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The Effect of StarD13 on Colorectal Cancer Proliferation and Invasion

By

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DEDICATION

To My Beloved Cousin Elie,

My Hero,

My Guardian Angel.

The Effect of StarD13 on Colorectal Cancer Malignancy and Invasion

Anita I. Nasrallah

ABSTRACT

Colon cancer is the cancer of the epithelial cells lining the colon. It is mainly divided into different stages according to invasiveness and metastatic ability of the tumor. Many mutations are acquired, leading to this malignancy. These occur in entities that greatly affect the cell cycle, cell signaling pathways, and cell movement, which all involve the action of Rho GTPases. The protein of our interest is DLC2, also known as StarD13 or START-GAP2, a GAP for Rho and Cdc42. Literature states that this protein is considered a tumor-suppressor in hepatocellular carcinoma. Previous work in our lab proved StarD13 to be a tumor suppressor in astrocytoma and in breast cancer. In this work, we studied the role of StarD13 in colon cancer. When overexpressed, StarD13 led to a decrease in cell proliferation in colon cancer cells. Consistently, knocking down StarD13 led to an increase in cell proliferation. This showed that, similarly to its role in astrocytoma and breast cancer, StarD13 seems to be a tumor suppressor in colon cancer as well. We were also interested in examining the role of StarD13 in cell motility. StarD13 knock down resulted in an inhibition of 2D cell motility. This is due to the inhibition of Rho, thus Rac-dependant focal complexes are not formed nor detached for the cells to move forward. However, StarD13 knock down led to an increase in 3D cell motility. Although StarD13 was indeed a tumor suppressor in our colon cancer cells, as seen by its effect on cell proliferation, it was needed for cancer cell invasion. Our study further describes the role of StarD13 as a tumor suppressor as well as a RhoGAP.

Keywords: StarD13, Colorectal Cancer, Cell Proliferation, Cell Motility, Cell invasion.

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LIST OF ABBREVIATIONS

HNPCC: Hereditary Nonpolyposis Colorectal Cancer

FAP: Familial Adenomatous Polyposis

Wnt: Wingless/integrated

APC: Adenomatous Polyposis Coli

DNA: Deoxyribonucleic acid

CTNNB1: Catenin Beta 1

NKD1: Naked cuticle 1

TCF7L2: Transcription factor 7-like 2

AXIN: Axis inhibition protein

PTEN: Protein tyrosine phosphatase

PI3K: Phosphatidylinositol 3-kinase

TP53: Tumor protein 53

BAX: Bcl associated x protein

TGF: Tumor growth factor

SMAD: Mothers against decapentaplegic (MAD) and small body size (SMA) genes

DCC: Deleted in colorectal cancer

RAF: Rapidly accelerated fibrosarcoma

RAS: Rat sarcoma

KRAS: Kristen rat sarcoma

BRAF: v-raf murine sarcoma viral oncogene homolog B1

SLC9A9: Solute carrier family 9 member 10

MSH: MutS homolog

TGFBR2: Tumor growth factor receptor 2

ACVR2A: Activin A receptor type IIA

ATM: Ataxia telangienctasia mutated

SOX: Sry-related HMG box

FAM123B: Family with sequence similarity 123B

MYC: Myelocytomatosis

CT: Computed tomography

PET: Positron Emission Tomography

MRI: Magnetic resonance imaging

COX-2: Cyclooxygenase-2

UFT: Tegafur-uracil

M2-PK: M2 Pyruvate Kinase

M: Mitosis phase

G1: Gap phase 1

S: Synthesis phase

G2: Gap phase 2

G0: Gap phase 0

CDK: Cyclin-dependent kinase

RTK: Receptor tyrosine kinase

GPCR: G-protein-coupled receptor

PI(4,5)P2: Phosphatidylinositol (4,5)-diphosphate

PI(3,4,5)P3: Phosphatidylinositol (3,4,5)-triphosphate

PH: Pleckstrin homology

Akt: Acutely transforming retrovirus

mTOR: Mammalian target of rapamycin

PDK1: Phosphoinositide-dependent kinase-1

Bad: Bcl associated death promoter

Bcl: B-cell lymphoma

GTPase: Guanosine triphosphatase

c-jun: JUN proto-oncogene

PARP: Poly-ADP-ribose polymerase

MAPK: Mitogen-activated protein kinase

pRb: Retinoblastoma protein

mdm2: mouse double minute 2

ECM: Extracellular matrix

MMP: Matrix metalloproteinase

TIMP: Tissue inhibitor of metalloproteinase

N-WASP: Neural wiskott-aldrich syndrome protein

Arp2/3: Actin-related protein 2/3

MT1-MMP: Membrane-type 1 matrix metalloproteinase

TES: Testin

LIM: Lin11, Isl-1, Mec-3

ROCK: Rho-associated coiled coil-containing protein kinase

LIMK: LIM kinase

SCAR: Suppressor of cyclic AMP

WAVE: WASP-family verprolin-homologous

VCA: Verpolin 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

GDI: Guanine nucleotide dissociation inhibitor

GEF: Guanine nucleotide exchange factor

GAP: GTPase activating protein

DH: Dbl homology

EGF: Epidermal growth factor

PDGF: Platelet-derived growth factor

LPA: Lysophosphatidic acid

Src: Schmidt-Ruppin A-2

Sos: Son of sevenless

FAK: Focal adhesion kinase

P130cas: Crk-associated substrate protein 130

Crk: CT10 regulator of kinase

DOCK180: Dedicator of cytokinesis 180

PKL: Paxillin kinase linker

PIX: p21 kinase-interacting exchange factor

GSK-3: Glycogen synthase kinase 3

mDia: Mammalian homolog of diaphanous

ERM: Ezrin-radixin-moesin

PAK: p21 activating kinase

CRIB: Cdc42/Rac interactive binding

MLCK: MLC kinase

MLC: Myosin light chain

ADF: Actin depolymerization factor

PIP5K: Phosphatidylinositol-4-phosphate 5-kinase

GIT: G protein-coupled receptor kinase-interacting

C3T: C3 transferase

CD-1: Cluster of differentiation 1

ATF-2: Activating transcription factor 2

NFκB: Nuclear factor κB

FTI: Farsenalytransferase inhibitor

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

START: STAR-related lipid transfer

DLC: Deleted in liver cancer

SAM: Sterile alpha motif

FAT: Focal adhesion targeting

Caco-2: Carcinoma of the colon

HT-29: Homo sapiens colon tumor

ATCC: American Type Culture Collection

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

dNTP: Deoxyrybonucleotide triphosphates

WST-1: Water-soluble tetrazolium salt

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ELISA: Enzyme-linked immunosorbent assay

BSA: Bovine serum albumin

GST: Glutathione S-transferase

RBD: Rhotekin binding domain

SEM: Standard error of the mean

FA: Focal adhesion

Chapter I

LITERATURE REVIEW

1.1. Colorectal Cancer

1.1.1. Introduction

Colorectal cancer, commonly known as bowel cancer, occurs in the colon, rectum, or appendix. Genetic analysis shows that tumors in these three locations are genetically of the same cancer (Cancer Genome Atlas Network, 2012). Colorectal cancer is the second most commonly diagnosed cancer in females, the third in males (World Health Organization, 2010), and the forth around the world (Jemal, Bray, Center, Ward, & Forman, 2011). More than a million cases are yearly detected globally (Cunningham, et al., 2010). It is widespread in developed countries, where around 60% of the cases were diagnosed (Ferlay, et al., 2010), most of which lead to death (Merika, Saif, Katz, Syrigos, & Morse, 2010).

There are several causes for the onset of colorectal cancers, which are nowadays better diagnosed and classified according to several criteria. Consistently, different treatments and prognostic measures are currently used to try and successfully cure this type of cancer (Cunningham, et al., 2010).

1.1.2. Causes and predisposition

Colorectal cancer occurs in a wide range of people. Most of them were previously, and might still be exposed to numerous risk factors. The rest are either genetically predisposed, or associated with other bowel diseases.

1.1.2.1. Risk factors

It is estimated that more than 80% of patients with colorectal cancer were exposed to a number of risk factors, such as male gender,

older age (Cunningham, et al., 2010), high intake of red meat or fat, smoking and obesity (Watson & Collins, 2011). The risk of alcohol increases at more than a drink per day (Fedirko, et al., 2011). Also, around 10% of these cases are related to insufficient activity (Lee, et al., 2012).

1.1.2.2. Genetics

Around 18% of all cases are patients with a family history. Thus, they have a two to three-fold risk increase than other people. Furthermore, three main genetic diseases are well associated with this type of cancer. The most common is known as the Lynch syndrome, or the hereditary nonpolyposis colorectal cancer (HNPCC) (Cunningham, et al., 2010). Familial adenomatous polyposis (FAP) (Half, Bercovich, & Rozen, 2009) and Gardner syndrome (Juhn & Khachemoune, 2010) are both as well strongly associated with this type of cancer.

1.1.2.3. Inflammatory Bowel Diseases

A third cause is the incidence of the Inflammatory Bowel Diseases, such as ulcerative colitis and Crohn's disease (Jawad, Direkzen, & Leedham, 2011). The longer the onset of these diseases (Xie & Itzkowitz, 2008), and the worse the inflammation (Triantafillidis, Nasioulas, & Kosmidids, 2009) will directly affect the risk of having colorectal cancer. However, only about 2% of this cancer is associated with the previously mentioned diseases (Jawad, Direkzen, & Leedham, 2011).

