Claus, Stowe, & Carter, 2001). Invasive breast carcinoma can be classified into progressive stages I through IV depending on its size and presence or absence at secondary sites, mainly lymph nodes. Inflammatory breast cancer (IBC) is a highly aggressive form of cancer that has disseminated to the dermal lymphatic systems reaching distant organs (van Golen, 2003).

1.1.3. Predisposition

It is estimated that about 10% of breast cancer cases are associated with family history (Lacroix, Toillon, & Leclercq, 2004; Ripperger, Gadzicki, Meindl, & Schlegelberger, 2009). The two major high penetrance breast cancer susceptibility genes are BRCA1 and BRCA2. Individuals with mutations in these two genes are at high risk to develop breast, ovarian and other cancers. The risk estimates for developing breast cancer range from 20 to 40% and the age of diagnosis is much younger in comparison to sporadic cases (Bordeleau, et al., 2011; Lacroix, et al., 2004). The human genome is typically very stable and resists many genetic alterations required for cancer development. However, the increase in the rate of mutation causes genetic instability, which is characteristic of BRCA gene abnormalities. Both BRCA1 and BRCA2 are relatively long genes capable of accumulating hundreds of different mutations that can alter the function of their corresponding proteins that are involved in the loss of the DNA repair (Bordeleau, et al., 2011; Lacroix, et al., 2004; Ripperger, et al., 2009).

In addition, there are several other predisposing susceptibility genes associated with an increased breast cancer risk. Such genes include ATM, TP53 and ARLTS1, which account only for a small portion of familial breast cancers (Ripperger, et al., 2009).

1.1.4. Tumor diagnosis and treatment

Breast cancer prevalence increased dramatically throughout the past few years. However patients' outcomes have improved due to the progress in major aspects of tumor diagnosis and treatment. Standard treatments include surgery, chemotherapy, radiation therapy, hormonal therapy and most recently targeted therapy (Stebbing, et al., 2012).

Targeting the pathways that are involved in promoting and sustaining growth and invasion of cancer cells is critical to achieve effective treatment of breast cancer (Schlotter, Vogt, Allgayer, & Brandt, 2008). Three major prognostic markers are routinely used to help define breast cancer therapy: Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth receptor (HER2). Recently, intensive effort has been employed in targeting key molecular markers involved in

tumor progression and prognosis in an attempt of developing practical, clinically relevant and effective treatment of patients (Hicks, 2012).

1.1.4.1.Molecular targeted therapy

During the past 25 years, targeting the estrogen receptor (ER) has been the most widely used molecular therapy approach in effectively curing and preventing breast cancer. Tamoxifen, a selective ER modulator is responsible for major improvements in survival rates and quality of life.

Other important biomarkers for targeted therapy include human epidermal growth receptor (HER2) and vascular endothelial growth factor (VEGF). Trastuzumb and bevacizumab have demonstrated high specificity and have been shown to improve response rates and survival of breast cancer patients. Moreover, when used in combination with chemotherapy these dugs have been shown to significantly reduce the risk of recurrence in early stages (Hicks, 2012; Schlotter, et al., 2008).

1.1.4.2. Signaling pathways as molecular therapy targets

In attempt to circumvent the resistance to both endocrine and cytostatic treatments currently in use, treatment with growth factor pathway inhibitors and their downstream effector signaling pathways is being implemented. Such approaches may push towards radical improvements in the current therapeutic strategies for breast cancer patients. Such targeted therapies opt to inhibit cell cycle progression, signal transduction, angiogenesis and induce apoptosis or cell death (Figure 1) (Schlotter, et al., 2008)

Breast cancer treatment targeting cell signaling pathways include: epidermal growth factor receptor (EGFR), G-protein-coupled receptor (GPCR), inhibitor of NF-κB kinase (IKK) tumor necrosis factor-related apoptosis inducing ligand (TRAIL), vascular epidermal growth factor receptor (VEGFR) and many others (Figure 1). Ultimately, such treatments result in modulating these critical cell-signaling pathways involved in cell survival, proliferation and motility (Hicks, 2012; Schlotter, et al., 2008).

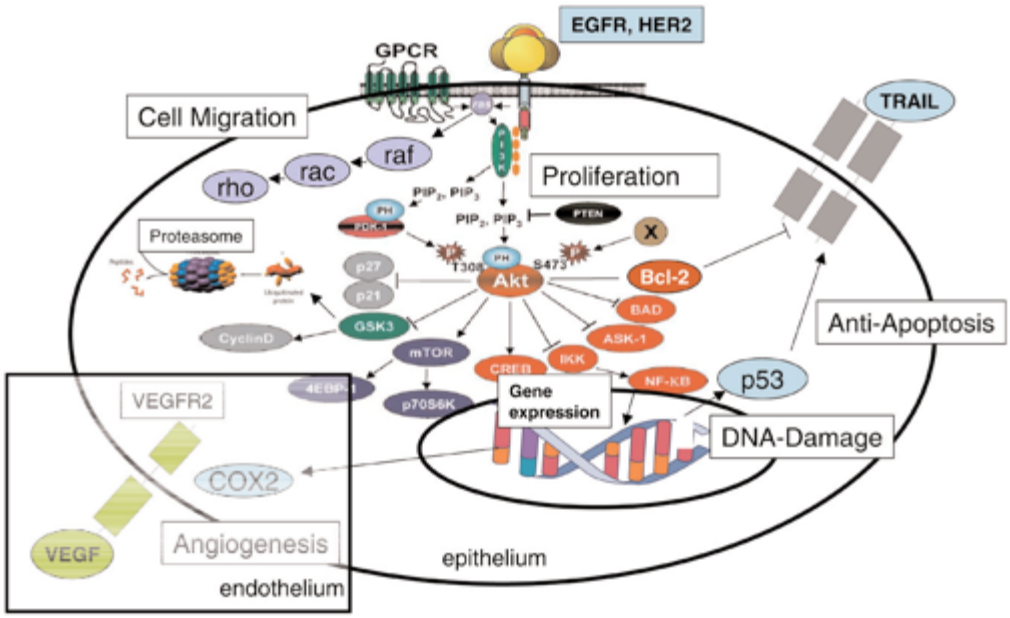


Figure 1: Cell signaling pathways: targets for breast cancer treatment. Major potential targets include EGFR, HER2 modulating cellular proliferation, GPCR resulting in altered cell migration, TRAIL modifying pro and anti-apoptotic elements and VEGR affecting angiogenesis. Source: (Schlotter, et al., 2008)

1.2. Cell survival, proliferation and apoptosis

1.2.1. Cell cycle progression

The link between cell survival, cell cycle and cell death has become an important molecular target in research throughout recent years. Normally the cell cycle of eukaryotic cells is divided into four non-overlapping phases. These include two preparatory gap phases G1 and G2 separated by DNA synthesis (S) phase and mitosis (M) phase (Maddika, et al., 2007)

During G1 phase, the cell prepares itself for the later DNA synthesis phase. Cells in this phase are diploid (2n) and tend to grow in size and produce new proteins required in subsequent phases (Collins & Garrett, 2005; Maddika, et al., 2007).

Following G1, the cell enters into S phase, in which DNA replication occurs. At the end of this phase each cell contains 2n chromosomes made up of two chromatids thus reaching a 4n DNA content (Maddika, et al., 2007; Schafer, 1998).

Before undergoing mitosis, cells carry on further growth and preparation and enter G2 phase. At the end of this phase cells are now ready to proceed to the M phase.

During M phase, cells undergo division or mitosis producing two daughter cells. These cells are diploid and genetically identical to each other and to the original mother cell. Cells that stop dividing or are not actively cycling tend to exit the cell cycle into a quiescent phase G0. (Collins & Garrett, 2005; Maddika, et al., 2007; Schafer, 1998)

Checkpoints are present at the end of each phase of the cell cycle that allow the arrest and activation of repair mechanism in case of any malfunction (Schafer, 1998). After passing a checkpoint, the cell becomes irreversibly committed to the next phase (Park & Lee, 2003). DNA damage or other critical malfunctions can activate cell cycle arrest and may also trigger the apoptotic cascade pathway leading to cell death (Maddika, et al., 2007). Thus apoptosis plays an essential role during the cell cycle progression preventing the propagation of damaged or unwanted cells (Maddika, et al., 2007; Park & Lee, 2003).

Regulated machinery exists to control the progression of the cell cycle to guarantee proper cell growth and proliferation. Cyclin-dependent kinases (CDKs) are key regulatory proteins that allow smooth transition from one phase to the other (Maddika, et al., 2007). CDKs are serine/threonine kinases that are activated at particular stages of the cell cycle (Collins & Garrett, 2005). These proteins are positively regulated by binding to respective cyclins (Park & Lee, 2003). Activating cyclins are produced at each phase of the cell cycle; these bind to corresponding CDKs targeting them to the nucleus whereby catalyzing the process of cell division. Cyclins undergo a cyclic expression during the cell cycle. Typically, before entering a new phase, cyclin of the previous phase is degraded and the apprpriate cyclin of the next phase is synthesized. Thus, the level of cyclins present in different phases is variable; however, CDKs remain constant (Schafer, 1998; Vermeulen, Van Bockstaele, & Berneman, 2003).

1.2.2. PI3-Kinase/Akt pathway controls cell survival

The Phosphatidylinositol 3-kinase (PI3-K) signaling pathway is one of the most important key survival pathways. It is activated by a myriad of cellular stimuli regulating fundamental cellular functions including proliferation, growth, gene transcription, cell cycle progression and apoptosis (Maddika, et al., 2007; Vivanco & Sawyers, 2002). These enzymes catalyze the phosphorylation of phosphoinositides and are classified according to substrate specificity and subunit organization (Dbouk & Backer, 2010; Engelman, Luo, & Cantley, 2006).

Class IA PI3-Ks are obligate heterodimers composed of a catalytic subunit (p110) and regulatory subunits (p85, p55 and p50) and are activated downstream of receptor tyrosine kinases (RTKs). Class IB, activated downstream of G-protein coupled receptor (GPCRs), are dimers composed of a p110 catalytic subunit and p101 or p87 regulatory subunits (Backer, 2008; Dbouk & Backer, 2010). Once activated PI3-Ks phosphorylate membrane PI(4,5)P2 producing PI(3,4,5)P3. This in turn creates an important docking site for a wide range of downstream effectors having the plecktrin homology (PH) domain. Thus activating numerous intracellular proteins involved in various interconnected signaling pathways that regulate cell survival, proliferation and motility (Maddika, et al., 2007; Vivanco & Sawyers, 2002; Yip, et al., 2004)

Akt is a downstream effector of PI3-K that controls cell survival through phospohrylating different substrates involved in survival and apoptotic pathways. It is recruited to the cell memrane through it PH domain which binds to PIP3. Akt is then phosphorylated and activated by PDK1 and mTOR kinases. One of the important targets of Akt is a pro-apoptotic protein of the Bcl-2 family, Bad. Phosphorylation of Bad by Akt activates and releases its associated apoptosis inhibitory protein thus blocking the apoptotic pathway (Figure2). Akt can also phosphorylate caspase-9 inducing a conformational change that results in the inhibition of its proteolytic activity, consequently blocking apoptosis and promoting cell survival (Maddika, et al., 2007; Vivanco & Sawyers, 2002)

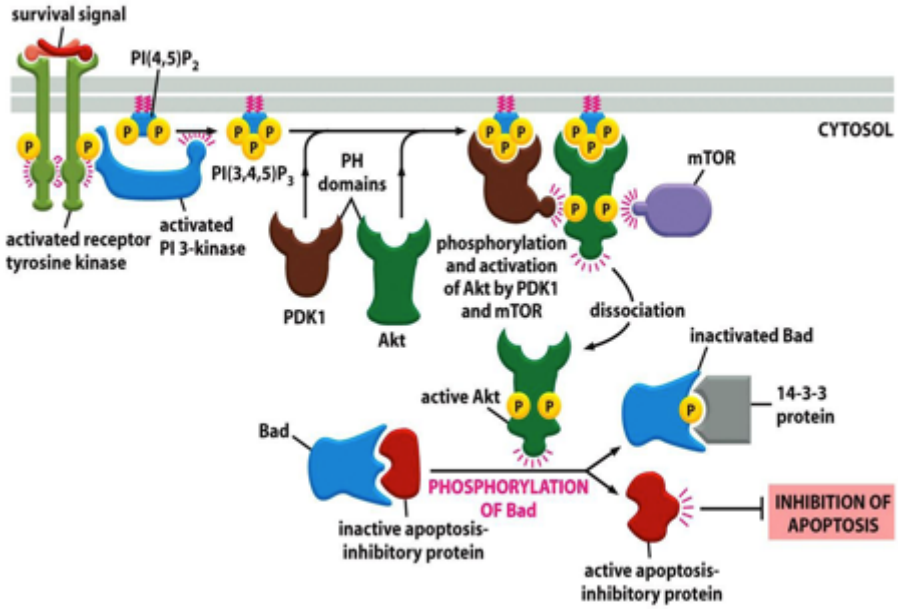


Figure 2: Akt promotes growth factor-related cell survival. Downstream of PI3-K, active Akt phosphorylates Bad making it dissociate from the inactive apoptosis inhibitory protein and losing its pro-apoptotic function. (Source: (Ramaswamy, et al., 1999)

1.2.3. Tumor suppressors and oncogenes

It is believed that tumor suppressors have evolved probably to protect multicellular organisms from random mutations affecting various cellular functions. This hypothesis is supported by the fact that critical tumor suppressor proteins or pathways are mutated in most human cancers. Normally, these proteins exert a negative regulatory role on cellular growth and proliferation. Thus, inactivation through deletions or mutations may allow the development of a malignant phenotype (Viallet & Minna, 1990).

p53 is one of the most important members of the tumor suppressor proteins (Levine, Momand, & Finlay, 1991). As a transcription factor, p53 has a crucial role in regulating cellular response to various stress signals through favoring cell cycle arrest, apoptosis or senescence. Consequently, in response to stress such as DNA damage, p53 enhances transcription of genes involved in cellular growth, apoptosis, angiogenesis and repair mechanisms. On the other hand, it can have direct non-transcriptional activities promoting survival (Figure 3) (Brown, Lain, Verma, Fersht, & Lane, 2009).

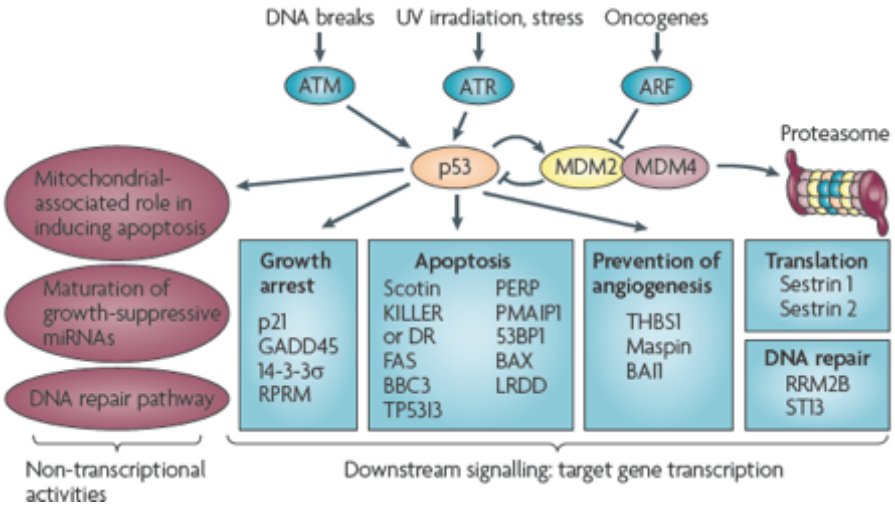


Figure 3: The p53 pathway. p53 is located at the center of a complex web of biological interactions that translates stress signals into cell cycle arrest or apoptosis. Upstream signaling to p53 increases its level and activates its function as a transcription factor in response to a wide variety of stresses, whereas downstream components execute the appropriate cellular response. (Source: (Brown, et al., 2009).

Oncogenes are gene products with putative role in promoting cellular transformation and initiating neoplastic growth. In general, mutations resulting in their constitutive activation of these proteins directly contribute to cellular transformation (Viallet & Minna, 1990). Oncogenes can be classified into different categories including growth factors, receptor tyrosine kinases, cytoplasmic kinases, regulatory GTPases and transcription factors (Croce, 2008). Consequently, the cellular location of these oncoproteins depicts their respective functions. For example, Ras proteins located in the inner membrane of the cell are involved in signal transduction. Others such as c-myc and c-jun are nuclear proteins implicated in transcription and regulation of the cell cycle (Evans, 1993; Maddika, et al., 2007). This implicates their cooperative role in surpassing growth leading to uncontrolled cell proliferation and ultimately carcinogenesis (Harrington, Fanidi, & Evan, 1994).

Therefore, in most cancer systems the antagonistic relationship between the tumor suppressors and oncogenes is delineated by the inactivation of the former and the constitutive activation of the latter (Viallet & Minna, 1990).

1.2.4. Apoptotic pathways

Apoptosis is a programmed cell death, which plays important roles in physiological processes including embryogenesis and immunology. Complex signaling pathways are involved in regulating apoptosis. There are two well-known distinct apoptotic pathways: the intrinsic pathway also known as p53-mitochondrial pathway; and the extrinsic pathway which is activated through the binding of external ligands to specific membrane receptors known as 'death receptors' (Munoz-Pinedo, 2012; Taylor, Cullen, & Martin, 2008).

Mitochondrial intrinsic pathway is regulated by members of the Bcl-2 family of proteins. These include anti-apoptotic Bcl-2 and pro-apoptotic Bax and Bad proteins.

Bcl-2 is known to be overexpressed in up to 80% of breast cancer patients. It is also associated with high expression of hormone receptors ER and PR. Bcl-2 inhibits the process of apoptosis through regulating mitochondrial membrane permeability. It inhibits the formation of secretory pores through which cytochrome c exit the mitochondria. Consequently, the assembly of caspases involved in the completing the apoptotic pathway is inhibited (Ruvolo, Deng, & May, 2001; Srivastava, et al., 1998).

Pro-apoptotic Bax and Bad proteins are responsible for the formation of pores through the membrane of the mitochondria leading to the release of cytochrome c. This in turn activates the assembly of caspases-3 and 9 resulting in the proteolytic cleavage of downstream PARP and triggering apoptosis (Munoz-Pinedo, 2012; Schlotter, et al., 2008).

1.2.5. Altered cell growth and cancer

Disturbances in the homeostatic regulation of multiple signaling pathways result in altered cellular growth and consequently promote carcinogenesis. Typically, mutations within critical processes will affect a cascade of signaling pathways leading to reduced cell death, prolonged cell survival and uncontrolled proliferation (Maddika, et al., 2007).

Disruption in the regulation of apoptosis prolongs the life of cells and thereby promoting carcinogenesis. Subsequently, apoptosis is inhibited in cancer cells, supposedly by the activation of anti-apoptotic proteins and the deregulation of the

homeostasis between tumor suppressors and oncogenes. The result is the prevention of cell death and prolonged survival, cancer cell characteristics (Schlotter, et al., 2008).

In addition to inhibiting apoptotic pathways, mutations may result in the constitutive activation of survival pathways. Notably, PI3-K/Akt and Ras/MAPK pathways are frequently disrupted in most human cancers. These alterations prolong cell survival and play a major role not only in cell transformation and tumor development but also in resistance to cancer treatments (Vivanco & Sawyers, 2002).

Being a highly organized and tightly regulated process, defects in the programmed regulation of cell cycle-progression can also result in cancerous growth and development (Collins & Garrett, 2005; Park & Lee, 2003). Oncogenes and tumor suppressor genes are known to be involved in mitogenic signal transduction affecting transcription and initiation of the cell cycle. Consequently, in transformed cells, many oncoproteins are always "turned on" and tumor suppressors or other inhibitory mechanisms are "turned off". P53, mdm2, pRb, p21 and many others are strictly involved in the regulation of the cell cycle. Hence, deregulation of these proteins promotes uncontrolled growth and development of neoplasia (Schafer, 1998).

1.3. Cell motility

1.3.1. Cell motility cycle

Cell motility is an essential cellular process involved in numerous physiological events including embryogenesis, wound healing, inflammation and tissue regeneration. Moreover, it is a central process for cancer cell invasion and metastasis. Cell migration usually occurs in response to a chemoattractant or a growth factor present around the cell, a process known as chemotaxis. A great effort in research has been directed towards understanding the molecular basis of cell motility in an attempt to find novel therapeutic targets that would inhibit tumor growth and metastasis (Lauffenburger & Horwitz, 1996).

Once a signal is detected, a migrating cell enters the cell motility cycle in an amoeboid-like manner. This starts with determining the direction of motion towards the chemoattractant. Then the cell extends a protrusion, towards the direction of motion, by initiating the polymerization of new actin filaments (Bailly, Condeelis, & Segall, 1998). The actin-rich protrusion then needs to be stabilized by the formation

of adhesions to the cell substratum. This provides anchorage to the cell to transmit a mechanical force used to pull its cell body forward towards the direction of motion. Simultaneously, the adhesion structures at the rear end of the cell are disassembled and the cell retracts its tail and moves forward (Figure 4) (Ananthakrishnan & Ehrlicher, 2007; Bailly, Yan, Whitesides, Condeelis, & Segall, 1998; J. S. Condeelis, et al., 2001).