1.1.3. Pathogenesis

Colorectal cancer originates from the epithelial lining, most often as a consequence of mutations in the Wnt signaling pathway. These mutations can be either acquired or inherited. They mostly occur in the intestinal gland stem cells (Ionov, Peinado, Malkhosyan, Shibata, & Perucho, 1993).

1.1.3.1. Tumor suppressor genes

In all colorectal cancer, *APC* is the most commonly mutated gene. It produces the APC protein, which prevents the accumulation of the β-catenin protein by binding to and degrading it. In the absence of APC protein, β-catenin highly accumulates in the cytoplasm, translocates to the nucleus, and binds to DNA, thus activating the transcription of several genes (Figure 1). These genes are responsible for stem cell renewal and differentiation. However, when improperly expressed at elevated levels they cause cancer (Markowitz & Bertagnolli, 2009).

Some colorectal cancers have high β-catenin levels due to mutations in its gene *CTNNB1*, and not in the *APC* gene. These mutations block the degradation of β-catenin. Other colorectal cancers have mutations in other APC analogues, such as NKD1, TCF7L2, AXIN1, or AXIN2. Another tumor suppressor, PTEN, normally inhibits the overexpressed oncogene PI3K. However if PTEN is mutated, it becomes deactivated (Markowitz & Bertagnolli, 2009).

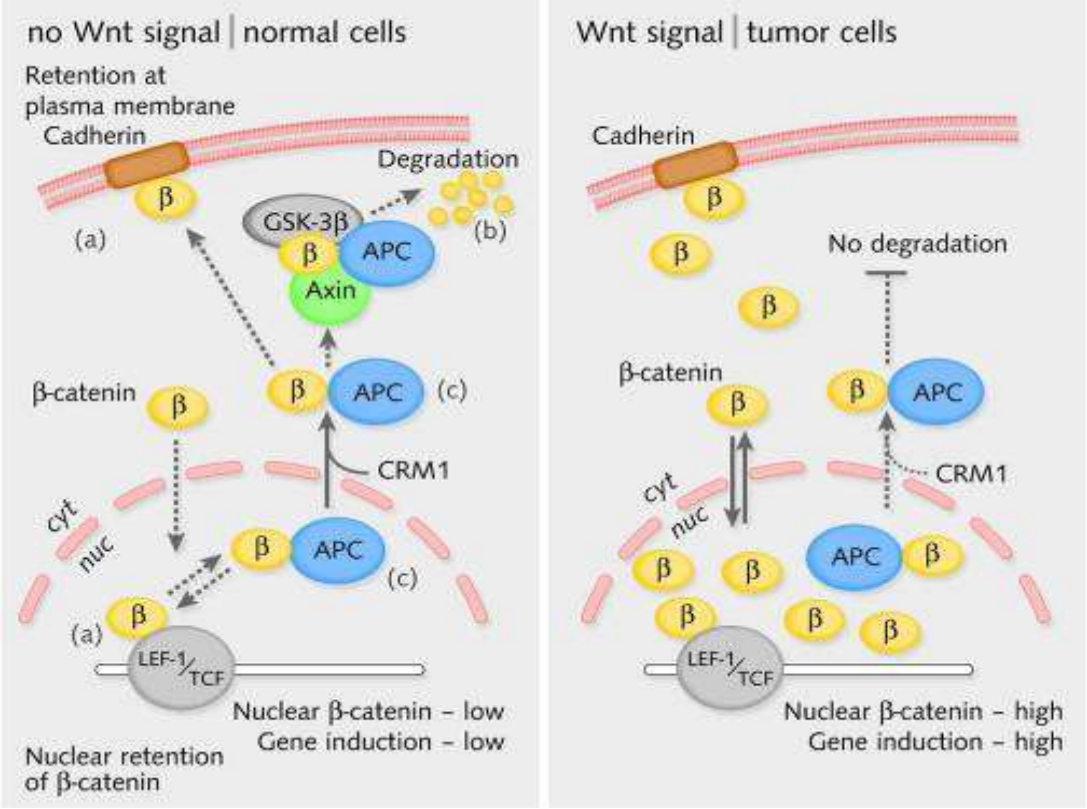

Figure 1: Nuclear-cytoplasmic shuttling of β-catenin. In normal cells, β-catenin is (a) maintained on the membrane, in the nucleus, and in the cytoplasm, (b) bound to

APC in the cytoplasm then degraded, and (c) shuttling in the cytoplasm and nucleus. In tumor cells, β-catenin is found in very high abundance, moving between the nucleus and cytoplasm independent of its binding to APC. Thus no degradation occurs. Source: **(Henderson & Fagotto, 2002)**

1.1.3.2. Apoptotic proteins

Other than the defects mentioned above, additional mutations must take place for the cells to acquire more cancerous characteristics. One of these mutations occurs in the p53 protein, which is produced by the *TP53* gene. This apoptotic protein monitors normal cell division. It kills the cells if they acquire any defect in the Wnt signaling pathway. Its mutation will change the tissue from a non-invasive adenoma into an invasive carcinoma (Chakravarthi, Krishnan, & Madhavan, 1999). In some cases, the gene encoding for BAX, another protective protein, is mutated, instead of the *TP53* gene (Markowitz & Bertagnolli, 2009).

Other apoptotic proteins are frequently mutated and deactivated in colorectal cancers. A major protein is the TGF-β, which in at least half of colorectal cancers has a deactivating mutation. In some cases, TGF-β is not the protein mutated, but its downstream protein SMAD (Markowitz & Bertagnolli, 2009). Another protein is the DCC protein, or Deleted in Colorectal Cancer. It acquires a deletion of its chromosomal segment (Mehlen & Fearon, 2004).

1.1.3.3. Oncogenes

Normal genes that encode proteins, known as oncoproteins, responsible for the regulation of cell growth and differentiation are known as proto-oncogenes. They are mainly involved in signal transduction. When activated, increased expression or mutations will result in the transformation into oncogenes, which are tumor-inducing agents (Chial, 2008). These are overexpressed in colorectal cancer, such as genes encoding the proteins PI3K, RAF, and RAS (Todd & Wong, 1999).

Under normal conditions and in response to growth factors, these proteins will stimulate the cell to divide. Acquired mutations will lead to the over-activation of cell proliferation. In some cases, the chronological order of mutations is crucial for the progression of cancer. To illustrate, if

at first a KRAS mutation occurred, this will lead to a self-limiting borderline lesion. On the other hand, if the KRAS mutation occurs after an APC mutation, it often leads to cancer (Vogelstein & Kinzler, 2004).

All the previously discussed mutations occur in one type of colorectal carcinomas, known as the hypermutated tumor type. These tumors have mutated forms of *BRAF*, *TCF7L2*, *SLC9A9*, *MSH3*, *MSH6*, *TGFBR2*, and *ACVR2A*. Genome-scale analysis reveals that the non-hypermutated tumor type contains mutated *ARIDIA*, *ATM*, *SOX9*, and *FAM123B*. What is in common among the genes in both carcinomas is their involvement in the TGF-β and WNT signaling pathways. This, in turn, will result in the hyper activity of the central player in colorectal cancer, MYC (Cancer Genome Atlas Network, 2012).

1.1.4. Classification of Colorectal Cancer

The most widely used classification system is the TNM Staging System. It is considered the most descriptive and precise. T stands for the depth of the tumor, and to which level did it penetrate the colon wall. N refers to the involvement of lymph nodes. M stands for the degree of metastases that took place or whether the tumor has spread or not (Yarbro, Page, Fiedling, Partridge, & Murphy, 1999).

Another less used system is the Duke's system (Dukes, 1932). It was then modified and became the Modified Dukes Staging System. This system classifies the tumors under four main categories, i.e. A, B, C, and D. Modified Duke Stage A is the tumors that only reach the mucosal wall. B is the ones that penetrate through and out of the wall. C is the advancement of the tumor into the lymph nodes. D is when tumors are depicted in other organs, such as the liver, lung and bone (Astler & Coller, 1954).

1.1.5. Symptoms and diagnosis

Signs and symptoms of colorectal cancer greatly depend on its location and ability to metastasize. These include fever, loss of appetite,

weight loss, constipation, and blood in stool. In people older than fifty, common symptoms are nausea, vomiting anaemia, and rectal bleeding (Alpers, Kalloo, Owyang, Powell, & Kaplowitz, 2009). It is important to note that the most evident symptoms are weight loss and rectal bleeding (Astin, Griffin, Neal, Rose, & Hamilton, 2011). Without them all other symptoms can be indicative of several different gastrointestinal diseases (Adelstein, Macaskil, Chan, Katelaris, & Irwig, 2011).

Colorectal cancers occurring on the right side of the colon, i.e. the ascending colon and cecum, usually cause severe fecal obstruction and anaemia. This is because these tumors tend to grow outward from a location of the bowel wall. However, left-sided tumors, i.e. tumors of the descending colon, cause constipation. These tumors are most likely circumferential (Jellema, et al., 2010).

The first step towards diagnosis is to take tumor biopsy during wither colonoscopy or sigmoidoscopy. After confirming the presence of the cancer, imaging tests are performed of the patient's chest, abdomen and pelvis, to determine the extent of the disease. These tests include CT scan, PET, and MRI. Based on these results, the physician can establish a clear idea of the stages of the cancer, depending on the TNM system of classification (Cunningham, et al., 2010).

1.1.6. Pathology of the tumor

After biopsy or surgery, a pathology report explicitly determines the cell type and grade of the tumor. In 95% of the cases, the colon cancer type is adenocarcinoma (Bafandeh, Khoshbaten, Eftekhar Sadat, & Farhanq, 2008). It originates from the glandular epithelium, invading the wall and infiltrating all layers. Tumor cells have irregular structures and might secrete mucus. Depending on the predominant cellular pleomorphism, gland architecture, and secretion of mucus, adenocarcinoma is separated into three differentiation levels: poorly, moderately, and well differentiated. Other than adenocarcinoma, rare types of colorectal cancer include squamous cell carcinoma and lymphoma (Danciu & Mihailovici, 2010).