Numerous intracellular proteins are involved in the regulation of the cell motility cycle. One of the most important family of proteins regulating this process is the Rho family GTPases. Members of this family play a crucial role in the reorganization of the actin cytosekeleton (J. Condeelis, 2001; van Golen, 2003).

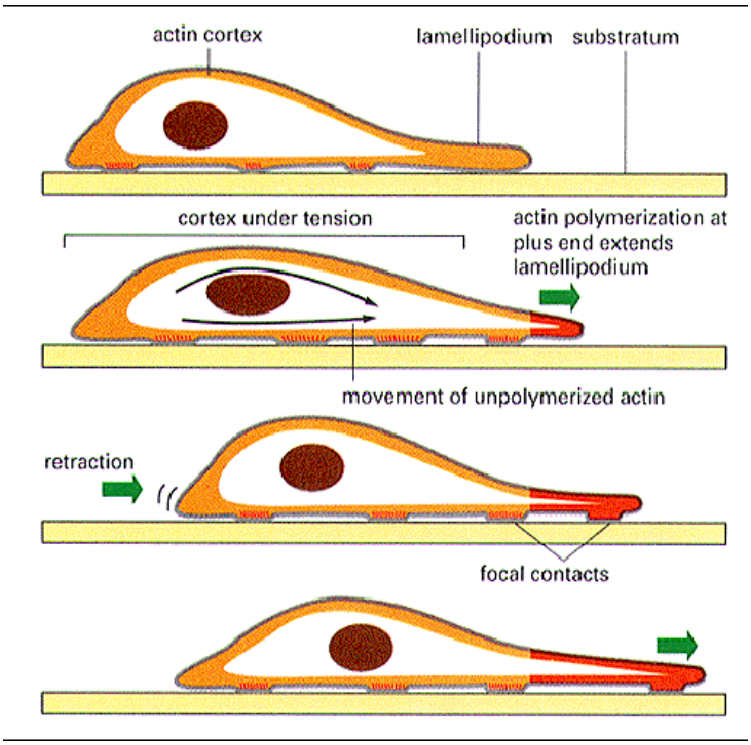


Figure 4: The cell motility cycle. A migrating cell first protrudes an extension in the direction of motion, forms adhesions to stabilize this protrusion, then detaches old adhesions at the rear end, and finally complete the cell cycle by retracting its tail and pulling its cell body forward. Source: (Childs, 2001).

1.3.2. Altered cell motility and cancer

Cell motility is a complex multistep process that integrates multiple intracellular signaling and regulatory pathways. Therefore, slight modifications in any step may dramatically affect normal cellular functions and result in cellular transformation and carcinogenesis. It is known that cell motility is essential for metastasis and without it tumors would be easily eradicated and/or surgically removed (Jiang, et al., 2009).

The acquisition of a motile phenotype is a critical step towards carcinogenesis and is required for a cell to gain metastatic competence. Thus, further descriptions of the molecular mechanisms regulating cancer cell motility would facilitate the development of specific and effective therapeutic treatments against metastasis and tumor cell invasion (Jiang, et al., 2009; van Golen, 2003).

1.4. Cell invasion

During cancer cell metastasis, cells emigrate from the primary site of the tumor into the bloodstream and are carried along to distant sites where they can extravasate out of blood vessels forming secondary neoplastic tissues (Gertler & Condeelis, 2011). To do so, cancer cells must penetrate the dense extracellular matrix (ECM) degrading the basement membrane and migrate towards blood and lymphatic vessels (Gertler & Condeelis, 2011; Kim, Liotta, & Kohn, 1993; Sahai, 2005).

Cellular invasion is defined as the transition from a primary tumor growth to a malignant phenotype. It is a multistep process that involves coordinated and well-organized intracellular and extracellular communications (Calvo & Sahai, 2011; Kim, et al., 1993).

1.4.1. ECM degradation

Initially, malignant cells transverse the underlying epithelial basement membrane and invade into the interstitial stroma of the extracellular matrix. This requires effective proteolysis of the ECM components including type IV collagen, glycoproteins and proteoglycans (Kim, et al., 1993). For this reason, cells secrete proteases and/or factors

that activate matrix proteases. These include matrix metalloproteinase's (MMPs), which are secreted as inactive pro-enzymes and activated extracellularly. Plasmin is one the factors needed to cleave secreted pro-enzymes and activate them. However, the secreted MMPs must overcome the presence of endogenous inhibitors (TIMPs) that may impede invasion process. Thus, the balance between active proteases and their inhibitors is crucial in determining the invasive phenotype of malignant cells (Aznavoorian, Murphy, Stetler-Stevenson, & Liotta, 1993; Gertler & Condeelis, 2011; Stetler-Stevenson, Aznavoorian, & Liotta, 1993).

After successful degradation of the ECM, cells are now free to migrate towards the circulatory system. This process is associated with tumor-induced angiogenesis, which not only promotes tumor expansion but also allows easy access to vascular tissues (Aznavoorian, et al., 1993). Moreover, the process of invasion and metastasis involves a complex network of interactions with surrounding host tissues and other components of the tumor microenvironment (Calvo & Sahai, 2011) This comprises numerous molecular pathways that steer and direct cellular migration (Mareel & Constantino, 2011). Finally, to successfully establish neoplastic tissue at distant sites, circulating tumor cells must be able to survive host immune system, extravasate out of the capillary and ultimately invade and proliferate in the tissue of the secondary site (Gertler & Condeelis, 2011; Kim, et al., 1993; Mareel & Constantino, 2011).

1.4.2. Molecular mechanism of invasion

In order to accomplish the successive steps during invasion, tumor cells are believed to form F-actin rich membrane protrusions known as invadopodia along with matrix metalloproteinase activity to degrade the dense extracellular barriers (Bravo-Cordero, et al., 2011). These structures extend vertically into the underlying ECM (J. Condeelis & Segall, 2003). Invadopodia are enriched with a network of actin filaments, actin binding proteins, matrix proteinases and regulatory proteins involved in actin polymerization and remodeling (Yamaguchi, et al., 2005).

Molecular mechanisms regulating the dynamics of invadopodia include signaling proteins such as Arp2/3, cofilin, cortactin and N-WASP, in addition to proteins that regulate matrix degradation such as MT1-MMP (Artym, Zhang, Seillier-Moiseiwitsch, Yamada, & Mueller, 2006; Yamaguchi, et al., 2005). Cofilin generates free barbed ends through the severing of actin filaments, leading to actin nucleation. Severing is

needed to expose free barbed ends since most filaments in resting cells are capped (Bravo-Cordero, et al., 2011; W. Wang, Eddy, & Condeelis, 2007). Cofilin is phosphorylated and inactivated by LIM and TES family kinases, by blocking its ability to bind to F-actin (Bravo-Cordero, et al., 2011). Studies have shown that the Rho family of small GTPases activate downstream kinase named ROCK that phosphorylates and activates LIM kinase (LIMK) (Olson & Sahai, 2009). (Figure 7) (Rho GTPases and their effectors are discussed later in details)

De novo nucleation is another mechanism by which barbed ends can be generated and this is mediated by the Arp2/3 complex, which provides a template for actin nucleation (Olson & Sahai, 2009). Arp2/3 is activated by the Wiskott-Aldrich syndrome protein (WASP) family. The WASP/SCAR/WAVE family of scaffold proteins directly bind to Arp2/3 via the VCA domain and are key regulators of actin polymerization (Olson & Sahai, 2009; Yamaguchi, et al., 2005).

Therefore, the regulation of actin dynamics in invadopodia is essential for tumor invasion and metastasis.

1.4.3. EMT

It is well known that the epithelial-mesenchymal transition is fundamental for tumor progression. Moreover, there is increasing evidence that the process of EMT is responsible for the dissemination of primary tumor epithelial cells to secondary metastatic sites and their acquisition of malignant phenotype (Thiery, 2002).

EMT of cancer cells is characterized by a downregulation of epithelial markers accompanied by an upregulation of mesenchymal proteins. This is reflected on the morphological basis depicted by an elongated polarized shape, pseudopodia formation, loss of cell-cell adhesion and increased motility (Morra & Moch, 2011; Thiery, 2002). Thus, a transient EMT process is required for cancer cell intravasation and dissemination.

Moreover EMT attenuates the immune response and overcomes additional safeguard mechanisms implemented against cancer cells including stimulation of apoptotic pathways and/or induction of premature senescence (Nieto, 2011). EMT also contributes to conferring resistance to radio/chemotherapy. However, once tumor cells reach a secondary site, it is important to revert back to epithelial phenotype (MET) in order to colonize and form metastases (Nieto, 2011; Thiery, 2002).

1.5. Rho family GTPases

Members of the Rho-family GTPases are small GTP-binding proteins (GTPases) that range between 20-40 KDa in size. These proteins play a vital role in cancer cell motility. All aspects of cellular motility and invasion including cellular polarity, cytoskeletal re-organization, and signal transduction pathways are controlled through the interplay between the Rho-GTPases (Sahai & Marshall, 2002; Tang, Olufemi, Wang, & Nie, 2008).

Activated Rho proteins can bind effector proteins and modulate cell behavior and morphology. Rho GTPases are implicated in cell migration through their ability to organize and regulate actin-containing structures. Frequent studies have shown that the Rho family GTPases regulate cell motility in breast cancer through their ability to mediate the remodeling of actin cytoskeleton as well as translating cellular signals from plasma membrane receptors to regulate focal adhesion, cell polarity, vesicular trafficking and gene expression (Tang, et al., 2008). Approximately 30% of human tumors possess a specific mutation in Ras oncogene leading to its protein level overexpression or constitutive activation. In contrast to Ras, no mutation in any of the Rho GTPases has been identified in breast cancer. Rather, these GTPases are often either overexpressed or hyperactive in breast cancer tissue. The variations in the levels of these Rho proteins might directly correlate with the advancement of breast cancer (Etienne-Manneville & Hall, 2002; Vega & Ridley, 2008). The three most characterized members of the Rho GTPases are Rho, Rac and Cdc42 which were found to be distinct in function from the other Rho proteins (Takai, Sasaki, & Matozaki, 2001).

1.5.1. General structure of Rho GTPases

All Rho GTPases have a consensus amino acid sequence at the N-terminal half that is responsible for specific interaction with GDP and GTP molecules and for a GTPase activity that hydrolyzes bound GTP into GDP and P_i.(Valencia, Chardin, Wittinghofer, & Sander, 1991) Four important domains are implicated in the binding and hydrolysis of GTP (Johnson, 1999). One of the most important domains is the effector or switch I domain, which is required for downstream functions of Rho

GTPases (Marshall, 1993). Rho proteins also have sequences at their COOH termini that undergo post-translational modifications with lipids, such as farnesyl, geranylgeranyl, palmitoyl and methyl moieties, and that are necessary for proper localization in the cell (Magee, et al., 1992).

1.5.2. Rho GTPases as binary switches

Rho GTPases switch between two conformations, a GDP-bound inactive state where they are sequestered and kept in the cytoplasm; and an active GTP-bound state (Sahai & Marshall, 2002; Vega & Ridley, 2008). Since Rho GTPases control many important signal transduction pathways, their activation is tightly regulated in the cell. The activity of Rho GTPases is regulated by nucleotide binding and by subcellular localization (Wennerberg & Der, 2004).

In response to a certain signal, replacing the bound GDP with a GTP molecule activates Rho GTPases. This induces a conformational change favoring the binding of the active Rho protein to downstream effectors. After activation of the effector, the GTP molecule is hydrolyzed to GDP rendering the GTPase back to its inactive form (Etienne-Manneville & Hall, 2002; Tang, et al., 2008). This constitutes a single cycle of activation/inactivation of Rho GTPases

Rho GTPases mediate the transduction of numerous intracellular signaling pathways affecting cell behavior and morphology. Thus, these critical proteins are implicated in many essential cellular processes including actin dynamics, gene transcription, cell cycle progression, cell adhesion, motility and invasion (Etienne-Manneville & Hall, 2002; Jaffe & Hall, 2005).

1.5.3. Rho GTPases and their regulation

The switch between active GTP-bound and inactive GDP-bound state is regulated by Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and Guanine-nucleotide dissociation inhibitors (GDIs). Figure 5 illustrates the model of Rho protein regulation.

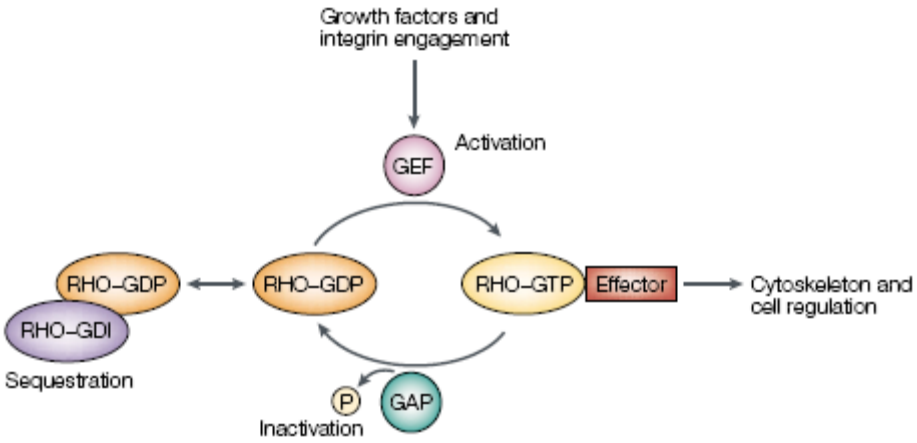


Figure 5: Regulation of Rho family proteins. Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and Guanine-nucleotide dissociation inhibitors (GDIs). Source: (Sahai & Marshall, 2002).

1.5.3.1 GEFs

These proteins have a Dbl homology (DH) domain and other domains such as Src homology 3 (SH3) and pleckstrin homology domain (PH). These regulators interact with phospholipids of the cell membrane and other proteins modulating the GDP-GTP exchange activity (Tang, et al., 2008).

During nucleotide exchange, the initial dissociation of GDP from the inactive form of Rho GTPases is considered the rate-limiting step. This reaction is very slow and is stimulated by a guanine nucleotide exchange factors (GEFs). Thus, GEFs activate Rho GTPases by mediating the exchange of GDP to GTP (Grise, Bidaud, & Moreau, 2009; Schmidt & Hall, 2002)

1.5.3.2 GAPs

Rho GTPases are negatively regulated by Rho GTPases activating proteins (GAPs). These proteins inhibit Rho GTPases by activating their

intrinsic GTPase activity. This leads to the hydrolysis of the bound GTP into GDP converting Rho GTPases back to their inactive conformation (Moon & Zheng, 2003).

In addition to activating GTP hydrolysis, GAPs may function as effectors of Rho GTPases to mediate other downstream effector functions (Grise, et al., 2009; Tang, et al., 2008)

1.5.3.3 GDIs

Guanine nucleotide dissociation inhibitors (GDIs) block both the GDP/GTP exchange and the GTP hydrolysis. These proteins prevent the dissociation of GDP from the inactive Rho proteins and their interaction with downstream effectors. GDIs can also bind to the active GTP-bound form preventing their interaction with GAPs. Moreover, GDIs modulate the cycling of GTPases between the cell membrane and the cytoplasm. Since the activity of Rho GTPases crucially depends on their translocation to the cell membrane, GDIs are considered important regulators with the ability to sequester GTPases in the cytoplasm by masking their hydrophobic region/domains (DerMardirossian & Bokoch, 2005; Garcia-Mata, Boulter, & Burridge, 2011; Grise, et al., 2009).

1.6. Signaling pathways of Rho GTPases

Rho family GTPases are activated in response to numerous extracellular stimuli captured by plasma membrane receptors. Hence, these proteins are involved in translating signals to regulate various cellular functions including cytoskeleton re-organization, cell-cell interaction, proliferation, cell adhesion, polarity, chemotaxis and many others (Figure 6) (Sahai & Marshall, 2002; Tang, et al., 2008).

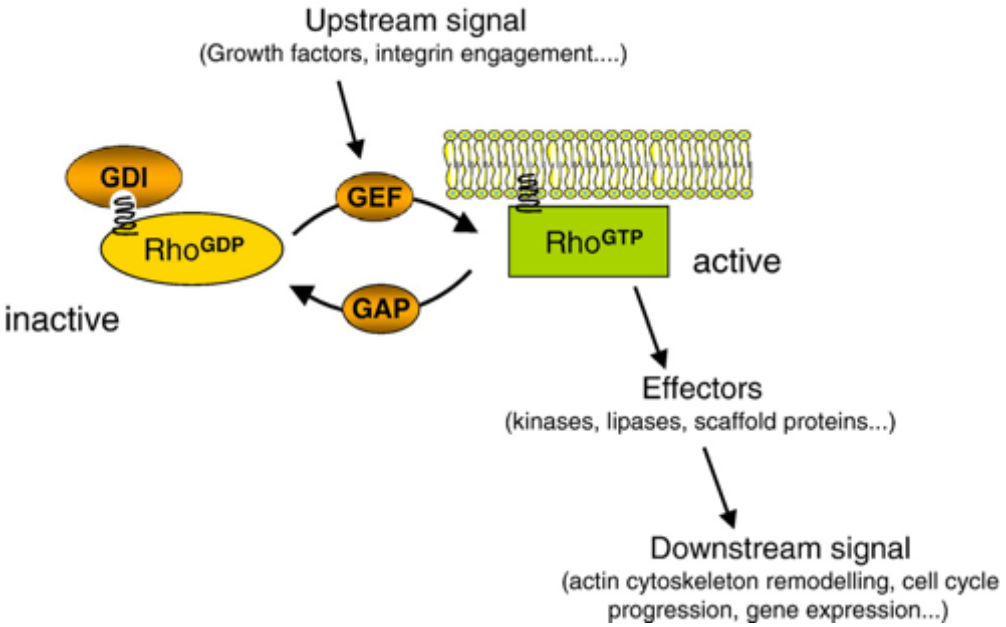


Figure 6: Signaling of Rho family proteins. Rho GTPases are activated in response to upstream signals such as growth factors and mitogens. This results in the transformation of GDP-bound Rho proteins to active GTP-bound state. Active GTPases in turn bind and activate downstream effectors, consequently translating various downstream signals including actin remodeling, cell cycle progression, transcription and many others. Source: (Grise, et al., 2009).

1.6.1. Upstream signaling

1.6.1.1.PI3K signaling

As mentioned earlier, Rho GTPase-activating proteins GEFs contain the pleckstrin homology (PH) domain, a 100-120 amino acid module that has a high binding affinity to phosphoinositides and can affect the catalytic DH domain of GEFs (Macias, et al., 1994). Binding to PI(4,5)P2 of the plasma membrane favors the interaction between PH domain of GEFs and the catalytic DH domain inhibiting its activity. PI3K phosphorylates PI(4,5)P2 producing PI(3,4,5)P3 that has high affinity to PH domain, thus releasing the DH domain and activating GEFs. In turn, GEFs can bind/activate Rho GTPases (Schmidt & Hall, 2002).

External stimuli such as LPA, PDGF, EGF and insulin have been shown to trigger the activation of Rho GTPases in a PI3K dependent manner. Treatment of fibroblasts with wortmanin, a PI3K inhibitor,

inhibited Rho and Rac mediated membrane ruffling in response to EGF (Nobes, Hawkins, Stephens, & Hall, 1995; Tang, et al., 2008). These data suggest that PI3K acts upstream of Rho GTPases to stimulate membrane ruffling in response to growth factors.

A well-characterized nucleotide exchanger, Vav, is known to be activated by PI3K. Vav is phosphorylated on tyrosine residues in the N-terminal region by Src and Syk kinases leading to the activation of its catalytic activity (Lopez-Lago, Lee, Cruz, Movilla, & Bustelo, 2000) An autoinhibitory constraint is imposed by its PH domain. However, when bound to PI(3,4,5)P3 produced by PI3K, the PH/DH interaction is weakened and this alleviates the inhibition (Bustelo, 2000; Crespo, Schuebel, Ostrom, Gutkind, & Bustelo, 1997)

The PH domain of Son of svenless (Sos) also binds to PIP3 relieving intramolecular inhibition. Sos is a GEF for both Ras and Rac. It forms a complex with a number of adaptor proteins downstream of receptor tyrosine kinases (Das, et al., 2000; Scita, et al., 1999).

Therefore, the activity of Rho GTPases is spatially regulated in many cellular functions following the subcellular localization of GEFs. PI3K activation plays an essential role in regulating the localization of GEFs through the production of PIP3 which binds to the PH domain of GEFs. Deletion of the PH domain in many GEFs results in the loss of in vitro activity, which can be restored by the addition of a CAAX motif that targets the protein to the plasma membane (Whitehead, et al., 1996; Whitehead, et al., 1999)

In addition, many studies in mammary tumors have shown that PI3K acts downstream of Rac and Cdc42 (Keely, Westwick, Whitehead, Der, & Parise, 1997). These findings suggest that PI3K and Rho GTPases are involved in a positive feedback loop that stimulates lamellipodia formation during cell motility.

1.6.1.2. Activation by adhesion

Numerous signaling pathways involving Rho GTPases are activated downstream of the cell adhesion to the ECM (DeMali & Burridge, 2003). In this respect, the activation of focal adhesion kinase (FAK) leads to the phosphorylation and activation of p130cas and paxillin (DeMali & Burridge, 2003; Zamir & Geiger, 2001). Phosphorylated p130cas activates Rac by forming a complex with adaptor proteins Crk and DOCK180, which is a GEF for Rac (Cary, Han, Polte, Hanks, & Guan, 1998; Klemke, et al., 1998; Matsuda, et al., 1996). Paxillin also forms a multi-component complex with the protein PKL and PIX, the latter being another Rac GEF (Bagrodia, Taylor, Jordon, Van Aelst, & Cerione, 1998).