Besides, the majority of colorectal cancer tumors are cyclooxygenase-2, or COX-2, positive. This enzyme is abundantly found in cancerous tissue of the colon. It aids in abnormal cell growth (Tsujii, et al., 1998).

1.1.7. Treatment and prognosis

As any other type of cancer, the treatment of colorectal cancer depends on its advancement. At early stages, surgery is mostly curative. At later stages when the cancer is metastatic, physicians tend to treat their patients by trying to prolong their life and keep them comfortable (Stein, Atanackovic, & Bokemeyer, 2011).

1.1.7.1. Surgery

Patients with localized colorectal cancer undergo surgeries to extract the tumor. It is either done by laparotomy or laparoscopy, which is a minimally invasive procedure. If other tumors are metastasized to the lungs or liver, they are removed surgically (Cunningham, et al., 2010).

1.1.7.2. Chemotherapy

In some cases, chemotherapy is used before surgery. This will help shrink the tumor before eradicating it. In other cases where the cancer has metastasized and entered the lymph nodes, chemotherapy is a must to help increase the life expectancy of the patient. Drugs may include oxaliplatin, irinotecan, leucovorin, UFT, capecitabine, or 5-fluorouracil. Monoclonal antibodies against molecular targets include cetuximab, panitumumab, or bevazicumab (Hoyle, et al., 2013). Understanding better the tumor biology and molecular pathways and mechanisms effectively led to the discovery of novel agents that specifically target molecular elements of cancer cells. This has helped improving the efficacy of drug-based chemotherapy that is nowadays combined with targeted monoclonal antibodies. Research is being extensively done to try and use cell signaling pathways as targets for colorectal cancer treatment, even though these pathways highly intercorrelate and crosstalk (Chung & Jang, 2013).

1.1.7.3. Radiation

Combining chemotherapy and radiation might be helpful. But, in most cases, this is not used as curative technique, since the bowels are highly sensitive to radiation (Devita, Lawrence, & Rosenberg, 2008).

1.1.7.4. Palliative care

At the stage when colorectal cancer becomes incurable, the best remedy is to improve the quality of life by alleviating the symptoms and reducing the complications (Wasserberg & Kaufman, 2007). These procedures may include stent placement or bypassing part of the intestine, and pain medications. This will help reduce bleeding, intestinal obstruction, and abdominal pain (Amersi, Stamos, & Ko, 2004).

Survival rates are directly linked to the type of cancer and its detection. Late stage cancers have five times less survival rates that early stage ones (Lin Koo, et al., 2013). To be on a safe side, follow-up is highly mandatory. This will help diagnose any new tumors that develop later, but had not originated from the original tumor (Simmonds, et al., 2006). Physical examinations are recommended, as well as blood tests and CT scans (Desch, et al., 2005). Close follow-up and intensive surveillance can reduce the five-year mortality rate of patients with colorectal cancer from 37% to 30% (Figueredo, et al., 2003; Renehan, Egger, Saunders, & O'Dwyer, 2002).

1.1.8. Prevention

The most important ways to prevent colorectal cancer are a healthy lifestyle, appropriate medication, and continuous screening.

1.1.8.1. Lifestyle

Dietary recommendations include reducing the consumption of red meat and increasing the intake of fruits, vegetables, fibers and whole grains (Doyle, 2007). Physical activity also helps reducing the risk of colorectal cancer (Harriss, et al., 2009).

1.1.8.2. Medication

People at high risk of having colorectal cancer are advised to take aspirin and celecoxib. They both appear to decrease the risk factor. Nevertheless, these drugs are not recommended to those at average risk (Cooper, et al., 2010). Vitamin D, especially its blood concentration, and Calcium intake are also associated with lower risks of colorectal cancer (Ma, et al., 2011; Yin, et al., 2011).

1.1.8.3. Screening

Several screening methods are applied nowadays, and they proved to be helpful in reducing death by the early detection (He & Efron, 2011). The three main tests are fecal occult blood testing of the stool, sigmoidoscopy, and colonoscopy (Qaseem, et al., 2012). The newest screening method is the M2-PK test to stool samples. The M2-PK enzyme biomarker is highly sensitive to colorectal cancer. It is able to detect bleeding and non-bleeding types, which a fecal occult blood test cannot do (Tonus, Sellinger, Koss, & Neupert, 2012).

1.2. Cell Survival, proliferation and apoptosis

1.2.1. Cell cycle progression

Recently, research has been shifted towards finding targeted therapies for cancer. The molecular basis of cell survival, cell cycle, and cell death is nowadays the hot topic. The cell cycle in eukaryotes is divided to two main phases: interphase and mitosis (M). The first is divided into gap 1 phase (G1), DNA synthesis phase (S), and gap 2 phase (G2) (Maddika, et al., 2007).

During the G1 phase, cells are diploid (2n). They produce proteins needed in later stages, and grow in size (Collins & Garrett, 2005). Next, cells enter the S phase, where DNA replication takes place. At this stage, cells double their DNA content and become 4n (Schafer, 1998). Then, cells enter the G2 phase. There, they undergo further cellular growth. At the end of this phase, cells become prepared for the M phase (Maddika, et al., 2007). During the M phase, most cells divide producing two identical diploid daughter cells. Others either stop dividing or are inactive by exiting the cell cycle and entering the G0 phase (Collins & Garrett, 2005; Maddika, et al., 2007).

At the end of each phase, checkpoints allow the activation and arrest of several repair mechanisms in cases of malfunction (Schafer, 1998). After the cell passes a checkpoint, it becomes irretrievably committed to continue through the subsequent phase (Park & Lee, 2003). Critical malfunctions such as DNA damage can lead to cell cycle arrest and trigger the apoptotic cascade pathway, resulting in programmed cell death. Hence, apoptosis is an important defence mechanism during the succession of cell cycle, which prevents the propagation of unwanted or damaged cells (Maddika, et al., 2007; Park & Lee, 2003).

In order to guarantee proper cellular growth and proliferation, regulated machinery control the cell cycle progression. One of the most important regulators is the family of Cyclin-dependant kinases or CDKs. These allow the smooth transition from a phase to another (Maddika, et al., 2007). They are serine/threonine kinases, and are only activated at specific periods of the cell cycle (Collins & Garrett, 2005). When binding to their respective cyclins, CDKs are positively regulated, and then targeted to the nucleus whereby cell division is catalyzed (Park & Lee, 2003). At every phase of the cell cycle, different cyclins are produced. They all undergo a cyclic expression. Normally, cyclins are degraded at the end of each phase, and other new cyclins of the next phase are produced. As a result, the levels of different cyclins change according to the phase the cell is in. However, CDK levels remain constant (Schafer, 1998; Varmeulen, Van Bockstaele, & Berneman, 2003).

1.2.2. PI3Kinase/Akt pathway controls cell survival

One of the most important survival pathways is the Phosphatidylinositol 3-kinase (PI3-K) signaling pathway. When activated by countless stimuli, this pathway regulates essential cellular functions, including apoptosis, cell cycle progression, gene transcription, growth and proliferation (Maddika, et al., 2007; Vivanco & Sawyers, 2002). As their name indicates, these phosphatidylinositol kinases catalyze the phosphorylation of phosphoinositides. They are classified according to their

subunit organization and their substrate specificity (Dbouk & Backer, 2010; Engelman, Luo, & Cantley, 2006).

PI3Ks are divided into two main classes: class IA and class IB. The first consists of heterodimer PI3Ks, with a p110 catalytic subunit and p50, p55, and p85 regulatory subunits. These are activated downstream of RTKs or receptor tyrosine kinases. The second class are dimers composed of a catalytic subunit p110 and regulatory subunits p87 and p101. PI3Ks of class IB are activated downstream of GPCRs or G-protein coupled receptors (Backer, 2008; Dbouk & Backer, 2010). Upon activation, PI3Ks will phosphorylate PI(4,5)P2, localized on the membrane, to produce PI(3,4,5)P3. In turn, this will create a docking site for many other downstream proteins with the PH or pleckstrin homology domain. All this will help activate various intracellular proteins that are involved in interconnected signaling pathways that regulate cell motility, proliferation, and survival (Figure 2) (Maddika, et al., 2007; Vivanco & Sawyers, 2002).