In addition to activating Rac, FAK also activates p190RhoGAP thus leading to the inhibition of RhoA. In many cells, during initial cell adhesion, FAK activity stimulates high Rac activity and low RhoA activity (O'Connor, Nguyen, & Mercurio, 2000).

1.6.2. Downstream signaling

1.6.2.1. Rho effectors

Downstream targets of Rho include the serine/threonine kinase p160ROCK which is mainly involved in the formation of stress fibers and focal adhesions (Struckhoff, Rana, & Worthylake, 2011). ROCK is known to phosphorylate myosin light chain (MLC) leading to actin-myosin contractility (Grise, et al., 2009; Tang, et al., 2008). At the same time, ROCK inhibits MLC dephosphorylation by inhibiting MLC phosphatases via their myosin binding subunit (MBS) (Kimura, et al., 1996). However, normal stress fiber formation also require the activity the mammalian homolog of diaphanous (mDia), another important Rho effector mediating actin nucleation (Alberts, 2001).

LIMK is another downstream effector of Rho, which phosphorylates the actin severing protein cofilin. As mentioned earlier (in section 1.4.2.), cofilin function is inhibited upon phosphorylation (Olson & Sahai, 2009).

Other Rho effectors include members of the ezrin-radixin-moesin (ERM) proteins. These proteins are found to associate with the cell plasma membrane where they mediate Rho-dependent actin cytoskeleton remodeling (Figure 7) (Takaishi, Sasaki, Kameyama, Tsukita, & Takai, 1995; Tang, et al., 2008).

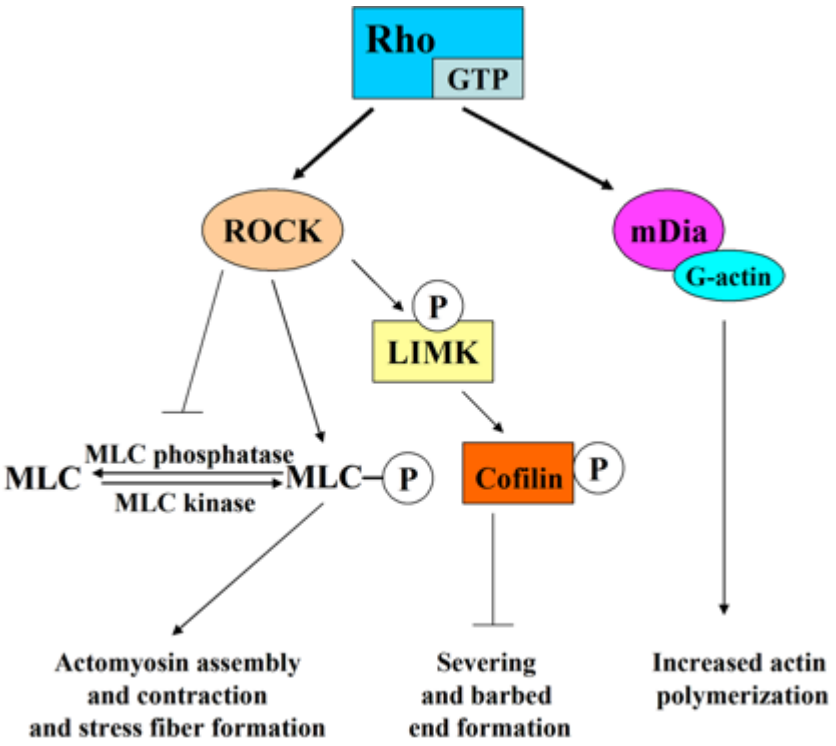


Figure 7: Downstream effectors of Rho. Explained in text in section 1.6.2.1.

1.6.2.2. Rac/Cdc42 effectors

Downstream signaling of Cdc42 and Rac include scaffold proteins belonging to the WASP/SCAR/WAVE family containing the VCA domain (Keely, et al., 1997; Tang, et al., 2008). These are key regulators of actin nucleation protein Arp2/3 complex that stimulates actin polymerization at the leading edge of the cell (Jaffe & Hall, 2005; Jiang, et al., 2009; Tang, et al., 2008; Yamaguchi, et al., 2005).

Some proteins are target effectors of both Rac and Cdc42. One of these proteins is the serine/threonine p21 activating kinase (PAK). PAK isoforms have an N-terminal regulatory domain and a C-terminal catalytic domain (Manser, Leung, Salihuddin, Zhao, & Lim, 1994). Moreover, all PAK proteins

share a common domain responsible for interaction with Rac and Cdc42, referred to as Cdc42/Rac interactive binding (CRIB) domain (Burbelo, Drechsel, & Hall, 1995; Manser, et al., 1994) Binding of active GTPase disrupts the autoinhibitory conformation of PAK and activates its catalytic domain by phosphorylation (Manser, et al., 1994). Active PAK phosphorylates MLCK thereby inactivating it and inhibiting MLC phosphorylation and contractility (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999). PAK is also targeted to adhesion complexes regulating focal adhesion turnover (Bokoch, 2003). In addition, PAK phosphorylates and activates LIMK (Figure 8) (Edwards, Sanders, Bokoch, & Gill, 1999). LIMK is therefore activated through ROCK and PAK pathways downstream of Rho and Cdc42/Rac respectively. This potentially results in the phosphorylation of cofilin inhibiting its actin-severing function and stabilizing actin polymerization at the leading edge of the cell (Burridge & Wennerberg, 2004)

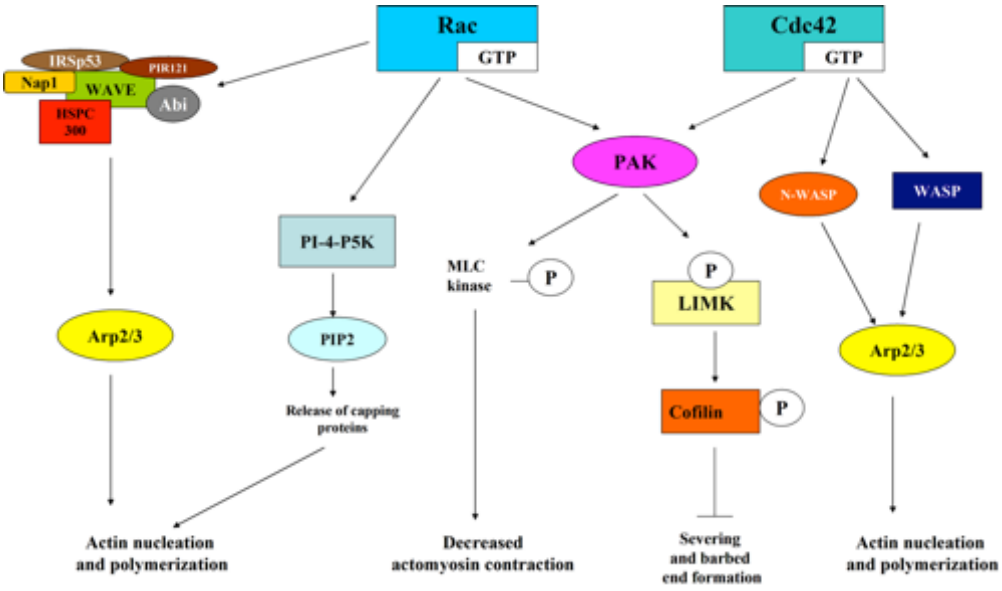


Figure 8: Downstream effectors of Rac and Cdc42. Explained in text in section 1.6.2.2.

1.7. Role of Rho GTPases in cell motility

In general, cell migration can be divided into separate successive steps: determining the direction of motion, cell polarization, lamellipodial protrusion, adhesion formation followed by cell body contraction and tail retraction (Figure 9) (Lauffenburger & Horwitz, 1996).

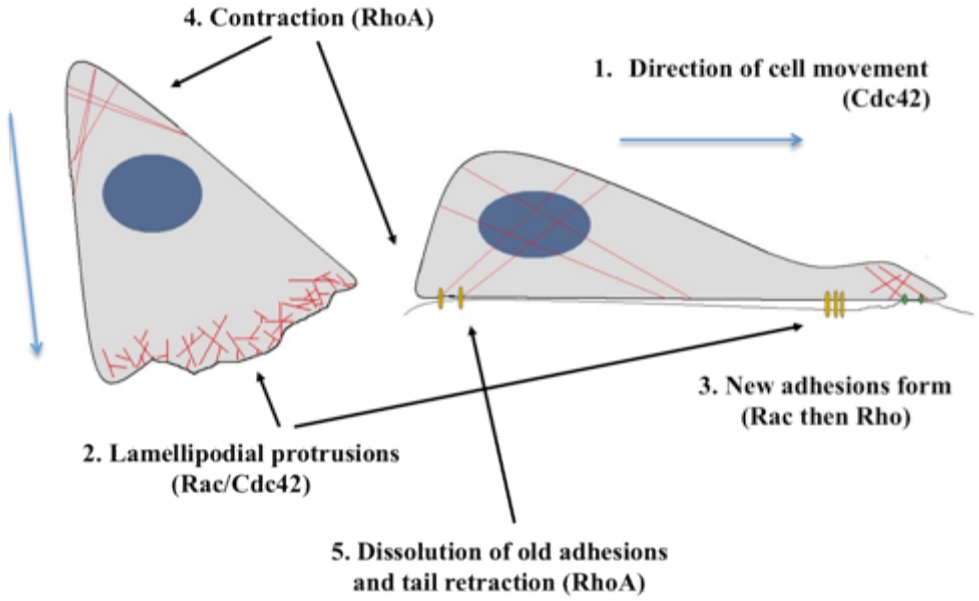


Figure 9: Rho GTPases in cell motility. A migrating cell enters cell motility cycle. Cdc42 determines the direction of motion. Rac induces lamellopodial formation at the leading edge by stimulating actin polymerization and focal complexes. Rho acts at the rear end leading to actin-myosin contractility and tail retraction.

1.7.1. Direction of motion and protrusion formation

The initial step during cell migration is the determination of direction of motion by generating membrane protrusions. These protrusions can be either spike-like filopodia or larger, broad lamellipodia (Tang, et al., 2008).

Filopodia are classically regarded as sensors for chemotactic cues required for direction sensing during cell migration. These protrusions extend out from the cell detecting and transmitting any environmental changes (Arjonen, Kaukonen, & Ivaska,

2011; Ridley, 2001). Cdc42 regulates the formation of filopodia by initiating actin polymerization through the activation of N-WASP (Nobes & Hall, 1999).

The lamellipodium is a meshwork of highly branched actin filaments at the cell edge (Small, Stradal, Vignal, & Rottner, 2002) The structure of the lamellipodia is known to be Rac-dependent involving a number of Rac downstream effectors (Figure 8) such as severing proteins ADF/cofilin (Van Troys, et al., 2008)and actin binding proteins like the Arp2/3 complex that is responsible for actin nucleation and branching. As well as the activation of PIP-5 kinase that produces PIP2 inhibiting actin-capping proteins. However these effectors are also downstream of Cdc42 (Figure 8). Consequently, Cdc42 is regarded as a potential regulator that drives Rac-dependent lamellipodia (El-Sibai, et al., 2007; Hall, 1998). In addition to its role in actin regulation, Cdc42 plays a crucial role in defining cell polarity with respect to the direction of motility, through the regulation of the microtubule cytoskeleton (Johnson, 1999)

1.7.2. Adhesion formation

Extended protrusions of migrating cells need to be stabilized by adhering to the ECM (J. S. Condeelis, et al., 2001). Cell adhesion to the ECM activates Rac and Cdc42 (as described in section 1.6.1.2.), which is required for cell spreading (Price, Leng, Schwartz, & Bokoch, 1998). It is therefore possible that there is continuous formation of new interactions between integrins and the ECM. The speed of cell migration depends on the composition of the substratum that dictates the relative levels of active Rho, Rac and Cdc42. Thus, a constant crosstalk between integrins and Rac is decisive for the cellular response to changing ECM composition (Nobes & Hall, 1999; Price, et al., 1998; Ridley, 2001; Tang, et al., 2008).

Rac stimulates the assembly of small punctate structures known as focal complexes that form behind the leading lamellipodium of the cell. However, these structures do not transmit adequate contractility during cell motility (Kaverina, Krylyshkina, & Small, 2002). Rac induces focal complex formation directly, through activating PAK (Figure 8), which in turn interacts with a complex of the exchange factor PIX, paxillin and GIT family of proteins. Also, Rac can indirectly contribute to the formation of focal complexes through antagonizing Rho activation (Rottner, Hall, & Small, 1999; Sander, ten Klooster, van Delft, van der Kammen, & Collard, 1999; Tang, et al., 2008). As the cell moves in the direction of motion, focal complexes can

either disassemble or mature into larger and more stabilized Rho-dependent structures known as focal adhesions (Arjonen, et al., 2011; Wolfenson, Henis, Geiger, & Bershadsky, 2009).

Focal adhesions provide anchorage for the cell thus conferring mechanical strength needed for the cell to contract its cell body and slide along the ECM (Wolfenson, et al., 2009).

Therefore, cell migration requires the focal complex/adhesions turn over regulated by the interplay between Rac and RhoA. Increasing Rho activation stabilizes focal adhesion attachments to the ECM, hence inhibiting cell motility (Cox, Sastry, & Huttenlocher, 2001; Sander, et al., 1999)

1.7.3. Cell body contraction and tail retraction

In a migrating cell, adhesion to the ECM alone is not sufficient. Cell body contractility and tail retraction are needed for the completion of the cell motility cycle (Figure 4).

Cell body contraction depends on actomyosin contractility, which is directly regulated by Rho. Rho acts via ROCK inducing contractility through the phoshporylation of MLC (Figure 7). This results in transmission of tension to the sites of adhesion. MLC is also regulated by MLC kinase (MLCK), thus it is likely that ROCK and MLCK act in concert to control cell contractility (Tang, et al., 2008). In addition to the ROCK pathway, RhoA is believed to negatively regulate cofilin leading to the inhibition of cell protrusions (El-Sibai, et al., 2008; Worthylake & Burridge, 2003). Thus, RhoA localizes to the rear of the cell inhibiting protrusions during motility.

Tail retraction is the final step of cell migration (Figure 9). At this point, adhesions must disassemble to ensure the completion of the cell motility cycle (Palecek, Huttenlocher, Horwitz, & Lauffenburger, 1998). Although the mechanism of tail detachment depends on the cell type and the strength of adhesion to the ECM, it is known that the reduction in RhoA activity could potentially inhibit retraction through reduced actomyosin contractility (Cox, et al., 2001; El-Sibai, et al., 2008; Worthylake & Burridge, 2003).

Therefore, the continuous interplay between Rho GTPases govern all aspects of cell motility including cell polarity, cytoskeleton re-organization, adhesion formation, cell contraction and tail retraction (Nobes, et al., 1995; Ridley, 2001)

1.7.4. Crosstalk between Rho GTPases

It was initially believed that Rho, Rac and Cdc42 play a defined role in regulating actin and adhesion dynamics during cell motility. However, this model is considered too simplistic due to crosstalk between the signaling pathways regulated by Rho GTPases (Khalil & El-Sibai, 2012). It was recently shown that RhoA for instance, is not only restricted to the generation of contractile force at the rear end of the cell, but also coordinates with Rac and Cdc42 at the cell edge in regulating actin cytoskeleton (El-Sibai, et al., 2008). Other studies have revealed an inverse relationship between Rho and Rac, where activation of Rac leads to the inactivation of Rho and vice versa (Sander, et al., 1999). This antagonism is explained in their antagonistic functions in cell adhesion (as described in section 1.7.2). Other examples where one family member negatively affects the activity of the other is through stimulating a GAP, or positively activating another through stimulating a GEF (Burridge & Wennerberg, 2004)

1.7.5. Altered role in cancer

The acquisition of motile and invasive phenotypes is the key component in developing metastatic competence. Both of these processes are strictly regulated by members of the Rho family GTPases (Sahai & Marshall, 2002). Studies have shown that constitutively activated Rho GTPases lead to the transformation of fibroblasts. In addition, genetic screening showed that Rho GTPases, particularly RhoA and RhoC, are found to be either overexpressed or hyperactive in many tumors including breast cancer (Sahai & Marshall, 2002; Tang, et al., 2008). Moreover, overexpression of RhoC was associated with 32% of invasive breast cancer and invasive ductal carcinoma (Kleer, et al., 2002; van Golen, 2003). In fact, forced expression of Rho proteins induced malignant transformation of human mammary epithelial cells resulting in an aggressive and highly motile phenotype. In addition, expressing a dominant-negative form of Rho inhibited cellular motility (Clark, Golub, Lander, & Hynes, 2000). Therefore, aberrant expression of Rho GTPases primarily contributes to cell

transformation and tumor development, given their role in the regulation of actin polymerization and motility and their interaction with numerous signaling pathways (Sahai & Marshall, 2002).

Dominant inhibitory/activating approaches have been used to describe the role of Rho GTPases in primary tumor growth and metastasis. Regulatory proteins GEFs and GAPs can cause dysfunctional activation/inhibition of Rho GTPases affecting cellular motility, invasion and ultimately metastasis (Bouzahzah, et al., 2001; M. Lin & van Golen, 2004). Moreover, as Rho GTPases are highly involved in promoting cellular transformation, many downstream effectors could be directly involved in tumor formation. For instance, direct inhibition of RhoA, through microinjection of C3T, or inhibiting its downstream effector ROCK using Y27632 leads to decreased motility and inhibition of focal complex maturation into focal adhesions (El-Sibai, et al., 2008).

There is also evidence that aberrant activation of Rho proteins can contribute to prolonged survival and prevent apoptosis. This is because of the ability of Rho GTPases to affect CDKs involved in regulating cell cycle progression promoting tumor initiation and growth (Tatsuno, Hirai, & Saito, 2000). Cyclin D1 is found to be overexpressed in 50% of breast cancers. Rho GTPases correlate to high expression of cyclin D1 through the activation of its promoter. Typically, Rac1 affects transcription of cyclin D1 through the activation of NF-κB and ATF-2 transcription factors that bind and activate cyclin D1 promoter (Guttridge, Albanese, Reuther, Pestell, & Baldwin, 1999; Joyce, et al., 1999). Thus, constitutively active forms of Rac1 stimulated transformation potential and cell proliferation through cyclin D1 overexpression (Joyce, et al., 1999). In addition, RhoA overexpression inhibits p21, a cyclin-dependent kinase inhibitor and an important tumor suppressor gene product (Liberto, Cobrinik, & Minden, 2002).

Given their integration in various pathways involved in cancer, Rho GTPase and their regulators are considered important therapeutic targets through the inhibition of cancer cell proliferation, motility and invasion. Several drugs have been shown to abrogate Rho GTPase functions. These drugs could directly target Rho proteins such as farsenalytrasferase inhibitors (FTIs) and strongylophorine-26 or could act through inhibition of their downstream effectors such as ROCK inhibitor Y-27632 (Du & Prendergast, 1999; McHardy, Warabi, Andersen, Roskelley, & Roberge, 2005; Tang, et al., 2008).

1.8. StarD13

1.8.1. Role as a tumor suppressor

DLC2 gene was first identified by Ching et al. (2003). It is located on position *13q12.3* and was found to be underexpressed in hepatocellular carcinoma (Ching, et al., 2003). DLC2 is commonly known as steriodogenic acute regulatory protein-related lipid transfer domain-containing protein 13 (StarD13). DLC2 shares 64% homology with DLC1, another member of the DLC family (Ullmannova & Popescu, 2006). StarD13 has an N-terminal SAM motif and a C-terminal START domain. It also harbors a RhoGAP domain, which is important to its function (Figure 10) (Ching, et al., 2003; Thorsell, et al., 2011; Ullmannova & Popescu, 2006). Overexpression of StarD13 was found to associate with significant decrease in cell growth and proliferation (Ching, et al., 2003). Moreover, DLC1, a closely related protein is found to be underexpressed in many types of cancer including lung, prostate, kidney, colon, breast, uterus, and stomach (Liao & Lo, 2008). This data suggests a potential role of StarD13 as a tumor suppressor (El-Sitt, et al., 2012)

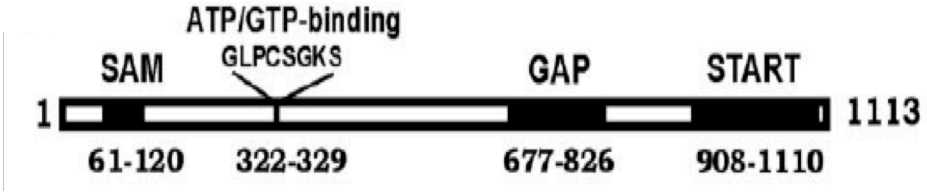


Figure 10: StarD13 structural domains. StarD13 harbors an N-terminal SAM domain followed by GTPase regulatory GAP domain and a C-terminal START domain. Source: (Thorsell, et al., 2011).