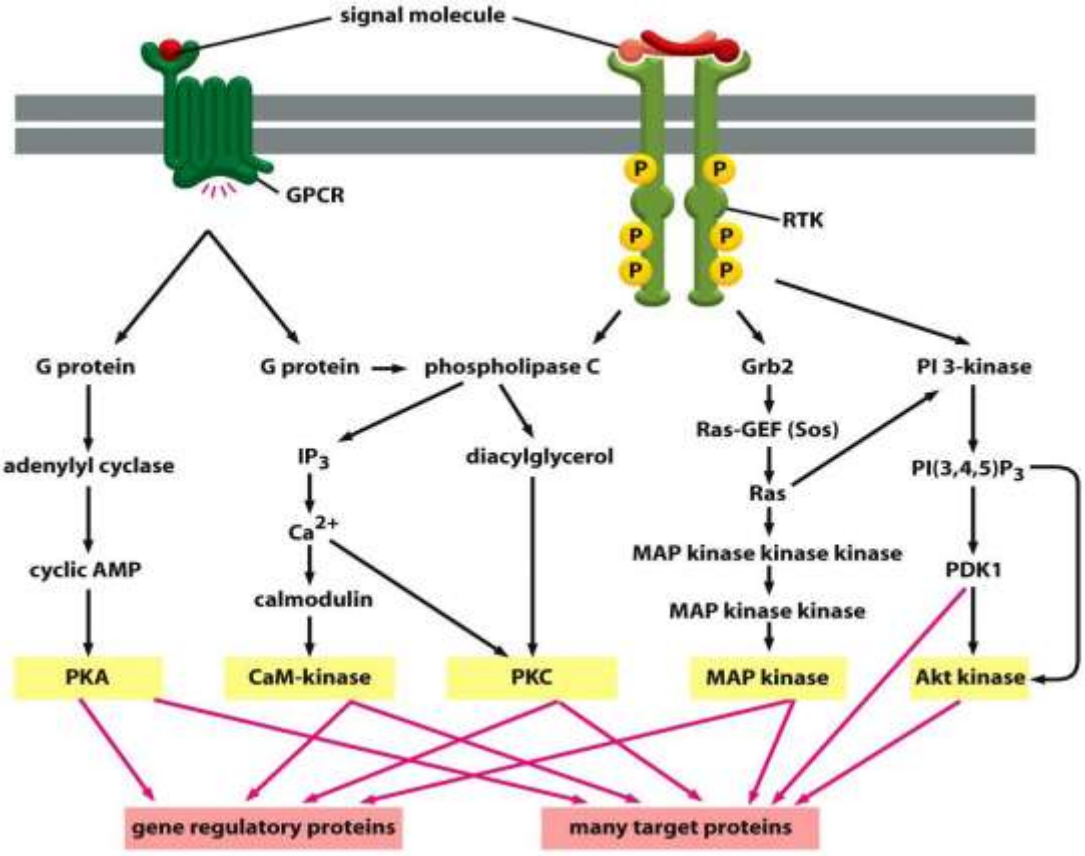

Figure 2: Plasma membrane receptors and protein kinases. Both classes of PI3Ks, downstream from GPCRs and RTKs play important roles in many signalling pathways, eventually affecting gene and protein regulation. Source: **(Kanoh & Rubin, 2010)**

One main downstream effector of PI3K is Akt. It controls cell survival by phosphorylating several substrates that are in turn involved in apoptotic and survival pathways. Its PH domain will help recruit it to the cell membrane, where it binds to PIP3. It is then phosphorylated and activated by mTOR and PDK1 kinases. The pro-apoptotic protein Bad, of the Bcl-2 family, is a main target of Akt. So, after Akt is activated, it phosphorylates Bad. This will release the associated apoptosis inhibitory protein and blocks the apoptotic pathway. In parallel, Akt can also phosphorylate caspase-9. This will induce the caspase-9 to undergo a conformational change, resulting in the inhibition of its proteolytic activity, and also blocking apoptosis and promoting cell survival (Figure 3) (Maddika, et al., 2007; Vivanco & Sawyers, 2002).

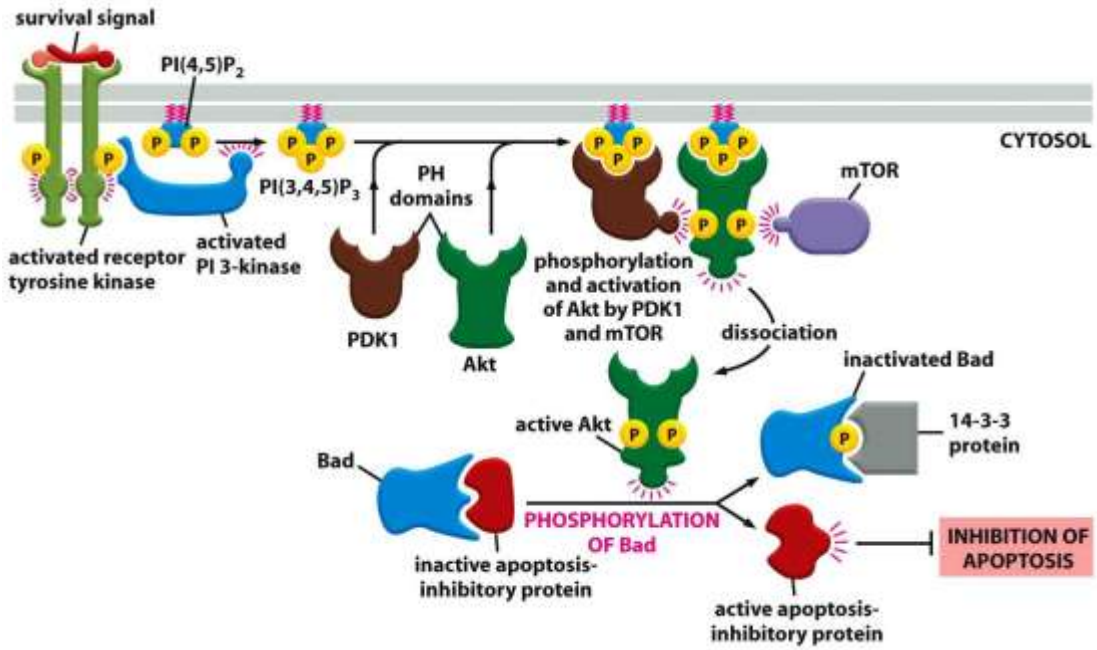

Figure 3: Akt promotes growth factor-related cell survival. Downstream from PI3K, activated Akt phosphorylates Bad, which dissociates form the inactive apoptosis-inhibitory protein. Bad will then lose its pro-apoptotic function, and apoptosis is inhibited. Source: **(Ramaswamy, et al., 1999)**

1.2.3. Tumor suppressors and oncogenes

Tumor suppressors are known as anti-oncogenes. They protect multicellular organisms from mutations randomly affecting many different

cellular functions, including cell differentiation and migration, mitogenic signaling, protein degradation and ubiquitination, cell cycle check point responses, DNA damage repair, and tumor angiogenesis (Sherr, 2004). Under normal conditions, tumor suppressors negatively regulate cellular proliferation. This is why the inactivation of these proteins allows the development of malignant phenotypes. This is supported by the fact that, in most human cancer cells, critical tumor suppressor genes are mutated (Viallet & Minna, 1990).

Among the most important members of the tumor suppressor proteins, p53, mainly a transcription factor, has a significant role in the regulation of cellular responses to a range of stress signals. Thus, the end result will favour apoptosis, cell cycle arrest, or senescence (Levine, Momand, & Finlay, 1991). In response to DNA damage, p53 will enhance the transcription of genes that are involved in repair mechanisms, angiogenesis, apoptosis, and cellular growth. It can also have non-transcriptional activities that promote cell survival (Brown, Lain, Verma, Fersht, & Lane, 2009). In turn, if DNA repair is defected, tumor suppressors are inactivated, and thus might lead to cancer such as hereditary and sporadic colon cancers (Markowitz S. , 2000).

Oncogenes are altered proto-oncogenes with acknowledged roles in the promotion of cellular transformation and initiations of neoplastic growth. Mutations resulting in the constitutive activation of these proteins lead to cellular transformation and alteration (Viallet & Minna, 1990). One system for classifying oncogenes is dividing them into five main categories: growth factors, tyrosine kinases (receptor and cytoplasmic), serine/threonine kinases, GTPases, and transcription factors (Croce, 2008). As we can notice, they were grouped according to their function and location in the cell, meaning that these two criteria interrelate. For example, proteins in the inner cell membrane are involved in signal transduction, such as Ras. Other oncoproteins c-myc and c-jun are involved in transcription and cell cycle regulation. These are nuclear proteins (Evans, 1993; Maddika, et al., 2007). Since oncoproteins are located in various sites of the cell and accordingly undergo different functions, this implicates their joint role in exceeding growth, eventually leading to unrestrained cell proliferation and finally carcinogenesis (Figure 4) (Harrington, Fanidi, & Evan, 1994).