1.8.2. StarD13 activity and localization

StarD13 specifically inhibits RhoA and Cdc42. This leads to the inhibition of the Rho-dependent actin stress fibers formation (Ching, et al., 2003). Moreover, START domain present at the N-terminus of StarD13 is important for targeting it to the mitochondria suggesting a possible role in the regulation of the mitochondrial membrane permeability and activation of apoptotic pathways (Ng, et al., 2006). In addition, studies on truncated mutants revealed its intracellular localization to focal adhesions by the presence of focal adhesion targeting domain (FAT) at its N-terminal portion. The FAT domain interacts with tensin2, a component of focal adhesions (Kawai, et al., 2009).

In addition, negative correlation was observed between StarD13 and RhoA in a RhoGAP manner. Thus, StarD13 was found to inhibit the function of RhoA through inhibiting Rho-mediated assembly of actin stress fibers (Ching, et al., 2003)

Taken together, it is likely that StarD13 is localized to focal adhesions of the cell through its N-terminal FAT domain, where it regulates the activity of RhoA, a process strictly correlated to cellular motility.

1.9. Purpose of the study

In this study we aimed at characterizing StarD13 in breast cancer in terms of its level of expression and its role in cellular proliferation, migration and invasion.

We first aimed to determine the level of expression of StarD13 in patient tissues representing different grades of breast cancer compared to normal tissues. Then, we wanted to study the effect on cellular proliferation, viability and cell cycle progression upon manipulating the level of StarD13 expression.

Next, we investigated its RhoGAP activity and localization in breast cancer cells. We then opted to determine the role of RhoA and StarD13 in cellular migration. Furthermore, we aimed at investigating the role of StarD13 and RhoA in the dynamics of cellular adhesion. And finally, we wanted to determine its effect on cell invasion.

Chapter II

MATERIALS & METHODS

2.1. Cell Culture

Human breast cancer cell lines (MCF-7 and MDA-MB231) obtained from ATCC, were cultured in DMEM medium supplemented with 10% FBS and 100U penicillin/streptomycin at 37°C and 5% CO_2 in a humidified chamber.

2.2. Antibodies and reagents

Goat polyclonal anti-StarD13 antibody was obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-RhoA, mouse monoclonal anti-Rac1, and mouse monoclonal anti-paxillin antibodies were purchased from Upstate biotechnology, Lake Placid, NY. Anti-goat and anti-mouse HRP-conjugated secondary antibodies were obtained from Promega. Fluorescent secondary antibodies (AlexaFluor 488) were obtained from Invitrogen. To visualize the actin cytoskeleton, cells were stained with Rhodamine phalloidin (Invitrogen). DAPI was also used to stain nuclei.

2.3. Cell transfection with siRNA

Goat FlexiTube siRNA for StarD13, RhoA, and Rac1 were obtained from Qiagen. The siRNAs used had the following target sequences: StarD13: 5'-CCCGCAATACGCTCAGTTATA-3', RhoA: 5'-TTCGGAATGATGAGCACACAA-3', and Rac1: 5'-ATGCATTTCCTGGAGAATATA-3'. The cells were transfected with the siRNA at a final concentration of 10 nM using HiPerfect (Qiagen) as described by the manufacturer. Control cells were transfected with siRNA sequences targeting GL2 Luciferase (Qiagen). After 72 hours, protein levels in total cell lysates were analyzed by western blotting using the appropriate antibodies or the effect of the corresponding knockdown was assayed.

2.4. Cell transfection with vectors

Cells were transfected with 5 μg GFP-StarD13, Dominant active RhoA, or control empty control vectors using Lipfectamine LTX with Plus reagent (Invitrogen) as described by the manufacturer. Cells were incubated with the transfection complexes for 4 hours then refed with DMEM supplied with 30% FBS. The experiments were carried on 24 hours following transfection.

The GFP-StarD13 and the RhoA constructs were generous gifts from respectively Dr. Hitoshi Yagisawa from the University of Hyogo, Japan and Dr. Hideki Yamaguchi from the Albert Einstein College of Medicine, New York, USA.

The constructs were transformed into One Shot TOP10 chemically competent E. Coli (Invitrogen), which were grown on a selective medium containing the appropriate antibiotic. The vectors were then extracted using MaxiPrep plasmid extraction kit from Qiagen.

2.5. Western blotting

Cell lysates were prepared by scraping the cells in a sample buffer consisted of 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl at a pH of 6.8. The resulting lysates were boiled for 5 minutes. Protein samples were separated by SDS-PAGE on 8% (for StarD13) or 15% (for RhoA and Rac) gels and transferred to PVDF membranes overnight at 30V. The membranes were then blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature and incubated with primary antibody at a concentration of 1:100 for 2 hours at room temperature. After the incubation with the primary antibody, the membranes were washed and incubated with secondary antibody at a concentration of 1:1000 for 1 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with western blotting chemiluminescent reagent ECL (GE Healthcare). The results were obtained on an X-ray film (Agfa Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.6. RT-PCR

Cells were grown in 6-well plate at density of $1x10^6$ cells/ml and were transfected by either control or StarD13 siRNA for 72hrs. Total RNA was extracted performed RNeasy extraction kit (Qiagen) according to manufacturer's instruction. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to amplify RNA of StarD13. 2μg of RNA was converted to cDNA using the OneStep RT-PCR kit (Qiagen) as described by the manufacturer. Briefly, gene-specific primers designed to detect cDNA were obtained from TIB-MolBiol with the following sequences: Forward: 5'-AGCCCCTGCCTCAAAGTATT-3', Reverse: 5'-AGCCCCTGCCTCAAAGTATT-3'. β-actin was used as a control with primers obtained from Sigma-Aldrich having the following sequences: Forward: 5'-ATGAAGATCCTGACCGAGCGT-3', Reverse: 5'-AACGCAGCTCAGTAACAGT-CCG-3'. Primers were used at a final concentration of 0.6μM. Primers were added to 5X Qiagen OneStep RT-PCR buffer providing a final concentration of 2.5mM $MgCl_2$ in the reaction mix. A final concentration of 400μM of each dNTP was added along with 2.0μl/reaction of enzyme mix. Final mastermix volume was adjusted to 50μl using RNase-free water. Thermal cycler conditions, for both reverse transcription and PCR, was programmed as follows: reverse transcription at 50°C for 30min, initial PCR activation step at 95°C for 15min, followed by 25 cycles of denaturation at 94°C for 1min, annealing at 52°C for 1min and extension at 72°C for 1min followed by a final extension step at 72°C for 10min. 10μl of the PCR products were run on 0.8% agarose gel stained with ethidium bromide at 100V for 30min. The resulting bands were visualized under UV light and photographed. β-actin was used as a loading control.

2.7. Antigen retrieval and immunohistochemistry

Human breast cancer tissues different grades were provided by Dr. Selim Nasser from Clemenceau Medical Center (CMC), Beirut. Tissue blocks were paraffin embedded and sectioned to 8 μm sections using a tissue microtome. Sections were deparaffinized in two changes of xylene 5min each then hydrated in two changes of 95% alcohol 2min each followed by 2 changes of 50% alcohol 2min each. Antigen retrieval was then performed in pre-heated Citra Plus (Biogenex) solution. Tissues were then fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X100 for 10

minutes. To decrease background fluorescence, tissues were rinsed with 0.1 M glycine then incubated with 0.1 M glycine for 10 minutes. For blocking, tissues were incubated 4 times with 1% BSA, 1% FBS in PBS for 5 minutes. Samples were stained with StarD13 primary antibody for 2 hours and with a fluorophore-conjugated secondary antibody for 2 hours. Tissue fluorescent images were taken using a 10X objective on a fluorescent microscope. For image analysis, all digital images were imported in image J software (National Institutes of Health, MA). The total fluorescence intensity of a fixed area from at least 10 different frames from each tissue was determined.

2.8. Trypan blue exclusion method

Cells were grown in 24 well plates (growth area: $2cm^2$) at a density of $2x10^6$ cells/ml. Depending on the experiment, cells were transfected with either StarD13 siRNA or GFP-StarD13 construct. Following treatment period, the supernatant from each well was collected, cells were washed with PBS, and the PBS washes were added to the supernatant of each well. Cells were then trypsinized and collected separately from the well contents and PBS. 20µl from each collection tube was mixed with 20µl of Trypan Blue. 10µl of this mixture was placed in a counting chamber under the microscope, and the number of living and dead cells was recorded accordingly. For each well, two countings were done separately, PBS washes/well supernatant and trypsinized cells. Under the microscope, dead cells appear blue, since they are permeable to Trypan Blue, while viable cells exclude the stain and thus appear bright. The percentage of dead cells was reported.

2.9. Cell proliferation reagent (WST-1)

Cells were seeded in 96 well plates (growth area: 0.6 cm^2) at a concentration of $1x10^6$ cells/ml. Depending on the experiment, cells were transfected with either StarD13 siRNA or GFP-StarD13 construct with appropriate controls. Following treatment period, 10 µl of Cell Proliferation Reagent (WST-1; Roche, Germany) was added to each well. The plates were incubated at in a humidified incubator (37°C) in 95% air and 5% CO_2 for 2 hours. WST-1 is a tetrazolium salt that on contact with metabolically active cells is cleaved to produce formazan dye by mitochondrial dehydrogenases. Quantitation of formazan is done colorimetrically at 450 nm. The absorbance of the each blank well was subtracted from the corresponding sample well.

The results were normalized to the corresponding controls, and the percent of cell proliferation was reported.

2.10. Immunostaining

The cells were plated on cover slips, and the appropriate treatment was applied. Cells were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X100 for 10 minutes. To decrease background fluorescence, cells were rinsed with 0.1 M glycine then incubated with 0.1 M glycine for 10 minutes. For blocking, cells were incubated 4 times with 1% BSA, 1% FBS in PBS for 5 minutes. Samples were stained with primary antibodies for 2 hours and with fluorophore-conjugated secondary antibodies for 2 hours. Fluorescent images were taken using a 60X objective on a fluorescent microscope.

2.11. Pull down assay

Cells were either transfected with GFP-StarD13 construct or an empty GFP construct as a control. Following treatment period, cells were lysed and the pull-down assay performed using the RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell BioLabs) following the manufacturer's instructions. Briefly, cell lysates were incubated with GST-RBD (for RhoA) or GST-PAK (for Rac1/Cdc42) for 1 hour at 4 °C with gentle agitation. Then, the samples were centrifuged, and the pellet washed for several times. After the last wash, the pellets were resuspended with sample buffer and boiled for 5 minutes. GTP-RhoA and GTP-Rac1/Cdc42 were detected by western blotting using anti-RhoA, anti-Rac1 and anti-Cdc42 antibodies provided in the kit. Total proteins were collected prior to the incubation with GST beads and used as a loading control.

2.12. Wound healing assay

Cells were grown to confluence on culture plates and a wound was made in the monolayer with a sterile pipette tip. After wounding, the cells were washed twice with PBS to remove debris and new medium was added. Phase-contrast images of the wounded area were taken at 0 and 24 hours after wounding. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated in μm/hr.

2.13. Motility assay

For motility analysis, images of cells moving randomly in serum were collected every 60 seconds for 2 hours using a 20X objective. During imaging, the temperature was controlled using a Nikon heating stage which was set at 37 °C. The medium was buffered using HEPES and overlayed with mineral oil. The speed of cell movement was quantified using the ROI tracker plugin in the ImageJ software, which was used to calculate the total distance travelled by individual cells. The speed is then calculated by dividing this distance by the time (120 minutes) and reported in μm/min. The speed of at least 15 cells for each condition was calculated. The net distance travelled by the cell was calculated by measuring the distance travelled between the first and the last frames.

2.14. Adhesion assay

96-well plates were coated with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37 °C then washed with washing buffer (0.1% BSA in DMEM). The plates were then blocked with 0.5% BSA in DMEM at 37 °C in a CO2 incubator for 1 hour. This was followed by washing the plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to 4x105 cell/ml. 50 μl of cells were added in each well and incubated at 37°C in a CO2 incubator for 30 minutes. The plates were then shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 minutes, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 minutes. Following the staining with crystal violet, the plates were washed extensively with water, and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550 μm using a plate reader.

2.15. Invasion assay

Cells were transfected with either control or StarD13 siRNAs and invasion assay was performed 48hrs following treatment period using the collagen-based invasion assay (Millipore) according to manufacturer's instructions. Briefly, 24hrs prior to assay, cells were starved with serum free medium. Cells were harvested, centrifuged and then resuspended in quenching medium (without serum). Cells were then brought to a

concentration of $1x10^6$ cells/ml. In the meantime, inserts were prewarmed with 300µl of serum free medium for 30min at room temperature. After rehydration, 250µl of media was removed from inserts and 250µl of cell suspension was added. Inserts were then placed in a 24-well plate, and 500µl of complete media (with 10% serum) was added to the lower wells. Plates were incubated for 24hrs at 37°C in a CO_2 incubator. Following incubation period, inserts were stained for 20min at room temperature with 400µl of cell stain provided with the kit. Stain was then extracted with extraction buffer (also provided). 100ul of extracted stain was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical Density was then measured at 560µm.

2.16. Statistical analysis

All the results reported represent average values from three independent experiments. All error estimates are given as ± SEM. The p-values were calculated by t-tests or chi-square tests depending on the experiment using the VassarStats: Website for Statistical Computation (http://vassarstats.net/).

Chapter III

RESULTS

3.1. Level of expression of StarD13 in breast cancer

Before studying the role of StarD13 in breast cancer cell, we first wanted to investigate its level of expression pattern in human breast cancer tissues. For this, breast cancer tissue sections were obtained from patients representing different grades. We then performed immunohistochemistry using an anti-StarD13 antibody (Figure 11A). The mean fluorescent intensity was then measured using ImageJ software. StarD13 showed a high expression level in non-invasive in situ carcinoma. Then, its level of expression decreased in grades I and II; however, as we moved on to higher grades of the tumor, StarD13 showed a significant increase in its level of expression (Figure 11B).

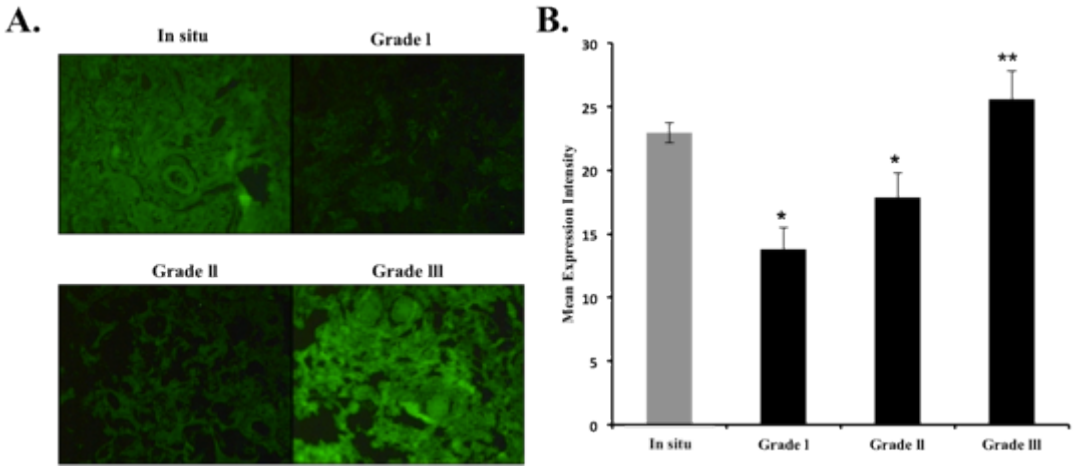


Figure 11: StarD13 expression levels in different grades of breast cancer. A) Representative fluorescent micrographs of formalin-fixed breast caner tissues were paraffin embedded and sectioned and then immunostained with anti-StarD113 antibody: in situ (upper left), grade I (upper right), grade II (lower left) and grade III (lower right). **B)** Quantitation of the immunohistochemistry in A. The mean fluorescent intensity/pixel was measured and expressed to the corresponding tissues. Data are the mean -/+ SEM from 3 different experiments (with 5 tissues each). *$p<0.0001$, **$p<0.04$.

3.2. StarD13 knockdown increases breast cancer cell viability

Next we wanted to investigate the role of StarD13 on cellular proliferation and viability. For this reason StarD13 was knocked down using small interfering siRNA. The resulting inhibition in the level of StarD13 was determined using western blot and RT-PCR in cells transfected with StarD13 siRNA as compared to control cells transfected with non-specific siRNA (Figure 12A). β-actin was used as a loading control. StarD13 knockdown resulted in a 40% decrease of dead cells as determined by trypan blue exclusion method (Figure 12B). Similarly, this was reflected in an increase of 15% in cell proliferation in cells transfected with StarD13 siRNA as opposed to control cells (Figure 12C) as determined using Wst-1 reagent.

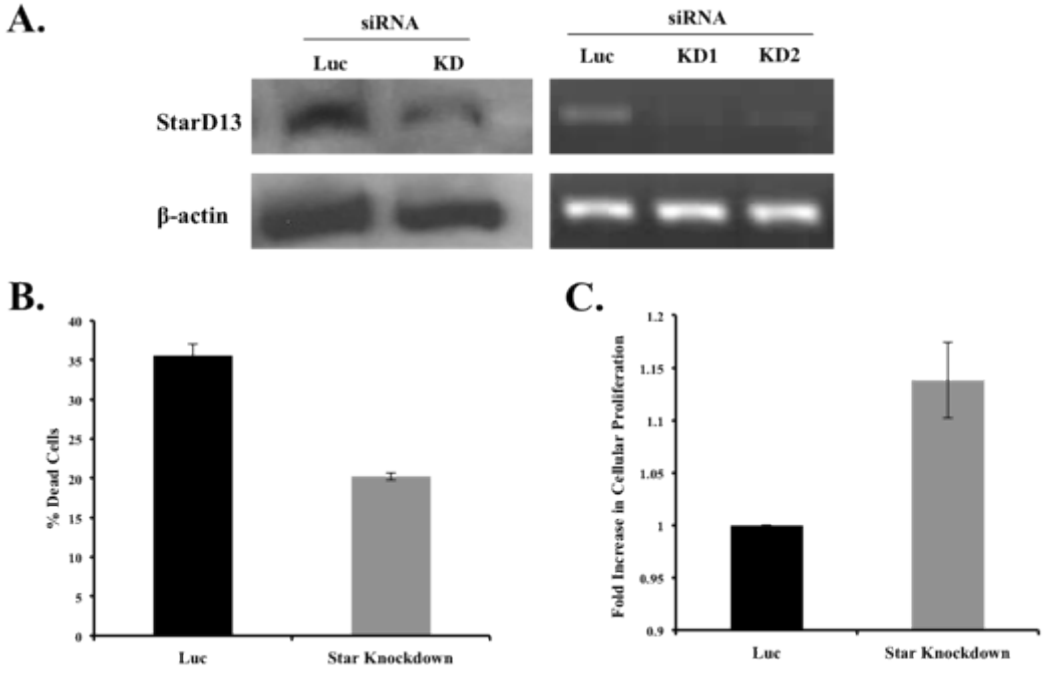


Figure 12: StarD13 under-expression increases cell viability. Cells were transfected with luciferase control siRNA or with StarD13 siRNA for 72 hours. **A)** The **c**ells were lyzed and immunoblotted by western blot analysis for StarD13 (left upper gel) or for actin (left lower gel) for loading control. RT-PCR was also performed to detect StarD13 levels using StarD13 primer (right upper gel) and actin as loading control (right lower gel) **B)** % of dead cells was determined using Trypan blue, results are shown as percent of total number of cells. **C)** Cell proliferation was determined using WST-1 reagent. Cell viability of siRNA-transfected cells was expressed as fold increase from control (luciferase-transfected). Data are the mean -/+ SEM from 3 different experiments.

3.3. StarD13 over-expression decreases breast cancer cell viability

Cells were transfected with a GFP-StarD13 construct and the resulting cell viability was determined as compared to cells transfected with GFP vector alone. Cells overexpressing StarD13 showed a drastic increase in the percentage of dead cells as compared to control cells as determined using Trypan blue (Figure 13A). On the other hand, there was a dramatic decrease in cellular proliferation as determined using the WST-1 reagent (Figure 13B).

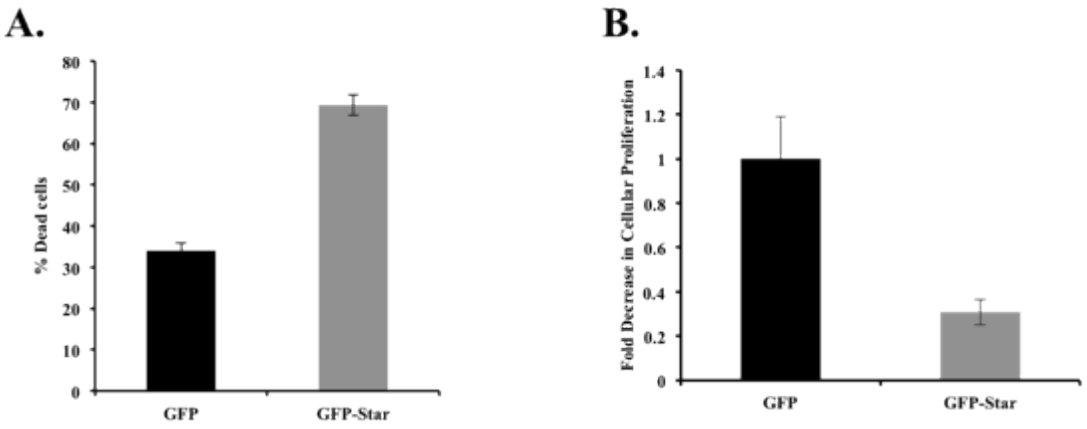


Figure 13: StarD13 over-expression decreases cell viability. Cells were transfected with GFP-StarD13 or GFP alone as control for 24 hours. **A)** The percentage of dead cells showed an increase of 50% as determined using Trypan blue. **B)** Cell proliferation of dramatically decreased in cells transfected cells with GFP-StarD13 construct as opposed to control cells. Data are the mean -/+ SEM from 3 different experiments.