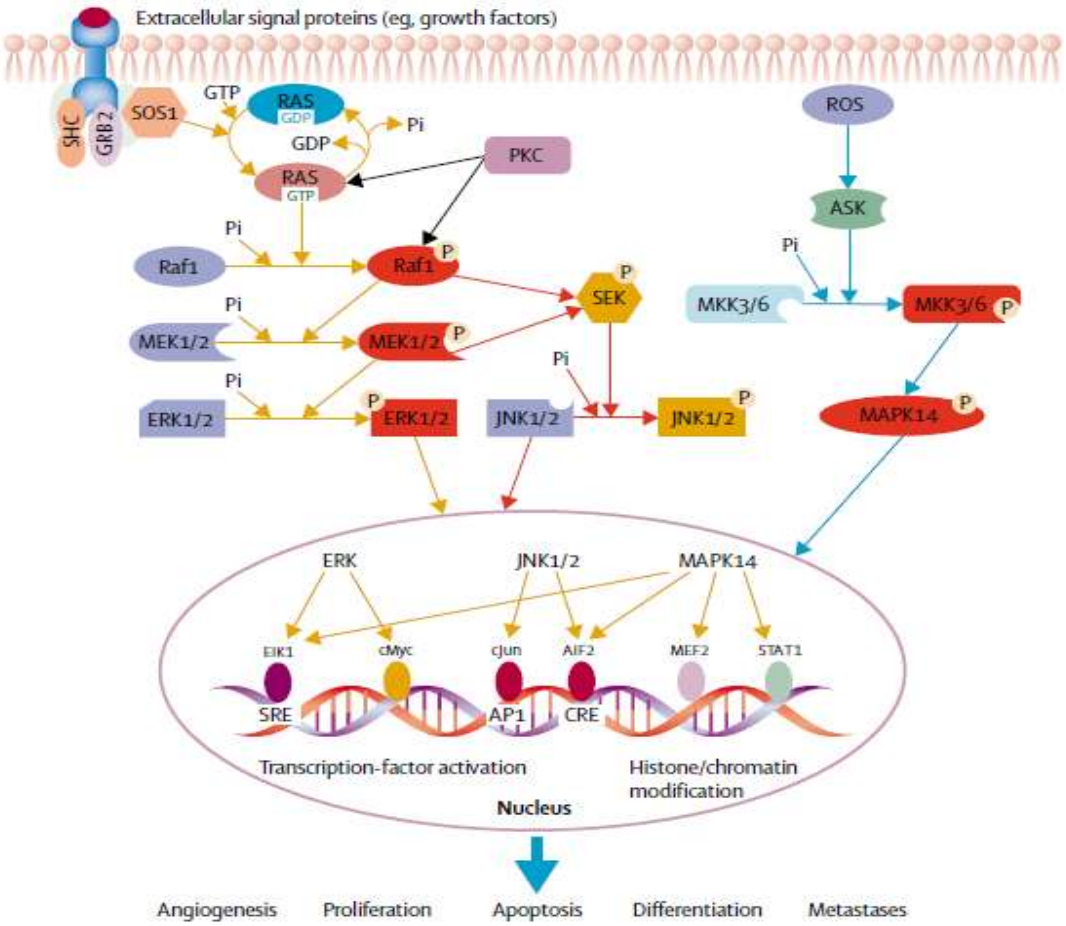

Figure 4: MAPK signaling in pathogenesis. Downstream effectors of the Ras pathway are key elements in cellular processes such as angiogenesis, proliferation, apoptosis, differentiation, and metastases. Source: **(Fang & Richardson, 2005)**

For the above mentioned reasons, tumor suppressors and oncogenes have antagonistic relationships in most cancer systems. This is illustrated by the inactivation of the first and the constitutive activation of the second (Viallet & Minna, 1990).

1.2.4. Apoptotic pathways

Apoptosis is termed programmed cell death. It results in morphological changes of cells and death. It plays important roles in embryogenesis, immunology, etc. Quite a number of complex signaling pathways are involved in apoptotic regulation, two of which are the intrinsic pathway or p53-mitochondrial pathway, and the extrinsic pathway activated by the binding of external ligands to specific membrane receptors called death

receptors (Muñoz-Pinedo, 2012; Taylor, Cullen, & Martine, 2008). In colorectal cancer, survivin is mostly responsible for apoptotic inhibition (Kawasaki, et al., 1998). The intrinsic pathway is mainly regulated by the Bcl family of proteins. They include pro-apoptotic Bax and Bad proteins, and anti-apoptotic Bcl-2 protein.

As compared to normal conditions, Bcl-2 is highly expressed in colorectal cancer patients. This phenomenon is also associated with the overexpression of several hormone receptors (Giannoulis, et al., 2004; Poinclous, et al., 2009). This is all significant, since Bcl-2 regulates the permeability of the mitochondrial membrane, thus indirectly restraining apoptosis. It inhibits secretory pore formation; through which cytochrome c exit the mitochondria. Subsequently, the apoptotic pathway would be incomplete due to the inhibition of caspase assembly (Ruvolo, Deng, & May, 2001; Srivastava, et al., 1998).

On the other hand, the pro-apoptotic proteins Bax and Bad are accountable for the formation of these pores in the mitochondrial membrane, allowing the release of cytochrome c into the cytoplasm. This will then activate the assembly of caspase-3 and caspase-9. The proteolytic cleavage of PARP, a downstream protein, takes place, thus triggering apoptosis (Muñoz-Pinedo, 2012). Both pro-apoptotic proteins are found to be down-regulated in patients with colorectal cancer, as compared to normal people (Jansson & Sun, 2002).

It is important to note that the levels of both pro and anti-apoptotic proteins of the Bcl family decrease significantly as the colorectal cancer tumor increases in size and further metastasizes (Giannoulis, et al., 2004; Jansson & Sun, 2002; Poinclous, et al., 2009; Yokota, 2000).

1.2.5. Altered cell growth and cancer

Typically, disturbing the homeostatic regulation of signaling pathways will definitely result in altered cell growth and eventually promoting carcinogenesis. Mutations and changes within these very critical processes affect a major cascade of pathways leading to uncontrolled proliferation, prolonged cell survival, and reduced cell death (Maddika, et al., 2007).

The disruption of apoptotic regulation prolongs cell life, promoting cancer. In cancer cells, apoptosis is inhibited by anti-apoptotic proteins. This is also achieved by upsetting the homeostasis between oncogenes and tumor suppressors. All this will result in prominent cancer cell characteristics, i.e. prolonged survival and prevention of cell death (Maddika, et al., 2007).

Along with inhibiting apoptotic pathways, mutations also result in the constitutive activation of survival pathways. Two of the most frequently disrupted survival pathways in humans cancers are the PI3-K/Akt and Ras/MAPK pathways. These mutations will also play major roles in tumor development and cell transformation, and most importantly in resistance to cancer treatments (Vivanco & Sawyers, 2002).

Since it is a tightly regulated and highly organized process, changes in the programmed regulation of the cell cycle succession might result in cancer development and growth (Collins & Garrett, 2005; Park & Lee, 2003). Being greatly involved in mitogenic signal transduction, tumor suppressor genes are "turned off" and oncogenes are "turned on" in transformed cancerous cells. Hence, the deregulation of proteins such as p53, p21, pRb, mdm2, and many others, promotes uncontrolled growth and development of tumors (Schafer, 1998).

1.3. Cell Motility

1.3.1. Cell motility cycle

Numerous physiological events, such as embryogenesis, tissue regeneration, inflammation, and wound healing, greatly depend on cellular motility. But also, cellular motility is crucial for cancer cell invasion and metastasis. It occurs usually as a response to growth factors or chemoattractants found in the ECM, around the cell. This process is known as chemotaxis (Lauffenburger & Horwitz, 1996). Due to its major role, cell motility has been a highly researched phenomenon. Scientists have been directing their studies towards understanding its molecular basis, as this

might lead to novel targeted therapeutic treatments inhibiting tumor growth, development, and metastasis (El Zouhairi, Charabaty, & Pishvaian, 2011).

Cell motility occurs in an amoeboid-like manner, after a signal is detected. It begins by determining the direction of motion, which is of course towards the chemoattractant. A protrusion is then extended from the cell towards the direction of motion. This is done by the polymerization of new actin filaments (Bailly, Condeelis, & Segall, 1998). The actin-rich protrusion is then stabilized by the adhesions formed to the cell substratum, providing anchorage to the cell. This will help transmit mechanical force, for the cell to pull its body forward and to the direction of motion. Concurrently, adhesion structures found at the rear edge of the cell will undergo disassembly, inducing the cell tail to retract moving the whole cell forward (Ananthakrishnan & Ehrlicher, 2007; Bailly, Yan, Whitesides, Condeelis, & Segall, 1998; Condeelis, et al., 2001). The family of Rho GTPases, including all its members, play a main role in regulating the cycle of cell motility, through the reorganization of the actin cytoskeleton (Condeelis J. S., 2001).

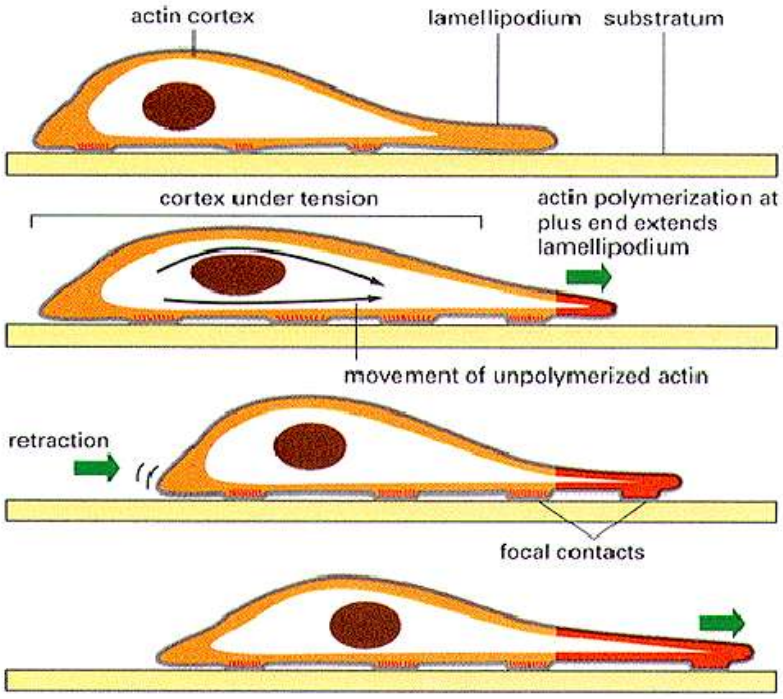

Figure 5: The cell motility cycle. First, signal-induced actin polymerization defines cell direction. Then, protrusions are extended, after which they become stabilized by adhesion. At the rear end, old adhesions detach, and the tail retracts and the cell body is pulled forward. Source: **(Childs, 2001)**

1.3.2. Altered cell motility and cancer

Cell motility is an intricate multistep process, integrating numerous regulatory and signaling pathways. Any slight deviation or malfunction at any step of the pathway may radically affect normal functions, resulting in transformation and carcinogenesis (Lauffenburger & Horwitz, 1996).