3.4. StarD13 knockdown downregulates tumor suppressors p53 and p21

In an attempt to find the mechanism by which StarD13 knockdown is increasing cellular proliferation, we immunoblotted for two important tumor suppressors p21 and p53. Cells were transfected with a StarD13 siRNA for 72 hours. Cells were lysed and total proteins extracted. Immunoblotting was then performed using anti-p21 and anti-p53 antibodies. Results show a decrease in protein expression levels of both p21 and p53 upon knocking down StarD13 (Figure 14).

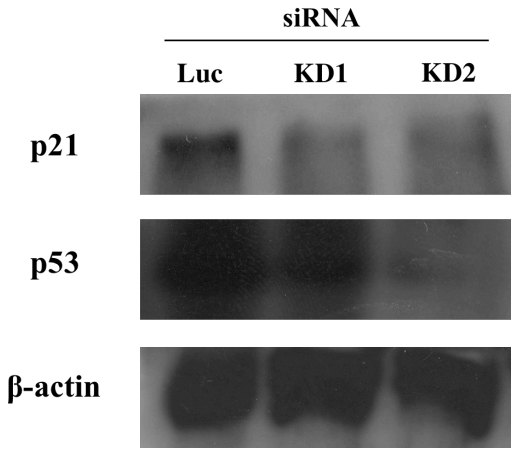


Figure 14: StarD13 knockdown down-regulates tumor suppressors p53 and p21. Cells were transfected with either luciferase control siRNA or with StarD13 siRNA for 72 hours. Cells were then lysed and immunoblotted by western blot analysis using anti-p21 (upper gel) or anti-p53 (middle gel) antibodies. Actin was used as a control (lower gel).

3.5. Role of StarD13 in breast cancer cells

3.5.1. StarD13 localizes to focal adhesions

As described previously, StarD13 localizes to focal adhesions in HeLa cells (Kawai, et al., 2009). To investigate the localization of StarD13 in cells, we transfected cells with GFP-StarD13 vector and detected its localization by fluorescence microscopy. Also, cells were fixed and immunostained using a fluorescent anti-StarD13 antibody (Figure 15)

3.5.2. StarD13 is a GAP for RhoA and Cdc42

StarD13 was also described to act as a GAP for RhoA and Cdc42 but not for Rac (Ching, et al., 2003). Hence, we were interested in determining the effect of the over-expression of StarD13 on the activation of these Rho GTPases in breast cancer cells. For this reason, a pull-down assay was performed to detect the level of activation of RhoA, Cdc42 and Rac1 in cells transfected with the GFP-StarD13 vector and compare it to the level of activation in control cells transfected with an empty vector. The results showed a 5-fold decrease in RhoA activation and a 2.5-fold decrease in the activity of Cdc42 in cells over-expressing StarD13 as compared to control cells (Figure 16A and B). However there was a relative increase in the activity of Rac1 (Data not shown).

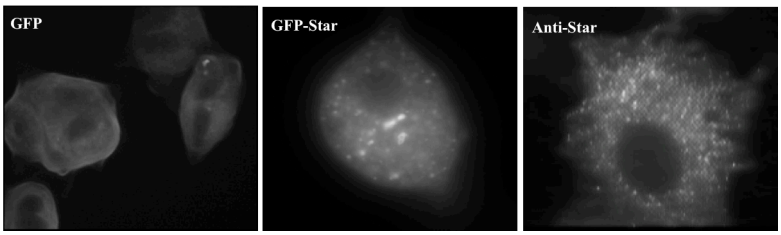


Figure 15: StarD13 localizes to focal adhesions. Cells were transfected with GFP-StarD13 (middle panel) or GFP alone (left panel). Also cells were fixed and immunostained using anti-StarD13 antibody (right panel). Micrographs shown were imaged using a 60x objective.

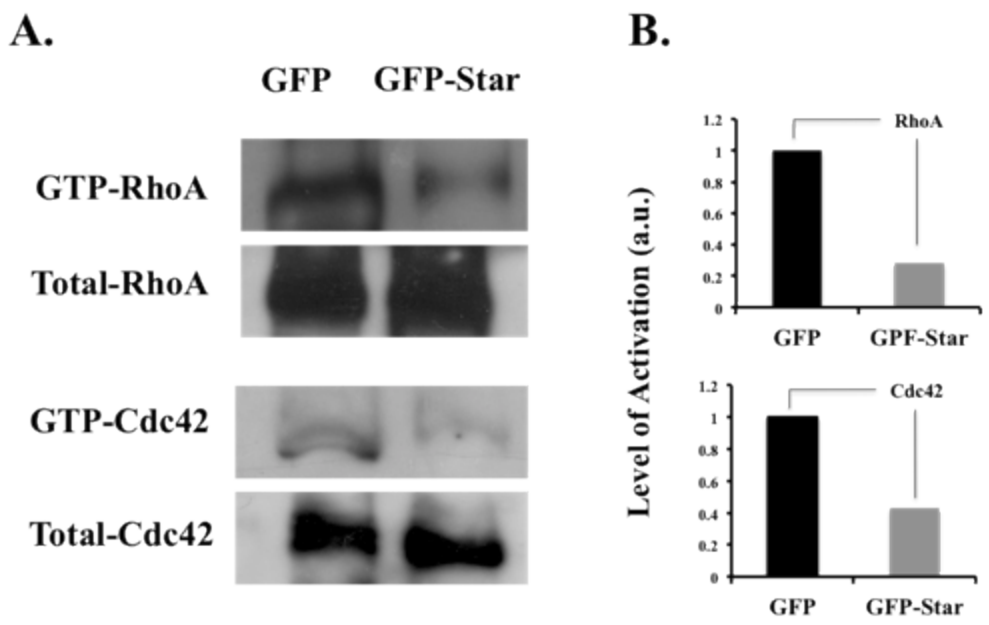


Figure 16: StardD13 is a specific GAP for Rho and Cdc42. **A)** Cells were transfected with either GFP alone (right lanes) or with GFP-StarD13 (left lanes). The cells were then lyzed and incubated with GST-RBD (Rhotekin binding domain) (upper pannels), or with GST-CRIB (Cdc42 and Rac interactive binding domain) (lower panels) to pull down active Rho and Cdc42 respectively. The samples were then blotted with Rho, and Cdc42 antibodies. The lower gels in each panel are western blots for the total cell lysates for loading control. **B)** The bands from the active RhoA and Cdc42 gels were quantitated using ImageJ and normalized to the amount of total proteins.

3.6. RhoA knockdown inhibits cell motility

After establishing that StarD13 localizes to focal adhesions of the cells and acts as a RhoGAP, we wanted to investigate the role of RhoA in breast cancer cell motility. For this purpose, RhoA was knocked down using a specific siRNA. The resulting expression level was detected by western blot in cells transfected with RhoA siRNA as compared to control cells transfected with non-specific siRNA (Figure 17A). The effect of RhoA knockdown was determined by wound healing assay and the cell speed was also assessed using time-lapse motility assay. Results showed a decrease of nearly three-folds in the rate of wound closure in cells transfected with RhoA siRNA (5.2 μm/hr) as compared to control cells (14.8 μm/hr) (Figure 17B and C). This was also reflected by a decrease in the average cell speed determined by time-lapse assay, from 0.41 μm/min in controls to 0.16 μm/min (Figure 18A and B; supplemental movie S1).

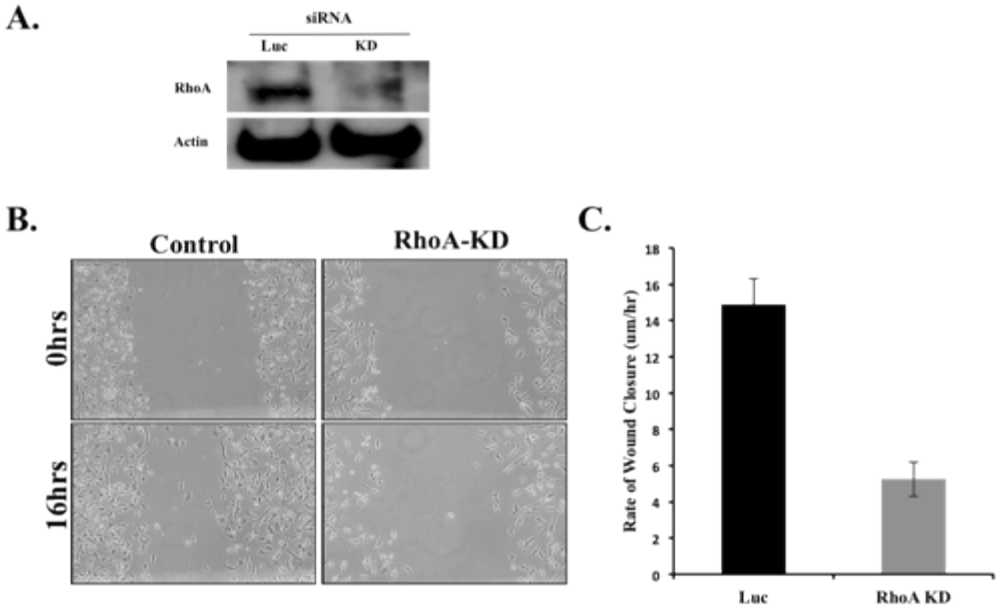


Figure 17: RhoA is required for cell motility-1. Cells were transfected with luciferase control siRNA or with RhoA siRNA for 72 hours. **A)** The cells were lyzed and immunoblotted by western blot analysis for RhoA (upper gel) or for actin (lower gel) for loading control. **B)** The luciferase siRNA-transfected and RhoA siRNA-transfected cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 16 hours (lower micrographs). **C)** Quantitation for B). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure for the luciferase and the Rho siRNA-transfected cells was calculated in μm/hr. Data are the mean -/+ SEM from 3 wound closure assays. The results had borderline significance with $p<0.05$.

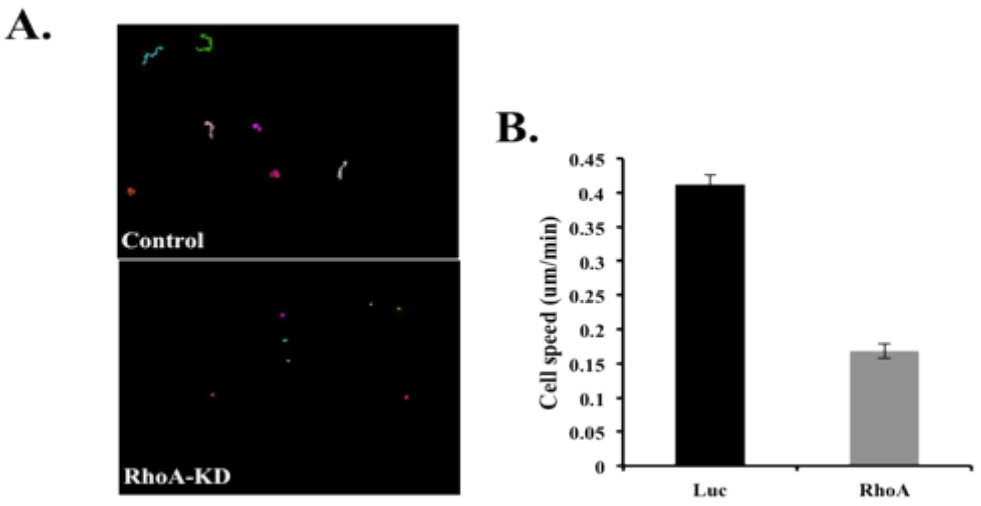


Figure 18: RhoA is required for cell motility-2. Cells were transfected with luciferase control siRNA or with RhoA siRNA for 72 hours. **A)** The net paths of projected 120 frames from 2 hour long time lapse movies of cells undergoing random motility in serum (different colors represent different cells) **B)** Quantitation of the cell speed from A) expressed in μm/min. Data are the mean -/+ SEM from 20 cells. The results were significant with $p<0.001$.

3.7. Role of StarD13 in cell motility

After establishing that StarD13 localized to focal adhesions where it has a GAP activity to Rho and Cdc42, we next wanted to investigate its role in cellular motility. For this reason, StarD13 was knocked down using siRNA oligonucleotides. The knockdown was confirmed by western blot and RT-PCR (Figure 12A). Results show that StarD13 knockdown decreased the rate of wound closure from 14 μm/hr to 7 μm/hr (Figure 19A and B). Furthermore, StarD13 knockdown significantly decreased the average speed of individual cells from 0.41 μm/min to 0.20 μm/min (Figure 19B and C). Altogether these results show that the knockdown of StarD13 inhibits breast cancer cell motility.

Looking at the morphology, cells were observed be stuck and not able to detach their tail in order to move forward (Figure 20A; supplemental movie S2)

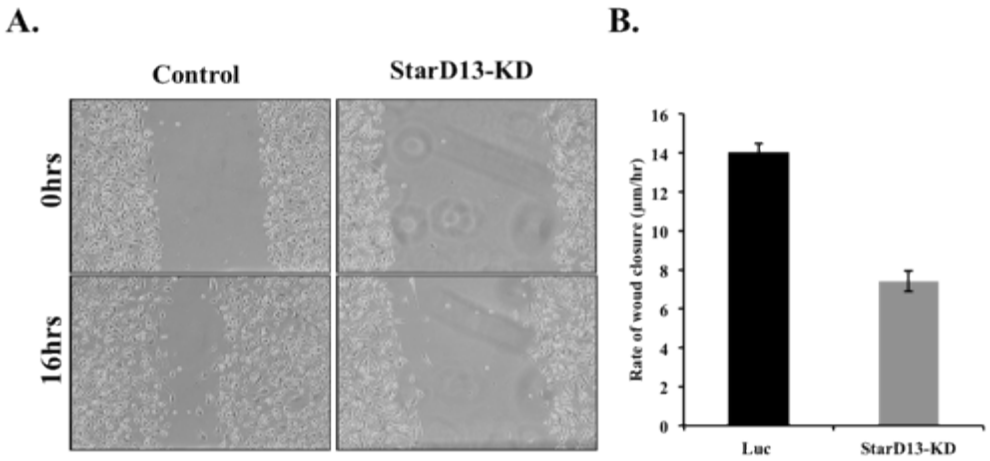


Figure 19: StarD13 is required for cell motility-1. Cells were transfected with luciferase control siRNA or with StarD13 siRNA for 72 hours. Cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 16 hours (lower micrographs). **B)** Quantitation for A). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure for the luciferase and the StarD13 siRNA-transfected cells was calculated in μm/hr. Data are the mean -/+ SEM from 3 wound closure assays. The results were significance with $p<0.01$.

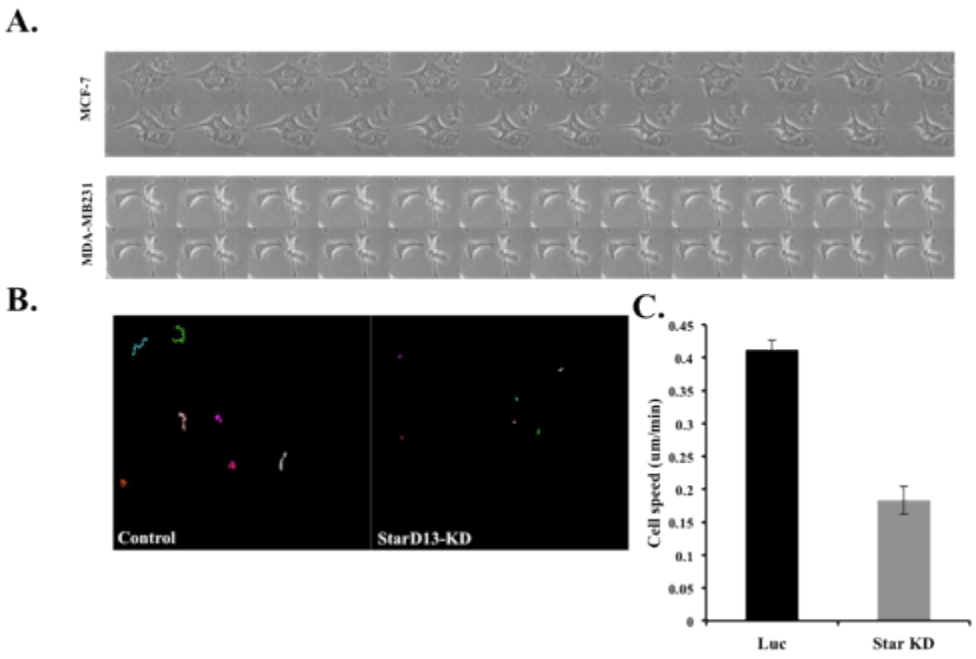


Figure 20: StarD13 is required for cell motility-2. Cells were transfected with luciferase control siRNA or with StarD13 siRNA for 72 hours. **A)** Montage of time-lapse movie (60 sec apart) showing StarD13 siRNA-transfected cells undergoing random motility in serum (MCF-7: upper panel, and MDAs: lower panel). **B)** The net paths of projected 120 frames from 2 hour long time lapse movies of cells undergoing random motility in serum (different colors represent different cells) **C)** Quantitation of the cell speed from A) expressed in μm/min. Data are the mean -/+ SEM from 20 cells. The results were significant with $p<0.001$.

3.8. Dominant active RhoA inhibits cell motility

After showing that RhoA knockdown inhibits cell motility and that StarD13 knockdown, where Rho is kept active, also inhibits cell motility it was of great interest to us to determine the effect of overexpressing a constitutively active form of RhoA. Thus, similar to StarD13 knockdown, dominant active RhoA suppressed cellular motility. This was observed through wound healing assay where the rate of wound closure was decreased from 12.3 μm/hr to 3.4 μm/hr (Figure 21).

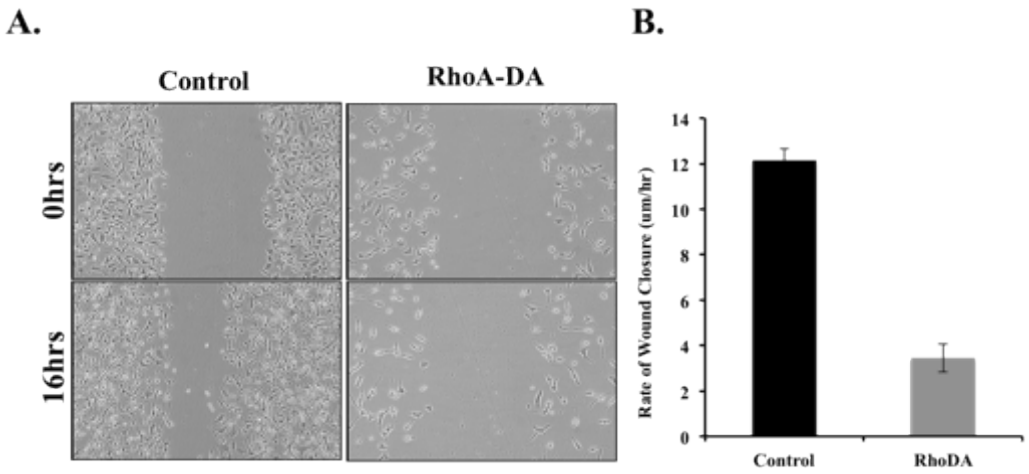


Figure 21: Constitutively active RhoA inhibits cell motility. Cells were left untransfected or transfected with a dominant active RhoA construct (RhoA DA). **A)** Cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 16 hours (lower micrographs). **B)** Quantitation for A). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated in μm/hr. Data are the mean -/+ SEM from 3 wound closure movies. The results were significant with $p<0.001$.

3.9. Dynamics of focal adhesions

After establishing the role of StarD13 in cell motility, we were interested in looking directly at focal adhesion. For this reason, we immunostained for Paxillin, a component of both focal complexes and focal adhesions (Nobes & Hall, 1999) using anti-paxillin antibody.

3.9.1. Rac is needed for the formation of focal complexes

Rac1 was knocked down using specific siRNA and stained for actin, nucleus and paxiliin. When observed under a fluorescent microscope, neither focal complexes nor focal adhesions were visible at the cell edge (Figure 22).

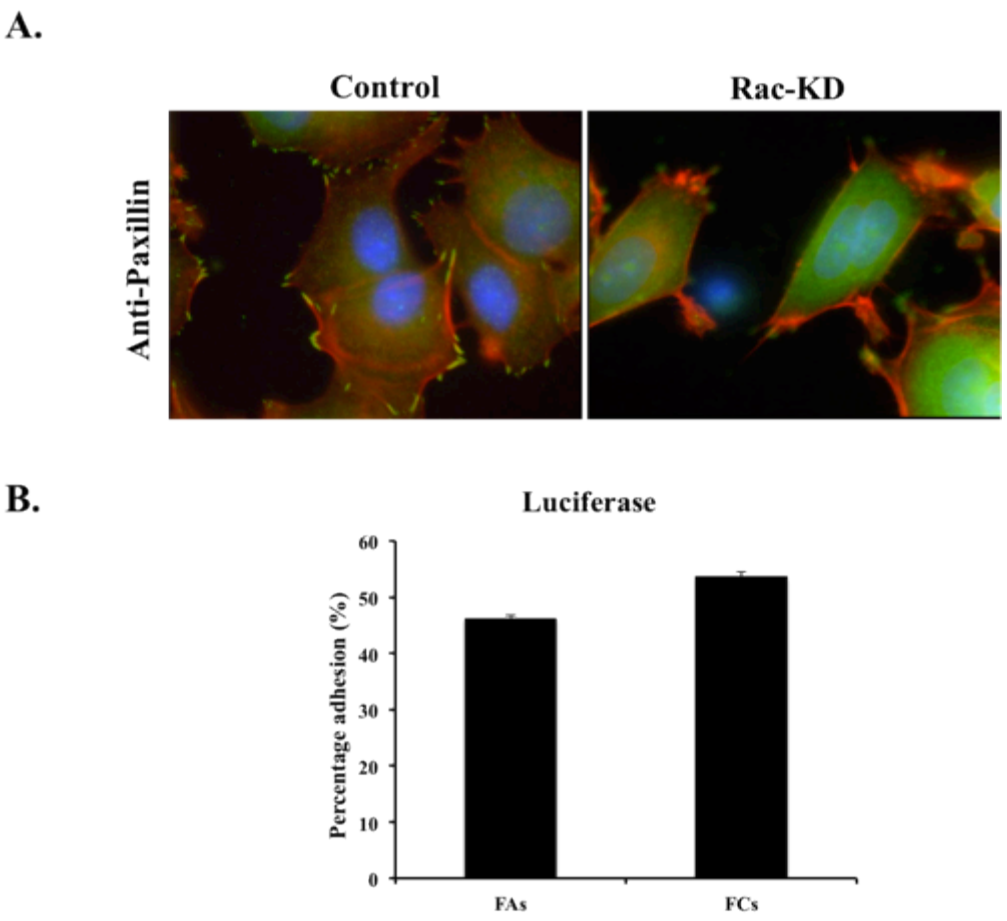


Figure 22: Rac is needed for formation of focal complexes. A) Representative micrographs of (MCF-7) cells that were transfected with either luciferase (left) or Rac siRNA (right) fixed and immunostained with anti-paxillin antibody. Cells were imaged using a 60x objective. **B)** Quantitation for A) represented as percentage of focal adhesions and focal complexes in control cells. Data are the mean -/+ SEM from 15 cells. Results showed borderline significance with $p<0.04$.

3.9.2. RhoA is needed for the maturation of focal adhesions

It was previously reported that RhoA is needed for the maturation of focal complexes into focal adhesions (Arjonen, et al., 2011; Wolfenson, et al., 2009). Thus, to verify this role in our system, RhoA was silenced it in breast cancer cells and stained for paxillin. Indeed, focal adhesions were less prevalent instead small punctate structures were highly present representing immature focal complexes (Figure 23A). This was reflected in a reduction of the percentage of focal adhesions from 46.3% in control cells to 20.9% in cells with RhoA knockdown. On the contrary, the percentage of focal complexes increased from 53.7% in control cells to 79.1% in cells transfected with RhoA siRNA (Figure 23B).

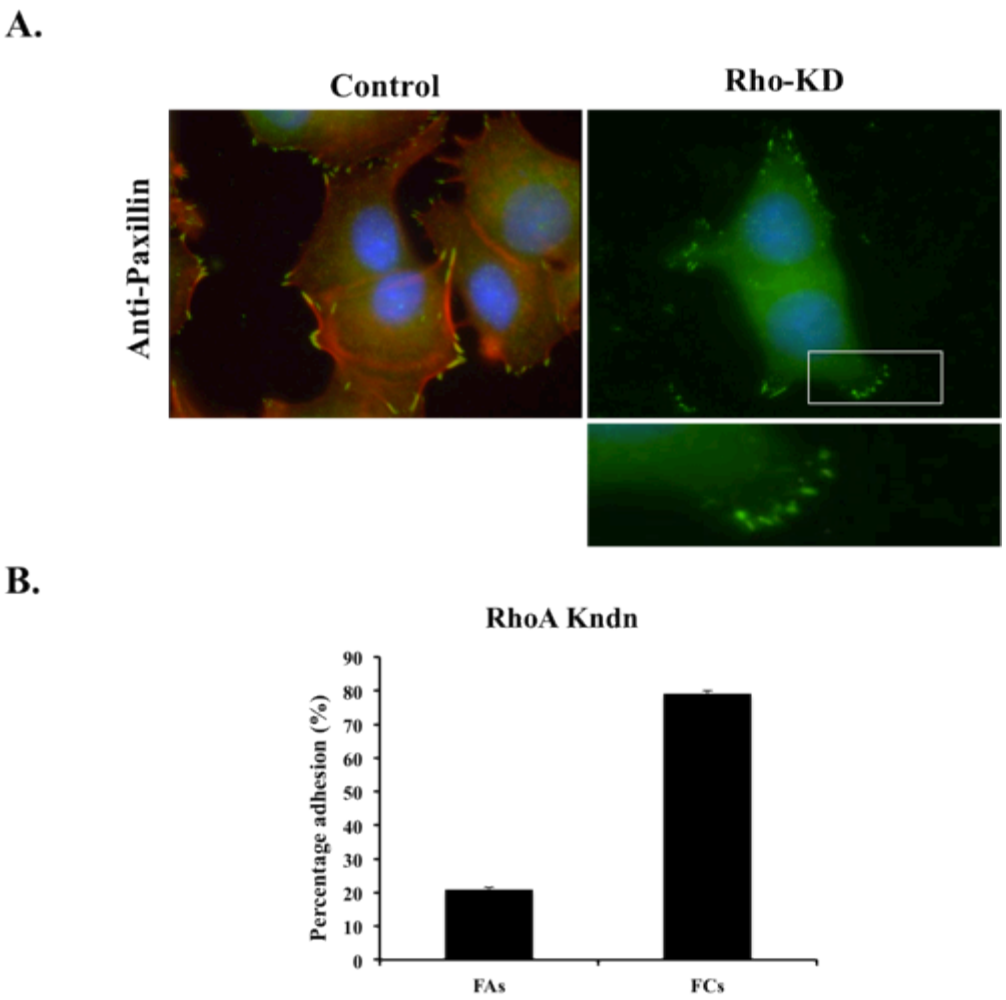


Figure 23: RhoA is needed for the maturation of focal complexes. A) Representative micrographs of (MCF-7) cells that were transfected with either luciferase (left) or RhoA siRNA (right) fixed and immunostained with anti-paxillin antibody. Cells were imaged using a 60x objective. **B)** Quantitation of A) represented as percentage of focal adhesions and focal complexes in cells with RhoA knockdown. Data are the mean -/+ SEM from 15 cells. The results were significant with $p<0.0001$.

3.9.3. StarD13 knockdown stabilizes focal adhesions

To investigate the effect of StarD13 on the dynamics of focal adhesions, we then transfected cells with StarD13 siRNA and stained for paxillin. Focal adhesions in these cells were more pronounced and more stable especially at the cell edges as compared to control cells (Figure 24A). Qunatitatively, 70.1% of adhesion structures were focal adhesions as opposed to 29.9% representing focal complexes (Figure 24B).

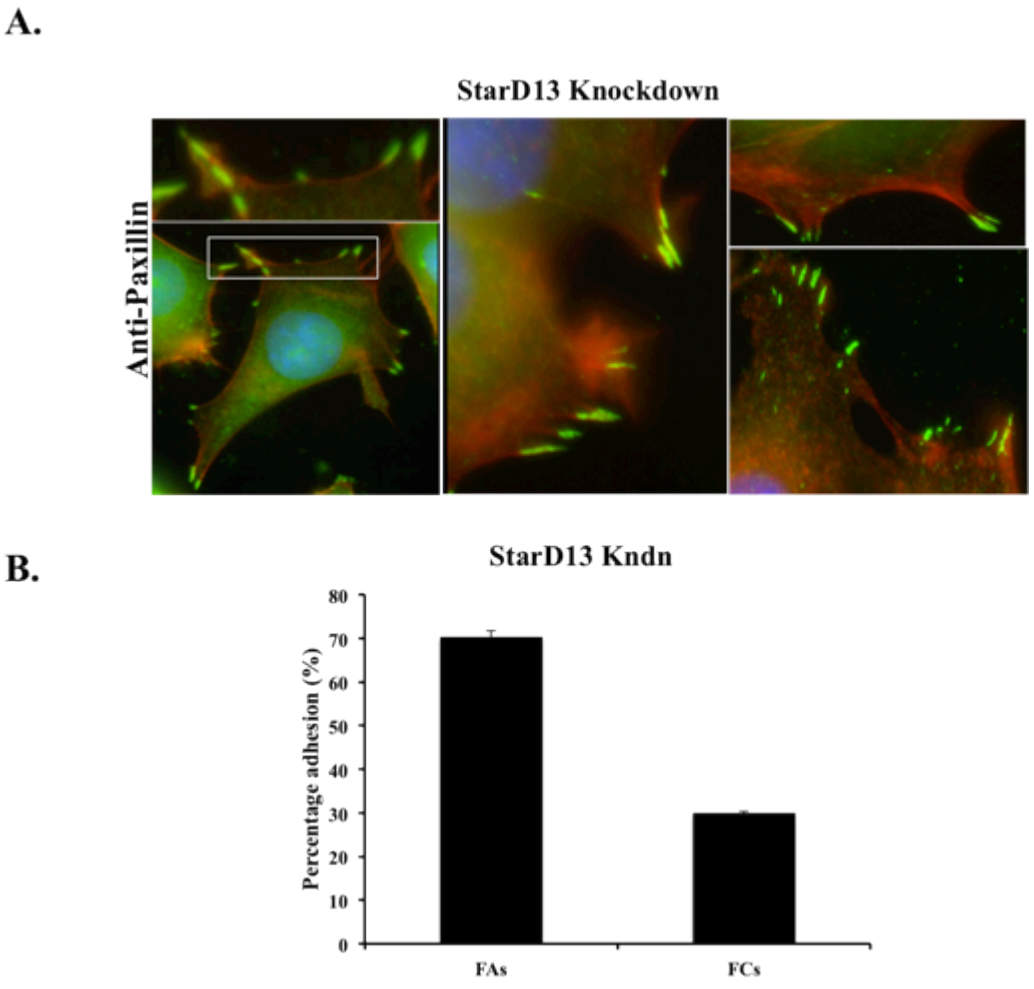


Figure 24: StarD13 knoockdown stabilizes focal adhesions. A) Representative micrographs of (MCF-7) cells that were transfected with StarD13 siRNA fixed and immunostained with anti-paxillin antibody. Cells were imaged using a 60x objective. **B)** Quantitation of A) represented as percentage of focal adhesions and focal complexes in cells with StarD13 knockdown. Data are the mean -/+ SEM from 15 cells. The results were significant with $p<0.0001$.

3.9.4. RhoDA mimics StarD13 phenotype stabilizing focal adhesions

In order to verify our hypothesis, similar to StarD13 knockdown, transfecting the cells with a dominant active form of RhoA lead to the stabilization of focal adhesions (Figure 25A). This was also reflected by a difference of more than 50% between the amounts of focal adhesions and focal complexes. The majority of adhesion structures in cells over-expressing a constitutively active RhoA were actually focal adhesions representing 77% as compared to 23% representing focal complexes (Figure 25B).

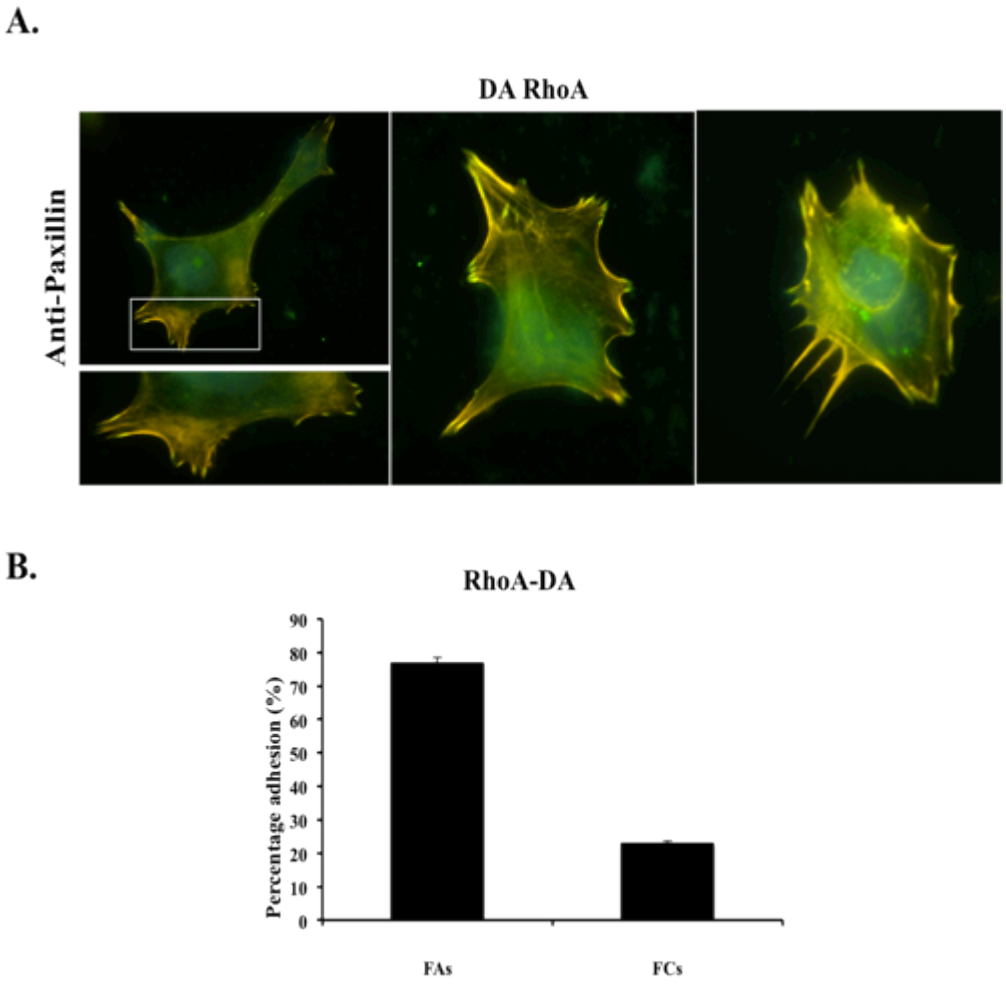


Figure 25: RhoDA mimics StarD13 knockdown phenotype A) Representative micrographs of (MCF-7) cells that were transfected with RhoA-DA contruct fixed and immunostained with anti-paxillin antibody. Cells were imaged using a 60x objective. **B)** Quantitation of A) represented as percentage of focal adhesions and focal complexes in cells with RhoA-DA knockdown. Data are the mean -/+ SEM from 15 cells. The results were significant with $p<0.0001$.

3.10. StarD13 knockdown increases cellular adhesion to collagen

Since it was shown to increase focal adhesion formation and stabilization, we next aimed at studying the effect of StarD13 knockdown on the adhesion of breast cancer cells to collagen. The results showed more than two-fold increase in the adhesion of cells with the StarD13 knockdown as compared to control cells (Figure 26A and B).

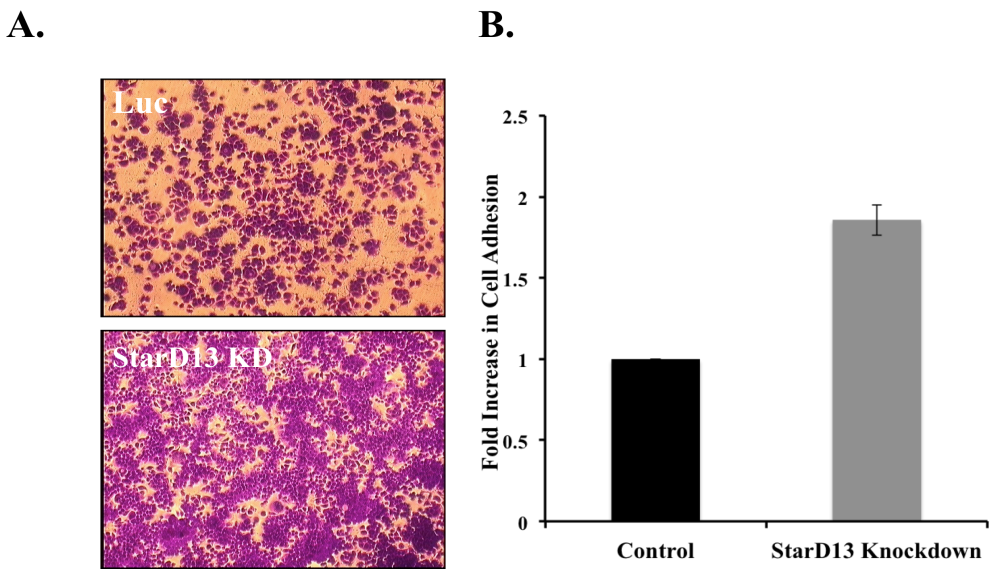


Figure 26: StarD13 knockdown increases cellular adhesion to collagen. A) Representative micrographs of cells fixed and stained with crystal violet to detect adhesion (as described in methods). **B)** Quantitation of A) Crystal violet was solubilized and the absorption of the plates was read at 550 μm using an ELISA plate reader. Data is measured in arbitrary units and normalized to the luciferase control. Data are the mean -/+ SEM from 3 experiments. The results were significant with $p<0.001$.

3.11. StarD13 knockdown increases cellular invasion

After establishing the role of StarD13 in 2D cell migration, we were interested in determining its role in 3D cell invasion. For this reason, we performed an in vitro collagen-based invasion assay using FBS as a chemoattractant. A chamber with serum free media in both wells was used as negative control. To our surprise, unlike its effect in 2D, there was nearly a half-fold increase in cell invasion in cells with StarD13 knockdown as compared to control cells (Figure 27A and B).

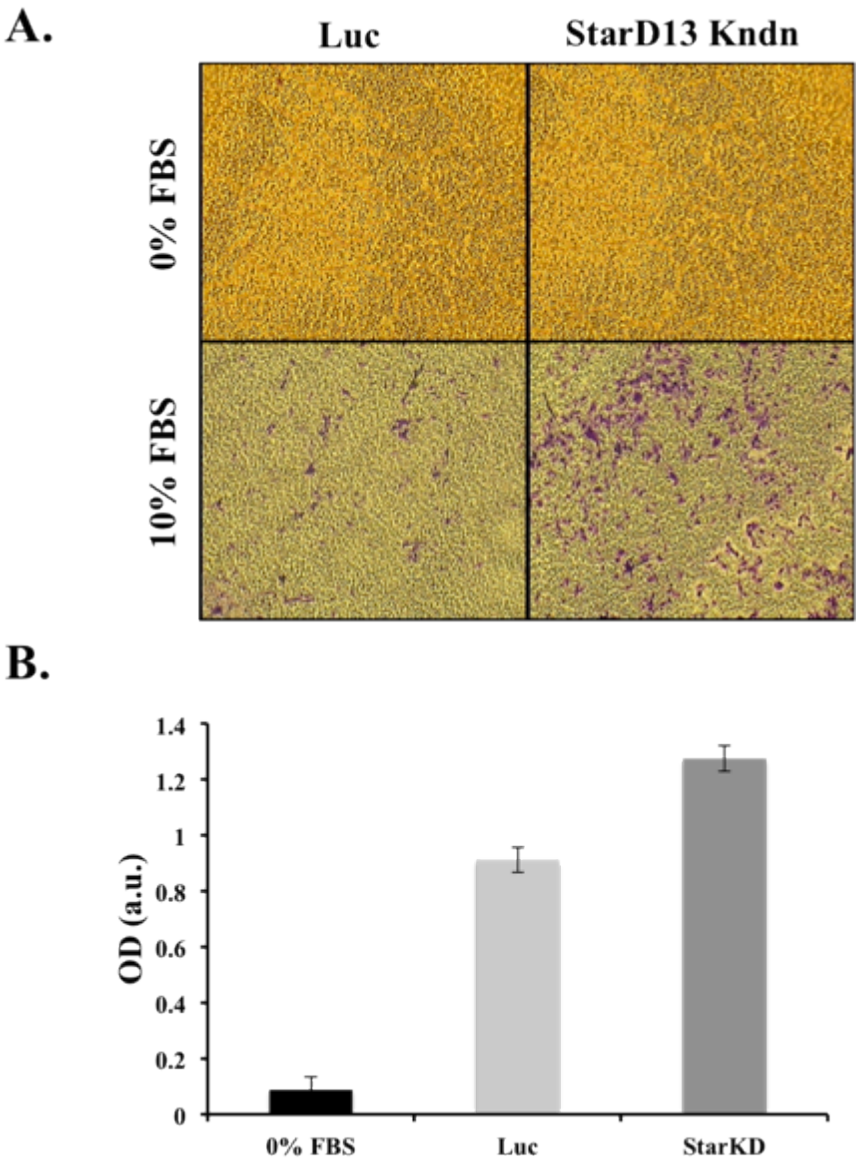


Figure 27: StarD13 knockdown increases cellular invasion. A) Representative micrographs of invaded cells on the bottom side of the membrane stained with cell stain according to assay instructions. Cells with StarD13 knockdown and control cells were allowed to invade towards 10% FBS for 24hrs. $1x10^6$ cells/ml were used in each assay. **B)** Cell stain was extracted and colorimetric measurements were taken at 560 μm. Data is measured in arbitrary units. Data are the mean -/+ SEM from 3 experiments. The results were significant with $p<0.02$.