Acquiring a motile phenotype is an important characteristic of cancerous cells. It is a critical step towards gaining metastatic competence. Thus, targeting cell motility processes will help in introducing novel therapeutic agents against metastatic and invasive tumors (Silva, 2004).

1.4. Cell Invasion

During metastasis, tumor cells migrate from the primary site to secondary sites. They must first degrade the basement membrane, penetrate the extracellular matrix (ECM), and then migrate towards the lymph and blood (Figure 6) (Gertler & Condeelis, 2011; Kim, Liotta, & Kohn, 1993; Sahai, 2005).

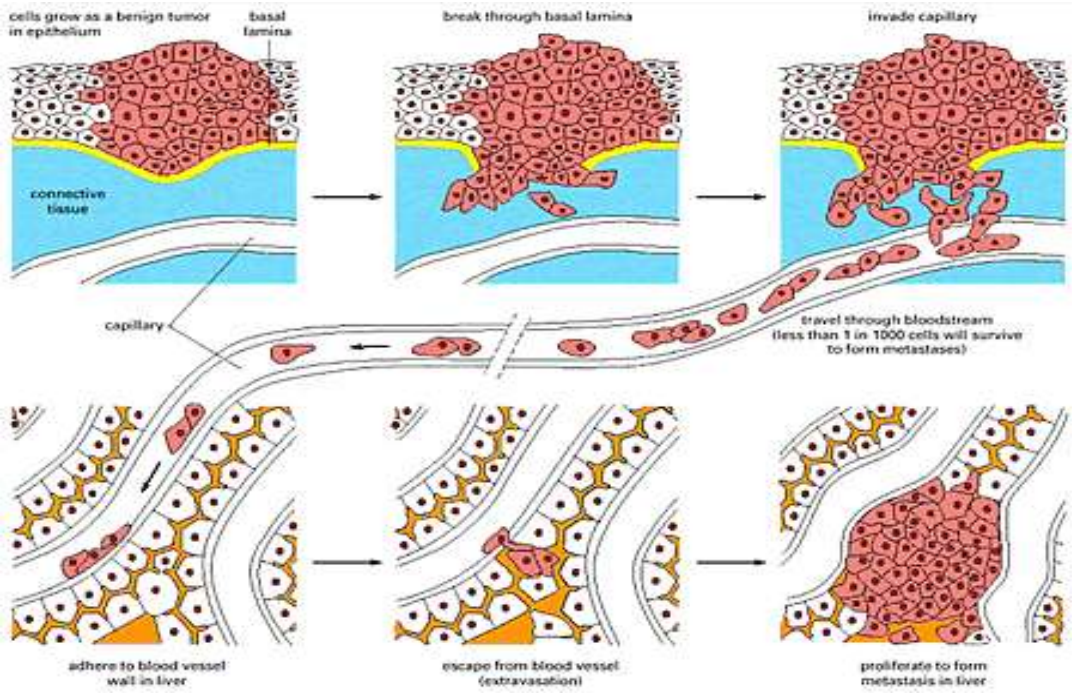

Figure 6: Process of invasion and metastasis. Cells first grow as benign tumors, invade through the basement membrane into the blood stream, where they travel, and adhere to a capillary wall. They will then extravasate and proliferate to form metastases. Source: **(Alberts, et al., 2002)**.

Cell invasion is referred to as the shift from primary benign tumors to malignant-acquired phenotypes. This involves the coordination and organization of both extracellular and intracellular communications (Calvo & Sahai, 2011).

1.4.1. ECM degradation

At first, the ECM must be successfully degraded at the site where cancer cells transverse the underlying basement membrane and invade to the interstitial stroma. Components of the ECM, such as proteoglycans, glycoproteins, and collagen type IV, must be effectively proteolysed (Kim, Liotta, & Kohn, 1993). Cancer cells would either secrete proteases or factors activating matrix proteases, such as matrix metalloproteinases (MMPs), which are associated with poor prognosis in colorectal cancer (Murray, Duncan, O'Neil, Melvin, & Fothergrill, 1996). These are secreted in their inactive pro-enzyme form, and are activated extracellularly by the help of plasmin. For this to successfully happen, MMPs must evade endogenous inhibitors, such as TIMPs. Therefore, there must be a homeostatic balance among active proteases and their inhibitors. This will determine the exact invasive phenotype of the tumor cells (Aznavoorian, Murphy, Stetler-Stevenson, & Liotta, 1993; Gertler & Condeelis, 2011; Stetler-Stevenson, Aznavoorian, & Liotta, 1993).

After successfully degrading the ECM, cells can now reach the circulatory system, by the help of a process called angiogenesis. This will facilitate the tumor in expanding and gaining easier access to vascular tissues (Aznavoorian, Murphy, Stetler-Stevenson, & Liotta, 1993). Continuously, components of the tumor environment will interact with the surrounding host tissue of the secondary site (Calvo & Sahai, 2011). This is done through a complex network of interconnected molecular pathways that highly influence cell migration (Brabletz, et al., 2005). Eventually, circulating cancer cells still have to escape the host immune system, extravasate, invade, and proliferate in the secondary site. Thus, neoplastic tissues will then be effectively established at distant sites (Gertler & Condeelis, 2011; Kim, Liotta, & Kohn, 1993; Brabletz, et al., 2005).

1.4.2. Molecular mechanism of invasion

During invasion, tumor cells must form membrane protrusions rich in F-actin. These are known as invadopodia. With the help of matrix metalloproteinases, successive steps during invasion would be accomplished by first degrading the dense barriers (Bravo-Cordero, et al., 2011). Invadopodia vertically extend into the ECM. They are enriched in actin filaments, regulatory and binding proteins, and matrix proteinases (Condeelis & Segall, 2003; Yamaguchi, et al., 2005).

The molecular mechanisms involved in invasion mainly regulate invadopodia dynamics and induce signaling pathways. These pathways comprise of various proteins, such as N-WASP, cortactin, Arp2/3, cofilin, and MT1-MMP (Artym, Zhang, Seillier-Moiseiwitsch, Yamada, & Mueller, 2006; Yamaguchi, et al., 2005).

Cofilin is a very important signaling protein. It generates free barbed ends after cutting actin filaments, leading to the nucleation of actin. Severing is crucial. It exposes the filaments, since most of them are covered and capped in resting cells. Cofilin is inactivated by TES and LIM families. These kinases phosphorylate and block cofilin's binding to F-actin (Bravo-Cordero, et al., 2011). It has been previously proven that the Rho family of small GTPases activate ROCK, a downstream kinase, which in turn activates the LIM kinase (LIMK) by phosphorylating it (Olson & Sahai, 2009).

Other than severing of actin filaments, barbed ends can be generated by a process called *de novo* nucleation. It is mediated by Arp2/3 complex, providing a template for actin nucleation (Olson & Sahai, 2009). The Arp2/3 complex is activated by the WASP family of proteins, Wiskott-Aldrich syndrome protein. Several scaffold proteins, WASP/SCAR/WAVE, have a VCA domain, through which they can directly bind to Arp2/3. All these proteins are key regulators for actin polymerization (Olson & Sahai, 2009; Yamaguchi, et al., 2005).

In all, the activation, inhibition, and regulation of the dynamics of actin, especially in invadopodia, are essential for the invasion and metastasis of tumors.

1.4.3. EMT

A very prominent fact is that tumor progression is mainly based on epithelial-mesenchymal transition (EMT). It is also well known that the EMT process is accountable for the spreading and propagation of primary cancerous cells to secondary metastatic sites (Thierry, 2002).

A series of events must take place accordingly for the EMT of cancer to progress. Epithelial markers must be downregulated, while mesenchymal proteins must be upregulated. Morphologically, this is portrayed by increased motility, loss of cell-cell adhesion, pseudopodia formation, and elongated polarized shapes (Morra & Moch, 2011; Thierry, 2002).

EMT favours the progression and stability of metastasized tumors, by overcoming safeguard mechanisms and attenuating the immune system. This is accomplished by overcoming apoptotic pathways and premature senescence (Nieto, 2011). In addition, EMT helps in acquiring resistance against radiotherapy and chemotherapy. However, in order for secondary site tumors to stabilize and colonize, they must revert back to their epithelial nature, a process known as MET (Nieto, 2011; Thierry, 2002).

1.5. Rho family GTPases

The family of Rho GTPases consists of small GTP-binding proteins, ranging between 20 and 40 KDa. They are key members in cancer cell motility and invasion. They play important roles in signal transduction, cytoskeleton re-organization, and cellular polarity (Boettner & Van Aelst, 2002). The gravity of their role lies in the fact that a simple biochemical idea is behind these biological complexities. By switching on only one single GTPase, a number of pathways will be activated co-ordinately. Thus, the harmonization of spatial and temporal switching of several GTPases is what makes this family prominent in eukaryotic cell biology (Etienne-Manneville & Hall, 2002). The three most considered Rho GTPases are Rho, Rac and Cdc42. They have distinct functions than other members of their family (Takai, Sasaki, & Matozaki, 2001; Vega & Ridley, 2008).