Chapter IV

DISCUSSION

Previous studies by Ching et al. (2003) identified StarD13 as a tumor suppressor gene in hepatocellular carcinoma cells. Later, Kawai et al. (2009) revealed that StarD13 actually localizes to focal adhesion in HeLa cells. However, in the current study, we provide an overall characterization of StarD13 in breast cancer in terms of expression, effect on cell proliferation and viability, localization, GAP activity and role in motility and invasion.

Looking at its level of expression by IHC we revealed that StarD13 is highly expressed in non-tumor in situ form of breast cancer and it is downregulated in grades I and II; confirming its role as a tumor suppressor. This was in accordance with previous studies where StarD13 was found to be under-expressed in several cancer types including lung, colon, gastric, ovarian, uterine, renal and rectal tumors (Ullmannova & Popescu, 2006). However to our utmost surprise, StarD13 showed a relatively high expression in highly metastatic forms of breast cancer. This was in accordance with a previous study conducted in our lab on astrocytoma where we showed StarD13 to over-expressed in grades III and IV as compared to grades I and II (El-Sitt, et al., 2012). This brought us to the hypothesis that although being a tumor suppressor, StarD13 might be needed for cellular motility. Thus, we considered an in vitro model of breast cancer cell lines to look at the effect of StarD13 on cellular proliferation and viability. The over-expression of StarD13 in breast cancer cell lines, using a GFP-construct led to an increase in the percentage of dead cells and a decrease in cell viability as determined by typan blue exclusion and WST-1 proliferation assay, respectively. Moreover, silencing StarD13 using specific siRNAs led to a decrease in cell death and an increase in cellular viability. This was consistent with a previous study in our lab done on astrocytoma cell lines. In this study, StarD13 was knocked down in astrocytoma cell lines using specific siRNA. Trypan blue exclusion method and WST-1 proliferation assay revealed a decrease in the percentage of dead cells and a relative increase in cellular viability. Conversely, an increase in dead cells and a decrease in cellular viability is observed upon transfecting asctrocytoma cells with StarD13-GFP construct (El-Sitt, et al., 2012). Therefore, consistent with the literature, StarD13 seems to play a tumor suppressor

function in breast cancer cells. However, in order to explain our IHC results, we next wanted to determine its probable role in cellular motility.

In an attempt to explain how the silencing of StarD13 is affecting cellular proliferation, we immunnoblotted for p21 and p53 tumor suppressor genes. Our results showed that silencing StarD13 down-regulated the level of expression of both p21 and p53 proteins. This suggests a possible mechanism by which StarD13 knockdown is promoting cellular proliferation through down-regulating p21 and p53 tumor suppressor genes.

As mentioned earlier, previous studies showed that StarD13 harbors a GAP domain(Ullmannova & Popescu, 2006) and localizes to focal adhesions (Kawai, et al., 2009). In our system, we confirmed StarD13 localization to focal adhesions in breast cancer cells. Moreover, we showed that it indeed functions as GAP where we observed a decrease in the activation of both RhoA and Cdc42 in cells over-expressing StarD13. Studies done in hepatocellular carcinoma showed that under-expression of StarD13 was associated with an over-expression of RhoA (Xiaorong, Wei, Liyuan, & Kaiyan, 2008) thus supporting our findings. These data collectively suggest that StarD13 functions in focal adhesion of the cells where it might have a role in regulating RhoA and affecting cellular motility.

Therefore, we were interested to determine first the effect of RhoA itself, on breast cancer cell motility. Our data showed that silencing RhoA resulted in the inhibition of cell migration. This indicates that RhoA might play a role in breast cancer progression and metastasis through contributing to cell motility. Our results are consistent with previous studies that showed that RhoA knockdown inhibits cell motility and invasion of MDA-MB231 cells (Wu, Wu, Rosenthal, Rhee, & Merajver, 2010). Vega et al. (2011) also showed that silencing of both RhoA and RhoC inhibited cellular motility in breast cancer cells. This implies that RhoA seems to play a positive role in promoting breast cancer cell motility. Moreover, inhibition of downstream effector ROCK inhibits cell motility in various cell systems (El-Sibai, et al., 2008; Ridley, 2001; Worthylake & Burridge, 2003).

Being a GAP for RhoA, we expected that StarD13 might be essential for cellular motility in breast cancer. Indeed, knockdown of StarD13 in breast cancer cell lines inhibited cell motility. This may explain the increase of StarD13 expression in grades III

obtained in IHC and verify our hypothesis that although being a tumor suppressor, this protein is needed for motility. Moreover, looking at their morphology, cells were stuck unable to detach their tail in order to retract their cell body and move forward (Figure 20A). This was consistent with previous studies in our lab, where StarD13 knockdown inhibited migration of astrocytoma cells. This was also reflected by an increase in cellular attachement through stabilization of focal adhesions. Also, StarD13 was shown to play an omportant role in the regulation of RhoA activity in astrocytoma cells (data not published). However, contradictory to our results, a previous study on normal endothelial cells reported that the inhibition of StarD13 led to an increase in cell migration (Y. Lin, et al., 2010). This discrepancy can be explained by the fact that this study was conducted in normal cell systems vastly different from breast cancer cells used in our study that typically display distinctive cellular morphology and altered signaling pathways.

RhoA has been extensively proven to be indispensible for the formation of focal adhesions (Arjonen, et al., 2011; Wolfenson, et al., 2009) and that increasing Rho activation stabilizes focal adhesions inhibiting cell motility (Cox, et al., 2001; Sander, et al., 1999). Thus, we formulated the hypothesis that StarD13 knockdown is keeping RhoA active in focal adhesions and it was of great interest to us to investigate the mechanism by which it is affecting cell motility. We started by looking at the dynamics of cellular adhesion following Rac and RhoA knockdowns. In cells with Rac knockdown, neither focal complexes nor focal adhesions were observed (Figure 21). This is in accordance with the fact that Rac is needed for the formation of focal complexes (Kaverina, et al., 2002). Moreover, in cell under-expressing RhoA showed inability to form mature focal adhesions (Figure 23). Similarly previous studies done on MTLn3 cells showed that inhibition of RhoA downstream effector ROCK blocked the maturation of focal adhesions in MTLn3 cells (El-Sibai, et al., 2008). However, silencing StarD13 led to the stabilization of focal adhesions and decrease in focal complexes (Figure 24). Hence, we suspected that cells with StarD13 knockdown, seem to have a constitutively active RhoA stabilizing cellular adhesion to the underlying substratum and impeding tail retraction resulting in inhibition of cell motility. It is known that the completion of the cell motility cycle requires the disassembly of focal adhesions, a process involving the inhibition of RhoA. Thus, we suspect that StarD13 actually plays a role in inhibiting RhoA leading to the detachment of the cell. Our data is strengthened by previous studies done on MDA-MB231 breast cancer cell lines where silencing RhoA

led to reduced stress fibers formation and a drastic decrease in focal adhesion formation (Wu, et al., 2010). This hypothesis was also reinforced by the increase of cellular adhesion to collagen I upon knocking down StarD13 (Figure 26). Relevantly, Wu et al. (2010) showed that silencing RhoA in MDA-MB231 cell lines decreased cellular adhesion to collagen I.

To further strengthen our hypothesis, we suspected that over-expressing a dominant active form of RhoA would mimic StarD13 knockdown phenotype in cell motility and adhesion. Indeed, transfecting the cells with a constitutively active RhoA inhibited cell motility and cells showed larger and more abundant focal adhesions relative to focal complexes (Figure 25). Similar studies involving the use of dominant active RhoA have demonstrated an inhibition of cellular motility (Banyard, Anand-Apte, Symons, & Zetter, 2000; Li, et al., 2010; Tkach, Bock, & Berezin, 2005; Vial, Sahai, & Marshall, 2003).

After determining the mechanism by which StarD13 might affect random 2D cell motility, it was intriguing to us to study its effect on cellular invasion in 3D. For this we transfected the cells with siRNA against starD13 and performed collagen-based transwell invasion assay. Knowing that StarD13 knockdown inhibited cellular motility in 2D it was assumed that it would also inhibit cell invasion. However, to our ultimate surprise, silencing StarD13 had a positive effect on cellular invasion, despite the fact that StarD13 knockdown stabilizes focal adhesions. This might be explained by the hypothesis that focal adhesions may play an unconventional role in cellular invasion. In fact, a recent report studied the contribution of focal adhesions to matrix degradation. Results revealed that several cell lines degraded underlying ECM specifically at focal adhesion sites. This process occurred through proteolytic activity of MMPs and not due to physical tension exerted by FAs onto the matrix (Y. Wang & McNiven, 2012). Moreover, other studies demonstrated that silencing RhoA leads to the inhibition of cellular invasion, particularly in breast cancer cell lines (Pille, et al., 2005; Wu, et al., 2010). This solidifies our data with starD13 knockdown where we typically have an increase in RhoA activity, thus promoting cellular invasion. However, as shown by Vega et al. (2011) depletion of RhoA in breast cancer cells resulted in the formation of elongated protrusions promoting cellular invasion. This implies that this aspect remains highly controversial and subject to various discrepancies. Moreover, it was previously discovered that in 3D matrices, tumor cells are able to switch between distinct modes of

motility (Sahai & Marshall, 2003). This pertains to interplay between different signaling requirements. Thus, cells can switch between a rounded blebbing movement and a more elongated protrusive fashion. Thus in our study, the depletion of StarD13 increased cellular adhesion to the ECM impeding 2D mesenchymal cellular migration; however, this was reflected in an increase in 3D movement. This suggests that when cells cannot move in an adhesion-dependent manner, they tend to switch to a more amoeboid fashion. This is also reflected by interplay between Rho/ROCK dependent and independent manner (Sahai & Marshall, 2003). Therefore, the ability of tumor cells to switch between modes of motility may limit the effectiveness of prospective inhibitory strategies targeting particular cell morphology, hence promoting the selection of a different mode to escape inhibition.

Prospective work includes investigating the mechanism by which StarD13 affects cellular viability and proliferation. This includes examining its effect on the cell cycle, in which we can actually detect whether StarD13 knockdown effect is through inducing cell cycle arrest or promoting cells to undergo apoptosis. Moreover, it would be interguing to us to study how StarD13 knockdown is promoting cellular invasion. This includes looking at markers for invasive phenotype including the production of MMPs and matrix degradation. This also involves examining the formation of invadopodial structures and the contribution of focal adhesios in matrix degradation process. This could even be taken a step further through the development of an *in vivo* 3D model and studying the role of StarD13 in tumor metastasis and invasion. This includes the role of StarD13 in intravasation and the ability of the tumor to reach secondary sites.

Chapter V

CONCLUSION

Although previously known as a tumor suppressor gene product, in the current study we described for the first time the role of StarD13 in breast cancer proliferation and motility. Our results showed that StarD13 negatively affects cellular proliferation. However, having a RhoGAP activity and localizing to focal adhesions of the cell, we showed that StarD13 actually plays an essential role in cellular motility. This correlated to the increase in its expression in metastatic forms of the tumor. In this context, looking at the dynamics of focal adhesion, StarD13 seemed to be involved in the inhibition of RhoA following the maturation of FAs that results in the detachment and forward movement of the cell. This is depicted below in our proposed model (Figure 28). Upon silencing StarD13 using siRNA, cells showed elongated tail morphology with stabilized focal adhesions and inhibitory cellular motility. However, this was not correlated in 3D mode, where we saw that cells with StarD13 knockdown seemed to have an enhanced invasive ability (Figure 29).

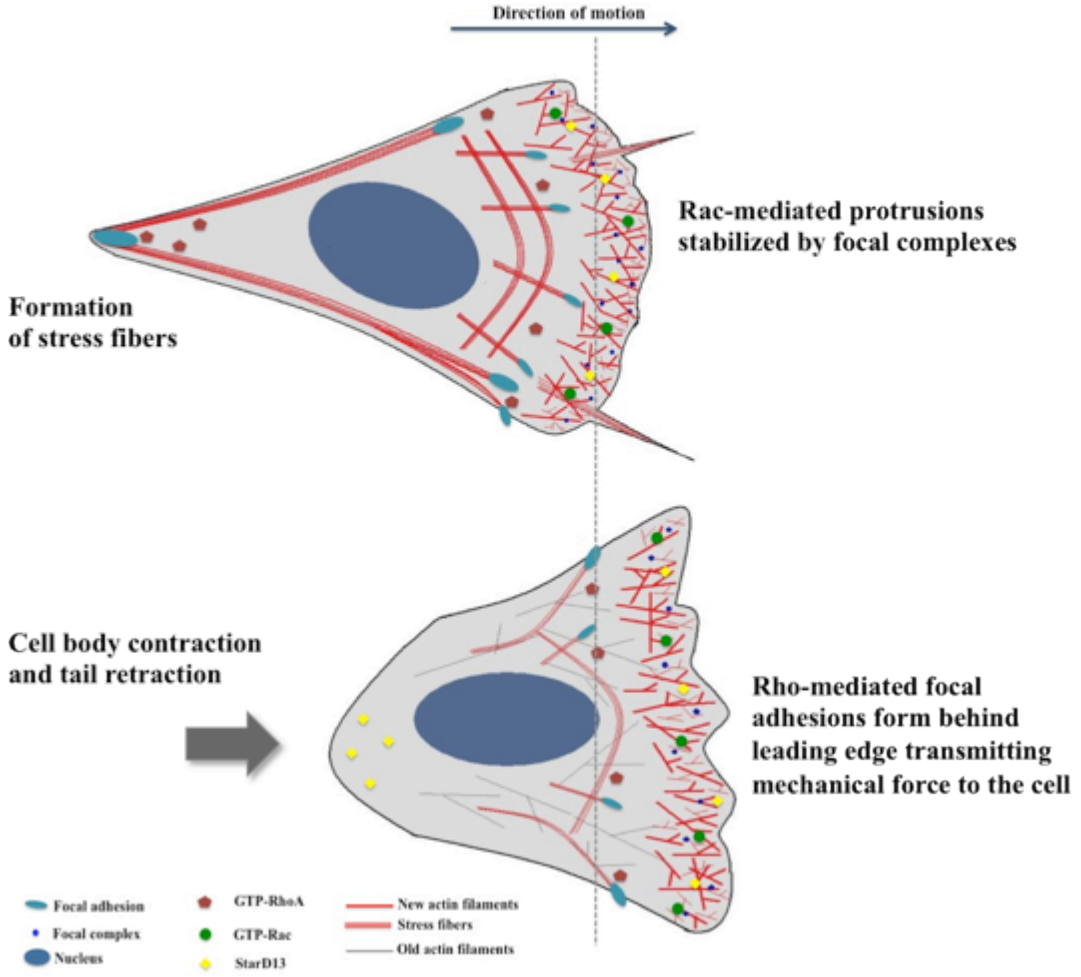


Figure 28: Model-1: Regulation of cell motility. Rac1 is active at the leading edge of the cell driving the formation of actin-rich lamellipodia and focal complexes. StarD13 keeps RhoA inactive at the front restricting it to an area behind the leading edge. RhoA is also active at the rear end of the cell inducing stress fibers formation. As the cell moves forward, RhoA activation causes the maturation of focal complexes into focal adhesions and leads to the contraction of the cell body (lower panel). StarD13 plays a role in the inactivation of RhoA, driving the disassembly of focal adhesions and allowing tail detachment.

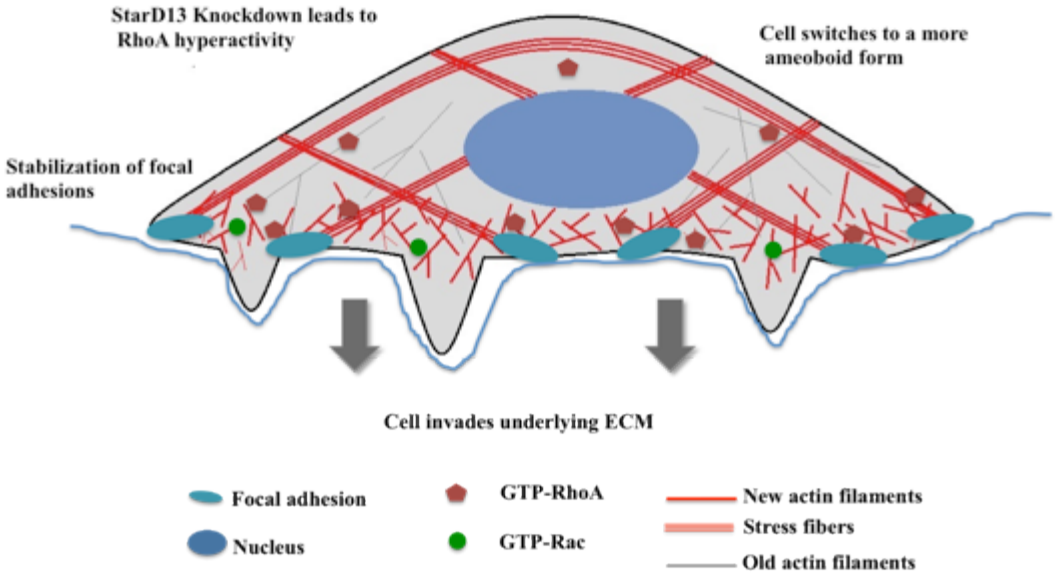


Figure 28: Model-2: StarD13 knockdown promotes cellular invasion. Upon knocking down StarD13, RhoA is kept highly active forming extensive stress fibers and stabilizing focal adhesions. The cell is therefore stuck and cannot move in 2D, this promotes transition into a more ameboid phenotype directing the cell towards degrading and invading the underlying ECM.

BIBLIOGRAPHY

Alberts, A. S. (2001). Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. *J Biol Chem, 276*(4), 2824-2830.

Ananthakrishnan, R., & Ehrlicher, A. (2007). The forces behind cell movement. *Int J Biol Sci, 3*(5), 303-317.

Arjonen, A., Kaukonen, R., & Ivaska, J. (2011). Filopodia and adhesion in cancer cell motility. *Cell Adh Migr, 5*(5), 421-430.

Artym, V. V., Zhang, Y., Seillier-Moiseiwitsch, F., Yamada, K. M., & Mueller, S. C. (2006). Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at invadopodia: Defining the stages of invadopodia formation and function. *Cancer Res, 66*(6), 3034-3043.

Aznavoorian, S., Murphy, A. N., Stetler-Stevenson, W. G., & Liotta, L. A. (1993). Molecular aspects of tumor cell invasion and metastasis. *Cancer, 71*(4), 1368-1383.

Backer, J. M. (2008). The regulation and function of Class III PI3Ks: Novel roles for Vps34. *Biochem J, 410*(1), 1-17.

Bagrodia, S., Taylor, S. J., Jordon, K. A., Van Aelst, L., & Cerione, R. A. (1998). A novel regulator of p21-activated kinases. *J Biol Chem, 273*(37), 23633-23636.

Bailly, M., Condeelis, J. S., & Segall, J. E. (1998). Chemoattractant-induced lamellipod extension. *Microsc Res Tech, 43*(5), 433-443.

Bailly, M., Yan, L., Whitesides, G. M., Condeelis, J. S., & Segall, J. E. (1998). Regulation of protrusion shape and adhesion to the substratum during chemotactic responses of mammalian carcinoma cells. *Exp Cell Res, 241*(2), 285-299.

Banyard, J., Anand-Apte, B., Symons, M., & Zetter, B. R. (2000). Motility and invasion are differentially modulated by Rho family GTPases. *Oncogene, 19*(4), 580-591.

Bokoch, G. M. (2003). Biology of the p21-activated kinases. *Annu Rev Biochem, 72*, 743-781.

Bombonati, A., & Sgroi, D. C. (2011). The molecular pathology of breast cancer progression. *J Pathol, 223*(2), 307-317.

Bordeleau, L., Lipscombe, L., Lubinski, J., Ghadirian, P., Foulkes, W. D., Neuhausen, S., … Narod, S. A. (2011). Diabetes and breast cancer among women with BRCA1 and BRCA2 mutations. *Cancer, 117*(9), 1812-1818.

Bouzahzah, B., Albanese, C., Ahmed, F., Pixley, F., Lisanti, M. P., Segall, J. D., … Pestell, R. G. (2001). Rho family GTPases regulate mammary epithelium cell growth and metastasis through distinguishable pathways. *Mol Med, 7*(12), 816-830.

Bravo-Cordero, J. J., Oser, M., Chen, X., Eddy, R., Hodgson, L., & Condeelis, J. (2011). A novel spatiotemporal RhoC activation pathway locally regulates cofilin activity at invadopodia. *Curr Biol, 21*(8), 635-644.

Brown, C. J., Lain, S., Verma, C. S., Fersht, A. R., & Lane, D. P. (2009). Awakening guardian angels: Drugging the p53 pathway. *Nat Rev Cancer, 9*(12), 862-873.

Burbelo, P. D., Drechsel, D., & Hall, A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem, 270*(49), 29071-29074.

Burridge, K., & Wennerberg, K. (2004). Rho and Rac take center stage. *Cell, 116*(2), 167-179.

Bustelo, X. R. (2000). Regulatory and signaling properties of the Vav family. *Mol Cell Biol, 20*(5), 1461-1477.

Calvo, F., & Sahai, E. (2011). Cell communication networks in cancer invasion. *Curr Opin Cell Biol, 23*(5), 621-629.

Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., & Guan, J. L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *J Cell Biol, 140*(1), 211-221.

Childs, G. V. (2001). The actin cytoskeleton. *University of Arkansas for Medical Sciences*. Retreived from http://www.cytochemistry.net/Cell-biology/actin_filaments_intro.htm

Ching, Y. P., Wong, C. M., Chan, S. F., Leung, T. H., Ng, D. C., Jin, D. Y., … Ng, I. O. (2003). Deleted in liver cancer (DLC) 2 encodes a RhoGAP protein with growth suppressor function and is underexpressed in hepatocellular carcinoma. *J Biol Chem, 278*(12), 10824-10830.

Clark, E. A., Golub, T. R., Lander, E. S., & Hynes, R. O. (2000). Genomic analysis of metastasis reveals an essential role for RhoC. *Nature, 406*(6795), 532-535.

Claus, E. B., Stowe, M., & Carter, D. (2001). Breast carcinoma in situ: Risk factors and screening patterns. *J Natl Cancer Inst, 93*(23), 1811-1817.

Collins, I., & Garrett, M. D. (2005). Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. *Curr Opin Pharmacol, 5*(4), 366-373.

Condeelis, J. (2001). How is actin polymerization nucleated in vivo? *Trends Cell Biol, 11*(7), 288-293.

Condeelis, J., & Segall, J. E. (2003). Intravital imaging of cell movement in tumours. *Nat Rev Cancer, 3*(12), 921-930.

Condeelis, J. S., Wyckoff, J. B., Bailly, M., Pestell, R., Lawrence, D., Backer, J., … Segall, J. E. (2001). Lamellipodia in invasion. *Semin Cancer Biol, 11*(2), 119-128.

Cox, E. A., Sastry, S. K., & Huttenlocher, A. (2001). Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. *Mol Biol Cell, 12*(2), 265-277.

Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S., & Bustelo, X. R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature, 385*(6612), 169-172.

Croce, C. M. (2008). Oncogenes and cancer. *N Engl J Med, 358*(5), 502-511.

Das, B., Shu, X., Day, G. J., Han, J., Krishna, U. M., Falck, J. R., … Broek, D. (2000). Control of intramolecular interactions between the pleckstrin homology and Dbl homology domains of Vav and Sos1 regulates Rac binding. *J Biol Chem, 275*(20), 15074-15081.

Dbouk, H. A., & Backer, J. M. (2010). A beta version of life: p110beta takes center stage. *Oncotarget, 1*(8), 729-733.

DeMali, K. A., & Burridge, K. (2003). Coupling membrane protrusion and cell adhesion. *J Cell Sci, 116*(12), 2389-2397.

DerMardirossian, C., & Bokoch, G. M. (2005). GDIs: Central regulatory molecules in Rho GTPase activation. *Trends Cell Biol, 15*(7), 356-363.

Du, W., & Prendergast, G. C. (1999). Geranylgeranylated RhoB mediates suppression of human tumor cell growth by farnesyltransferase inhibitors. *Cancer Res, 59*(21), 5492-5496.

Edwards, D. C., Sanders, L. C., Bokoch, G. M., & Gill, G. N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat Cell Biol, 1*(5), 253-259.

El-Sibai, M., Nalbant, P., Pang, H., Flinn, R. J., Sarmiento, C., Macaluso, F., … Backer, J. M. (2007). Cdc42 is required for EGF-stimulated protrusion and motility in MTLn3 carcinoma cells. *J Cell Sci, 120*(19), 3465-3474.

El-Sibai, M., Pertz, O., Pang, H., Yip, S. C., Lorenz, M., Symons, M., … Backer, J. M. (2008). RhoA/ROCK-mediated switching between Cdc42- and Rac1-dependent protrusion in MTLn3 carcinoma cells. *Exp Cell Res, 314*(7), 1540-1552.

El-Sitt, S., Khalil, B. D., Hanna, S., El-Sabban, M., Fakhreddine, N., & El-Sibai, M. (2012). DLC2/StarD13 plays a role of a tumor suppressor in astrocytoma. *Oncol Rep*, (in press).

Engelman, J. A., Luo, J., & Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet, 7*(8), 606-619.

Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature, 420*(6916), 629-635.

Evans, V. G. (1993). Multiple pathways to apoptosis. *Cell Biol Int, 17*(5), 461-476.

Garcia-Mata, R., Boulter, E., & Burridge, K. (2011). The 'invisible hand': Regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol, 12*(8), 493-504.

Gertler, F., & Condeelis, J. (2011). Metastasis: Tumor cells becoming MENAcing. *Trends Cell Biol, 21*(2), 81-90.

Grise, F., Bidaud, A., & Moreau, V. (2009). Rho GTPases in hepatocellular carcinoma. *Biochim Biophys Acta, 1795*(2), 137-151.

Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G., & Baldwin, A. S., Jr. (1999). NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol, 19*(8), 5785-5799.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science, 279*(5350), 509-514.

Harrington, E. A., Fanidi, A., & Evan, G. I. (1994). Oncogenes and cell death. *Curr Opin Genet Dev, 4*(1), 120-129.

Hicks, D. G. (2012). Targeted therapies in breast cancer: Tailoring treatment to the molecular drivers of disease progression. *MLO Med Lab Obs, 44*(1), 36, 38-39.

Jaffe, A. B., & Hall, A. (2005). Rho GTPases: Biochemistry and biology. *Annu Rev Cell Dev Biol, 21*, 247-269.

Jiang, P., Enomoto, A., & Takahashi, M. (2009). Cell biology of the movement of breast cancer cells: Intracellular signalling and the actin cytoskeleton. *Cancer Lett, 284*(2), 122-130.

Johnson, D. I. (1999). Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol Mol Biol Rev, 63*(1), 54-105.

Joyce, D., Bouzahzah, B., Fu, M., Albanese, C., D'Amico, M., Steer, J., … Pestell, R. G. (1999). Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway. *J Biol Chem, 274*(36), 25245-25249.

Kaverina, I., Krylyshkina, O., & Small, J. V. (2002). Regulation of substrate adhesion dynamics during cell motility. *Int J Biochem Cell Biol, 34*(7), 746-761.

Kawai, K., Seike, J., Iino, T., Kiyota, M., Iwamae, Y., Nishitani, H., … Yagisawa, H. (2009). START-GAP2/DLC2 is localized in focal adhesions via its N-terminal region. *Biochem Biophys Res Commun, 380*(4), 736-741.

Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., & Parise, L. V. (1997). Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature, 390*(6660), 632-636.

Khalil, B. D., & El-Sibai, M. (2012). Rho GTPases in primary brain tumor malignancy and invasion. *J Neurooncol*, (in press).

Kim, Y. S., Liotta, L. A., & Kohn, E. C. (1993). Cancer invasion and metastasis. *Hosp Pract (Off Ed), 28*(5), 92-96.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., … Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science, 273*(5272), 245-248.

Kleer, C. G., van Golen, K. L., Zhang, Y., Wu, Z. F., Rubin, M. A., & Merajver, S. D. (2002). Characterization of RhoC expression in benign and malignant breast disease: A potential new marker for small breast carcinomas with metastatic ability. *Am J Pathol, 160*(2), 579-584.

Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., & Cheresh, D. A. (1998). CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. *J Cell Biol, 140*(4), 961-972.

Lacroix, M., Toillon, R. A., & Leclercq, G. (2004). Stable 'portrait' of breast tumors during progression: Data from biology, pathology and genetics. *Endocr Relat Cancer, 11*(3), 497-522.

Lauffenburger, D. A., & Horwitz, A. F. (1996). Cell migration: A physically integrated molecular process. *Cell, 84*(3), 359-369.

Levine, A. J., Momand, J., & Finlay, C. A. (1991). The p53 tumour suppressor gene. *Nature, 351*(6326), 453-456.

Li, L., Li, J., Wang, J. Y., Yang, C. Q., Jia, M. L., & Jiang, W. (2010). Role of RhoA in platelet-derived growth factor-BB-induced migration of rat hepatic stellate cells. *Chin Med J (Engl), 123*(18), 2502-2509.

Liao, Y. C., & Lo, S. H. (2008). Deleted in liver cancer-1 (DLC-1): A tumor suppressor not just for liver. *Int J Biochem Cell Biol, 40*(5), 843-847.

Liberto, M., Cobrinik, D., & Minden, A. (2002). Rho regulates p21(CIP1), cyclin D1, and checkpoint control in mammary epithelial cells. *Oncogene, 21*(10), 1590-1599.

Lin, M., & van Golen, K. L. (2004). Rho-regulatory proteins in breast cancer cell motility and invasion. *Breast Cancer Res Treat, 84*(1), 49-60.

Lin, Y., Chen, N. T., Shih, Y. P., Liao, Y. C., Xue, L., & Lo, S. H. (2010). DLC2 modulates angiogenic responses in vascular endothelial cells by regulating cell attachment and migration. *Oncogene, 29*(20), 3010-3016.

Lopez-Lago, M., Lee, H., Cruz, C., Movilla, N., & Bustelo, X. R. (2000). Tyrosine phosphorylation mediates both activation and downmodulation of the biological activity of Vav. *Mol Cell Biol, 20*(5), 1678-1691.

Macias, M. J., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M., & Oschkinat, H. (1994). Structure of the pleckstrin homology domain from beta-spectrin. *Nature, 369*(6482), 675-677.

Maddika, S., Ande, S. R., Panigrahi, S., Paranjothy, T., Weglarczyk, K., Zuse, A., … Los, M. (2007). Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. *Drug Resist Updat, 10*(1-2), 13-29.

Magee, A. I., Newman, C. M., Giannakouros, T., Hancock, J. F., Fawell, E., & Armstrong, J. (1992). Lipid modifications and function of the ras superfamily of proteins. *Biochem Soc Trans, 20*(2), 497-499.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., & Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature, 367*(6458), 40-46.

Mareel, M., & Constantino, S. (2011). Ecosystems of invasion and metastasis in mammary morphogenesis and cancer. *Int J Dev Biol, 55*(7-9), 671-684.

Marshall, M. S. (1993). The effector interactions of p21ras. *Trends Biochem Sci, 18*(7), 250-254.

Matsuda, M., Ota, S., Tanimura, R., Nakamura, H., Matuoka, K., Takenawa, T., … Kurata, T. (1996). Interaction between the amino-terminal SH3 domain of CRK and its natural target proteins. *J Biol Chem, 271*(24), 14468-14472.

McHardy, L. M., Warabi, K., Andersen, R. J., Roskelley, C. D., & Roberge, M. (2005). Strongylophorine-26, a Rho-dependent inhibitor of tumor cell invasion that reduces actin stress fibers and induces nonpolarized lamellipodial extensions. *Mol Cancer Ther, 4*(5), 772-778.

Moon, S. Y., & Zheng, Y. (2003). Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol, 13*(1), 13-22.

Morra, L., & Moch, H. (2011). Periostin expression and epithelial-mesenchymal transition in cancer: a review and an update. *Virchows Arch, 459*(5), 465-475.

Munoz-Pinedo, C. (2012). Signaling pathways that regulate life and cell death: Evolution of apoptosis in the context of self-defense. *Adv Exp Med Biol, 738*, 124-143.

Ng, D. C., Chan, S. F., Kok, K. H., Yam, J. W., Ching, Y. P., Ng, I. O., … Jin, D. Y. (2006). Mitochondrial targeting of growth suppressor protein DLC2 through the START domain. *FEBS Lett, 580*(1), 191-198.

Nieto, M. A. (2011). The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu Rev Cell Dev Biol, 27*, 347-376.

Nobes, C. D., & Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol, 144*(6), 1235-1244.

Nobes, C. D., Hawkins, P., Stephens, L., & Hall, A. (1995). Activation of the small GTP-binding proteins rho and rac by growth factor receptors. *J Cell Sci, 108*(1), 225-233.

O'Connor, K. L., Nguyen, B. K., & Mercurio, A. M. (2000). RhoA function in lamellae formation and migration is regulated by the alpha6beta4 integrin and cAMP metabolism. *J Cell Biol, 148*(2), 253-258.

Olson, M. F., & Sahai, E. (2009). The actin cytoskeleton in cancer cell motility. *Clin Exp Metastasis, 26*(4), 273-287.

Palecek, S. P., Huttenlocher, A., Horwitz, A. F., & Lauffenburger, D. A. (1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J Cell Sci, 111(*7), 929-940.

Park, M. T., & Lee, S. J. (2003). Cell cycle and cancer. *J Biochem Mol Biol, 36*(1), 60-65.

Pille, J. Y., Denoyelle, C., Varet, J., Bertrand, J. R., Soria, J., Opolon, P., … Li, H. (2005). Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Mol Ther, 11*(2), 267-274.

Price, L. S., Leng, J., Schwartz, M. A., & Bokoch, G. M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell, 9*(7), 1863-1871.

Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., … Sellers, W. R. (1999). Regulation of G1 progression by the PTEN tumor

suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A, 96*(5), 2110-2115.

Ridley, A. J. (2001). Rho GTPases and cell migration. *J Cell Sci, 114*(15), 2713-2722.

Ripperger, T., Gadzicki, D., Meindl, A., & Schlegelberger, B. (2009). Breast cancer susceptibility: Current knowledge and implications for genetic counselling. *Eur J Hum Genet, 17*(6), 722-731.

Rottner, K., Hall, A., & Small, J. V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr Biol, 9*(12), 640-648.

Ruvolo, P. P., Deng, X., & May, W. S. (2001). Phosphorylation of Bcl2 and regulation of apoptosis. *Leukemia, 15*(4), 515-522.

Sahai, E. (2005). Mechanisms of cancer cell invasion. *Curr Opin Genet Dev, 15*(1), 87-96.

Sahai, E., & Marshall, C. J. (2002). RHO-GTPases and cancer. *Nat Rev Cancer, 2*(2), 133-142.

Sahai, E., & Marshall, C. J. (2003). Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol, 5*(8), 711-719.

Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., & Collard, J. G. (1999). Rac downregulates Rho activity: Reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J Cell Biol, 147*(5), 1009-1022.

Sanders, L. C., Matsumura, F., Bokoch, G. M., & de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science, 283*(5410), 2083-2085.

Schafer, K. A. (1998). The cell cycle: A review. *Vet Pathol, 35*(6), 461-478.

Schlotter, C. M., Vogt, U., Allgayer, H., & Brandt, B. (2008). Molecular targeted therapies for breast cancer treatment. *Breast Cancer Res, 10*(4), 211.

Schmidt, A., & Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: Turning on the switch. *Genes Dev, 16*(13), 1587-1609.

Scita, G., Nordstrom, J., Carbone, R., Tenca, P., Giardina, G., Gutkind, S., … Di Fiore, P. P. (1999). EPS8 and E3B1 transduce signals from Ras to Rac. *Nature, 401*(6750), 290-293.

Small, J. V., Stradal, T., Vignal, E., & Rottner, K. (2002). The lamellipodium: Where motility begins. *Trends Cell Biol, 12*(3), 112-120.

Srivastava, R. K., Srivastava, A. R., Korsmeyer, S. J., Nesterova, M., Cho-Chung, Y. S., & Longo, D. L. (1998). Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. *Mol Cell Biol, 18*(6), 3509-3517.

Stebbing, J., Filipovic, A., Ellis, I. O., Green, A. R., D'Silva, T. R., Lenz, H. J., … Giamas, G. (2012). LMTK3 expression in breast cancer: Association with tumor phenotype and clinical outcome. *Breast Cancer Res Treat, 132*(2), 537-544.

Stetler-Stevenson, W. G., Aznavoorian, S., & Liotta, L. A. (1993). Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu Rev Cell Biol, 9*, 541-573.

Struckhoff, A. P., Rana, M. K., & Worthylake, R. A. (2011). RhoA can lead the way in tumor cell invasion and metastasis. *Front Biosci, 16*, 1915-1926.

Takai, Y., Sasaki, T., & Matozaki, T. (2001). Small GTP-binding proteins. *Physiol Rev, 81*(1), 153-208.

Takaishi, K., Sasaki, T., Kameyama, T., Tsukita, S., & Takai, Y. (1995). Translocation of activated Rho from the cytoplasm to membrane ruffling area, cell-cell adhesion sites and cleavage furrows. *Oncogene, 11*(1), 39-48.

Tang, Y., Olufemi, L., Wang, M. T., & Nie, D. (2008). Role of Rho GTPases in breast cancer. *Front Biosci, 13*, 759-776.

Tatsuno, I., Hirai, A., & Saito, Y. (2000). Cell-anchorage, cell cytoskeleton, and Rho-GTPase family in regulation of cell cycle progression. *Prog Cell Cycle Res, 4*, 19-25.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: Controlled demolition at the cellular level. *Nat Rev Mol Cell Biol, 9*(3), 231-241.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer, 2*(6), 442-454.

Thorsell, A. G., Lee, W. H., Persson, C., Siponen, M. I., Nilsson, M., Busam, R. D., … Lehtio, L. (2011). Comparative structural analysis of lipid binding START domains. *PLoS One, 6*(6), e19521.

Tkach, V., Bock, E., & Berezin, V. (2005). The role of RhoA in the regulation of cell morphology and motility. *Cell Motil Cytoskeleton, 61*(1), 21-33.

Ullmannova, V., & Popescu, N. C. (2006). Expression profile of the tumor suppressor genes DLC-1 and DLC-2 in solid tumors. *Int J Oncol, 29*(5), 1127-1132.

Valencia, A., Chardin, P., Wittinghofer, A., & Sander, C. (1991). The ras protein family: Evolutionary tree and role of conserved amino acids. *Biochemistry, 30*(19), 4637-4648.

Van Golen, K. L. (2003). Inflammatory breast cancer: Relationship between growth factor signaling and motility in aggressive cancers. *Breast Cancer Res, 5*(3), 174-179.

Van Troys, M., Huyck, L., Leyman, S., Dhaese, S., Vandekerkhove, J., & Ampe, C. (2008). Ins and outs of ADF/cofilin activity and regulation. *Eur J Cell Biol, 87*(8-9), 649-667.

Vega, F. M., & Ridley, A. J. (2008). Rho GTPases in cancer cell biology. *FEBS Lett, 582*(14), 2093-2101.

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif, 36*(3), 131-149.

Vial, E., Sahai, E., & Marshall, C. J. (2003). ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. *Cancer Cell, 4*(1), 67-79.

Viallet, J., & Minna, J. D. (1990). Dominant oncogenes and tumor suppressor genes in the pathogenesis of lung cancer. *Am J Respir Cell Mol Biol, 2*(3), 225-232.

Vivanco, I., & Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer, 2*(7), 489-501.

Wang, W., Eddy, R., & Condeelis, J. (2007). The cofilin pathway in breast cancer invasion and metastasis. *Nat Rev Cancer, 7*(6), 429-440.

Wang, Y., & McNiven, M. A. (2012). Invasive matrix degradation at focal adhesions occurs via protease recruitment by a FAK-p130Cas complex. *J Cell Biol, 196*(3), 375-385.

Wennerberg, K., & Der, C. J. (2004). Rho-family GTPases: It's not only Rac and Rho (and I like it). *J Cell Sci, 117*(8), 1301-1312.

Whitehead, I. P., Khosravi-Far, R., Kirk, H., Trigo-Gonzalez, G., Der, C. J., & Kay, R. (1996). Expression cloning of lsc, a novel oncogene with structural similarities to the Dbl family of guanine nucleotide exchange factors. *J Biol Chem, 271*(31), 18643-18650.

Whitehead, I. P., Lambert, Q. T., Glaven, J. A., Abe, K., Rossman, K. L., Mahon, G. M., … Der, C. J. (1999). Dependence of Dbl and Dbs transformation on MEK and NF-kappaB activation. *Mol Cell Biol, 19*(11), 7759-7770.

Wolfenson, H., Henis, Y. I., Geiger, B., & Bershadsky, A. D. (2009). The heel and toe of the cell's foot: A multifaceted approach for understanding the structure and dynamics of focal adhesions. *Cell Motil Cytoskeleton, 66*(11), 1017-1029.

Worthylake, R. A., & Burridge, K. (2003). RhoA and ROCK promote migration by limiting membrane protrusions. *J Biol Chem, 278*(15), 13578-13584.

Wu, M., Wu, Z. F., Rosenthal, D. T., Rhee, E. M., & Merajver, S. D. (2010). Characterization of the roles of RHOC and RHOA GTPases in invasion, motility, and matrix adhesion in inflammatory and aggressive breast cancers. *Cancer, 116*(11 Suppl), 2768-2782.

Xiaorong, L., Wei, W., Liyuan, Q., & Kaiyan, Y. (2008). Underexpression of deleted in liver cancer 2 (DLC2) is associated with overexpression of RhoA and poor prognosis in hepatocellular carcinoma. *BMC Cancer, 8*, 205.

Yamaguchi, H., Lorenz, M., Kempiak, S., Sarmiento, C., Coniglio, S., Symons, M., … Condeelis, J. (2005). Molecular mechanisms of invadopodium formation: The role of the N-WASP-Arp2/3 complex pathway and cofilin. *J Cell Biol, 168*(3), 441-452.

Yip, S. C., El-Sibai, M., Hill, K. M., Wu, H., Fu, Z., Condeelis, J. S., … Backer, J. M. (2004). Over-expression of the p110beta but not p110alpha isoform of PI 3-kinase inhibits motility in breast cancer cells. *Cell Motil Cytoskeleton, 59*(3), 180-188.

Zamir, E., & Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci, 114*(Pt 20), 3583-3590.