1.5.1. General structure of Rho GTPases

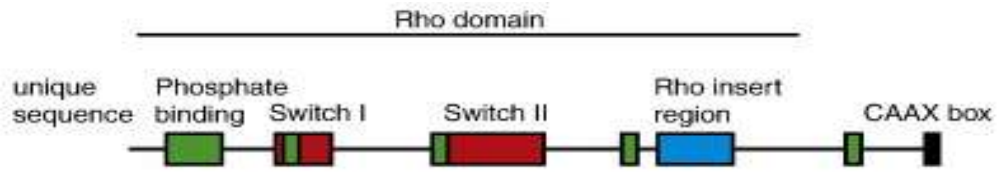

Figure 7: Primary structure of Rho GTPases. Source: (Gad & Aspenström, 2010)

All members of the Rho GTPase family have a constant sequence of amino acids at their N-terminal where they bind to GDP and GTP, acquiring their GTPases activity to hydrolyze them (Johnson, 1999; Valencia, Chardin, Wittinghofer, & Sander, 1991). An important Rho binding domain is Switch I, responsible for downstream effectors (Marshall, 1993). At their C-terminal, the CAAX box undergoes post-translational changes important for their proper localization in the cell (Magee, et al., 1992) (Figure 7).

1.5.2. Rho GTPases as binary switches

The tight control over the activity of Rho GTPases is based on their subcellular localization and nucleotide binding (Wennerberg & Der, 2004). Thus, they switch between an inactive form when bound to GDP and localized in the cytoplasm, and an active form when bound to GTP and recruited to the plasma membrane (Vega & Ridley, 2008) (Figure 8).

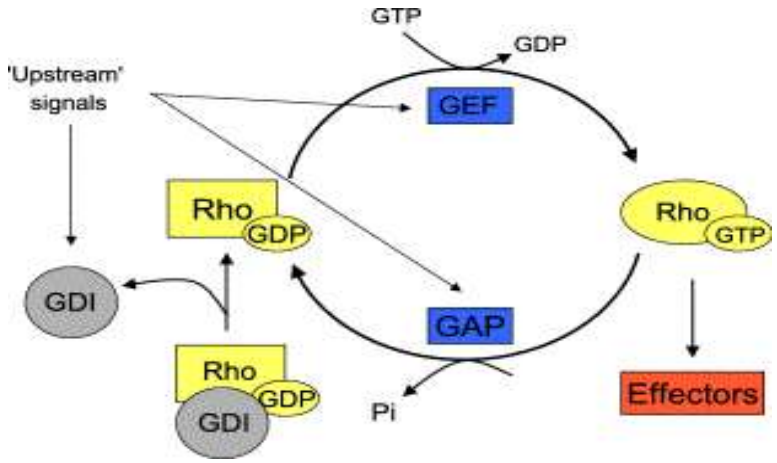

Figure 8: The Regulation of Rho family of proteins. GDI: guanine nucleotide dissociation inhibitor, GEF: guanine nucleotide exchange factor, and GAP: GTPase activating protein. Source: **(Raftopoulou & Hall, 2004)**.

Certain upstream signals will activate the Rho GTPases, by phosphorylating its GDP to GTP, inducing a conformational change that favours downstream effectors to bind to Rho GTPases. Then, GTP is hydrolyzed back to GDP and the GTPase returns back to its inactive form, completing one cycle of GTPases activation and inactivation (Etienne-Manneville & Hall, 2002; Jaffe & Hall, 2005).

1.5.3. Rho GTPases and their regulation

As shown in figure 8, the regulation of a Rho protein and its switch from the inactive to the active form is done by GEFs, GAPs, and GDIs.

1.5.3.1. GEFs

The primary dissociation of GDP from inactive Rho GTPases is known to be the rate-limiting step. It is a very slow reaction that must be stimulated by GEFs. They have several important domains, such as the pleckstrin homology domain (PH), Src homology 3 domain (SH3), and Dbl homology domain (DH). GEFs alter the GDP-GTP exchange, by interacting with phospholipids of proteins at the cell membrane. Therefore, GEFs activate Rho GTPases (Grise, Bidaud, & Moreau, 2009; Schmidt & Hall, 2002).

1.5.3.2. GAPs

As their name indicates, GAPs are proteins that activate the intrinsic GTPase activity of Rho GTPases. This will hydrolyze the GTP into GDP, thus transforming Rho GTPases back to their inactive form. Also, GAPs may mediate other downstream functions of Rho GTPases (Grise, Bidaud, & Moreau, 2009). Hence, GAPs negatively regulate Rho GTPases, rendering them inactive (Moon & Zheng, 2003).

1.5.3.3. GDIs

GDIs play a very important role. They inhibit GEFs and GAPs. They prevent the GDP dissociation of inactive Rho GTPases, and the interaction of active forms with GAPs. Continuously, they aid in the cycling of Rho GTPases between the cell membrane and the cytoplasm. In the cytoplasm, GDIs mask the binding domains of GTPases, thus sequestering them away from the cell membrane, which is the site of their activation. This is crucial, since temporal and spatial properties are key characteristics for the regulation of Rho GTPases (DerMardirossian & Bokoch, 2005; Garcia-Mata, Boulter, & Burridge, 2011; Grise, Bidaud, & Moreau, 2009).

1.6. Signaling pathways of Rho GTPases

Many plasma membrane receptors capture extracellular stimuli and activate the Rho family of GTPases. Growth factors and mitogens are upstream signals regulating Rho GTPases, which activate downstream effectors (Figure 8). In turn, these proteins translate the signals, regulating countless functions like chemotaxis, polarity, cell adhesion, proliferation, cell-cell interaction, cytoskeleton re-organization, etc (Sahai & Marshall, 2002).

1.6.1. Upstream signaling

1.6.1.1. PI3K signaling

GEFs have a PH domain with high affinity to phosphoinositides, such as PI(4,5)P2 found on the plasma membrane (Macias, et al., 1994). PI(4,5)P2 binds to GEF, favouring the interaction between PH and DH domains of GEF. It is then phosphorylated by PI3K forming PI(3,4,5)P3, inducing its binding to the PH domain. This leads to the dissociation of DH catalytic domain, which in turn will activate the GEF. Consecutively, GEF will bind and activate Rho GTPases (Schmidt & Hall, 2002).

Several studies have shown that PI3K works upstream of Rho GTPases. Insulin, EGF, PDGF, and LPA are external signals triggering the activation of Rho GTPases through the PI3K pathway. This was shown by treating fibroblasts with wortmannin, which is a PI3K inhibitor. Rho and Rac, members of the Rho GTPase family, were inhibited, suggesting that in response to growth factors, GTPases are downstream of PI3K (Nobes, Hawkins, Stephens, & Hall, 1995).

A renowned GEF, also activated by PI3K, is Vav. It is activated by Src kinases, which in turn activates its catalytic domain (Lopez-Lago, Lee, Cruz, Movilla, & Bustelo, 2000). This is achieved when Vav binds PI(3,4,5)P3, thus weakening the PH/DH interaction. The auto-inhibition exerted by the PH domain is alleviated (Bustelo, 2000; Crespo, Schuebel, Ostrom, Gutkind, & Bustelo, 1997).

Another important GEF is Son of sevenless (Sos). It binds to PIP3 through its PH domain, removing auto-inhibition. Downstream of receptor tyrosine kinases (RTKs), Sos binds many adaptor proteins (Das, et al., 2000).

The subcellular localization of GEFs is important for Rho GTPase activation. This spatial regulation is achieved by active PI3K producing PIP3, rendering the loss of the PH domain. Another motif targeting the protein to the plasma membrane is CAAX (Etienne-Manneville & Hall, 2002).

Conducted research has shown that Rho GTPases and PI3K are highly involved in cancer cell motility, by stimulating lamellipodia formation. This is further proved by showing that Rac and Cdc42 are upstream of PI3K (Keely, Westwick, Whitehead, Der, & Parise, 1997).

1.6.1.2. Activation by adhesion

Other than upstream signals, cell-ECM adhesion also stimulates Rho GTPase activation. Focal adhesion kinases (FAKs) are activated, leading to paxillin and p130cas phosphorylation and activation (DeMali & Burridge, 2003; Zamir & Geiger, 2001).

Upon phosphorylation, p130cas forms complexes with Crk and DOCK180, a GEF for Rac (Cary, Han, Polte, Hanks, & Guan, 1998; Klemke, et al., 1998; Matsuda, et al., 1996). Also, when phosphorylated, paxillin complexes with PKL and PIX, another GEF for Rac (Bagrodias, Taylor, Jordan, Van Aelst, & Cerione, 1998).

Other than activating Rac, FAK inhibits RhoA by activating p190RhoGAP. This is why in most cells low RhoA activity but high Rac activity are stimulated by FAK activation (O'Connor, Nguyen, & Mercurio, 2000).

1.6.2. Downstream signaling

Major downstream effectors of the PI3K signaling pathway are small GTPases, such as Rho, Rac, and Cdc42. These are important regulators of the cell cytoskeleton, by promoting actomyosin assembly, stress fiber formation, actin nucleation and polymerization, etc (Ananthakrishnan & Ehrlicher, 2007; Etienne-Manneville & Hall, 2002; Zhou & Sinder, 2006).

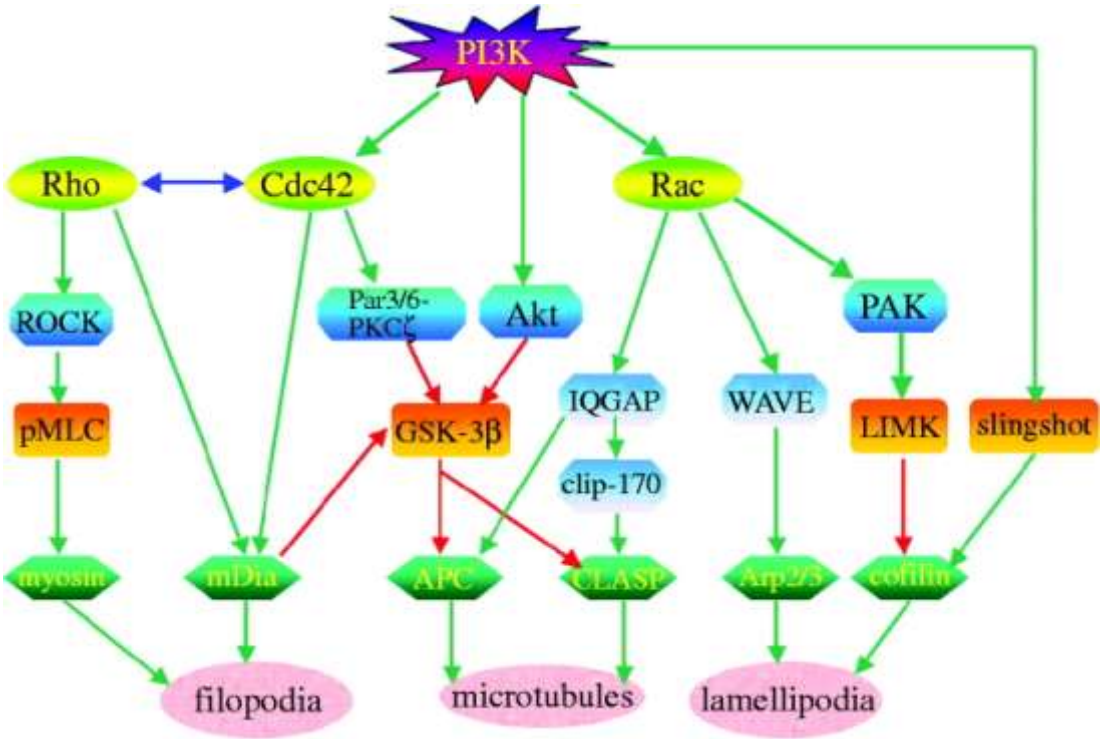

Figure 9: Downstream effectors of Rho, Rac, and Cdc42. Explained in sections 1.6.2.1 and 1.6.2.2. Red arrows indicate inhibition, while green arrows indicate activation. Source: (Zhou & Sinder, 2006).

1.6.2.1. Rho Effectors

Rho is known to be indirectly regulated by the PI3K pathway. One of its main effectors is p160ROCK, the serine/threonine kinase involved in focal adhesions and stress fibers formation. Through ROCK, the myosin light chain (MLC) is phosphorylated, and actin dynamics are regulated by the contractility of actin and myosin (Figure 9) (Grise, Bidaud, & Moreau, 2009; Kimura, et al., 1996; Struckhoff, Rana, & Worthylake, 2011). Also, studies have shown that Rho can inactivate GSK-3 through mammalian homolog of diaphanous (mDia) activation, leading to actin nucleation and stress fiber formation (Alberts A. S., 2001).

Another downstream protein is LIMK. It phosphorylates cofilin, rendering it inactive as mentioned previously. Cofilin is known to be a protein that severs actin (Olson & Sahai, 2009).

Members of the ERM family of proteins, known as ezrin-radixin-meosin proteins, are Rho effectors. They are associated with the plasma membrane, where active Rho is translocated, and actin cytoskeleton remodelling take place (Takaishi, Sasaki, Kameyama, Tsukita, & Takai, 1995).

1.6.2.2. Rac/Cdc42 Effectors

The VCA is a consensus domain found in all downstream effectors of Rac and Cdc42. It is found in the WASP/SCAR/WAVE family of proteins (Keely, Westwick, Whitehead, Der, & Parise, 1997).

PAK is a serine/threonine p21 activating kinase. It is an effector of Rac and Cdc42. These proteins have a catalytic domain at their C-terminal, and a regulatory domain at their N-terminal (Manser, Leung, Salihuddin, Zhao, & Lim, 1994). They also have a common domain where Rac and Cdc bind, called the CRIB domain, or the Cdc42/Rac interactive binding domain (Burbelo & Drechsel, 1995). When active GTPases bind to PAK, the regulatory domain is disrupted and the catalytic domain is activated by phosphorylation, rendering PAK active

(Manser, Leung, Salihuddin, Zhao, & Lim, 1994). Active PAK has a couple of important roles. It inactivates MLCK by phosphorylating it, consecutively activating MLC by inhibiting its phosphorylation (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999). Another role is to phosphorylate and activate LIMK, when targeted to adhesion complexes (Figure 9) (Bokoch, 2003; Burridge & Wennerberg, 2004; Edwards, Sanders, Bokoch, & Gill, 1999).

1.7. Role of Rho GTPases in 2D and 3D cell migration

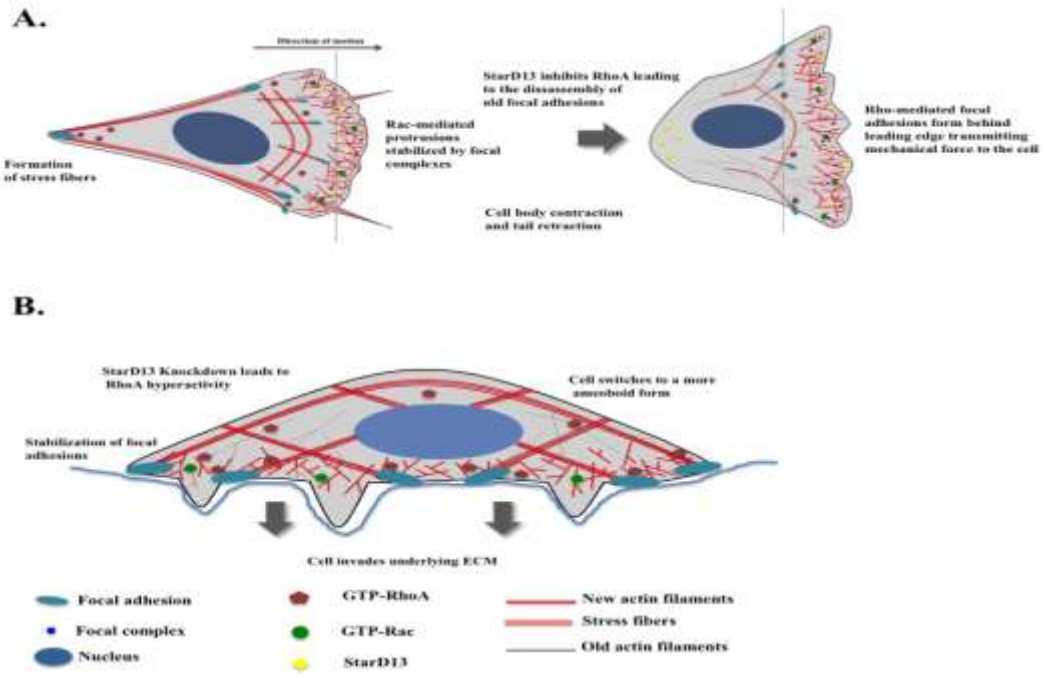

Figure 10: Rho GTPases in cell motility. A: 2D cell motility. B: 3D cell motility.

Cell motility cycle involves several consecutive steps, including: direction of motion, cell polarization, formation of protrusions, adhesion, cell body contraction, and retraction of the tail (Lauffenburger & Horwitz, 1996).

1.7.1. Direction of motion and protrusion formation

The determination of the direction of motion is the initial step in the motility cycle. Membrane protrusions are generated and can be either lamellipodia or filopodia.

At the cell edge, a lamellipodium is a meshwork of actin filaments (Small, Stradal, & Rottner, 2002). It involves effectors downstream to Rac, such as the Arp2/3 complex, ADF/cofilin (Van Troys, et al., 2008), and PIP-5-K activation. Thus, these lamellipodia are Rac-dependent. Yet, these are also downstream of Cdc42. Thus, Cdc42 is known to be a possible regulator, driving Rac-dependent lamellipodia (El-Sibai, et al., 2007; Hall, 1998).

Another form of protrusions is the filopodia, which functions as a sensor for chemoattractants during migration. They pull out from the cell to detect any environmental alterations (Arjonen, Kaukonen, & Ivaska, 2011; Ridley, 2001). By activating N-WASP, actin polymerization occurs, leading to the formation of filopodia. This is regulated by Cdc42 (Nobes & Hall, 1999).

In addition, Cdc42 regulates cell polarity and direction of motion through the microtubule cytoskeleton (Johnson, 1999).

1.7.2. Adhesion formation

After the formation of protrusions, these must stabilize and adhere to the ECM, thus activating Rac and Cdc42 required for cell spreading (Condeelis J. S., 2001; Price, Leng, Schwartz, & Bokoch, 1998). Relative levels of active Rho GTPases, i.e. Rho, Rac, and Cdc42, is an indication of the composition of the substratum, which in turn signifies the speed of cell migration. Hence, the interaction between Rac and integrins is crucial for the cell to respond with respect to the changing substratum composition (Nobes & Hall, 1999; Price, Leng, Schwartz, & Bokoch, 1998; Ridley, 2001).

The interplay between Rac and RhoA defines adhesion formation of cell (Rottner, Hall, & Small, 1999). The former is responsible for the formation of primary focal complexes by activating PAK, which consecutively interact with PIX, paxillin, and GIT proteins. Another pathway for the formation of these focal complexes behind the lamellipodia is to antagonize Rho activation (Sander, ten Klooster, van Delft, van der Kammen, & Collard, 1999). These complexes are not enough to transmit contractility during cell movement (Kaverina, Krylyshkina, & Small, 2002). Therefore, focal complexes will mature into more stabilized Rho-dependent focal

adhesions (Arjonen, Kaukonen, & Ivaska, 2011; Wolfenson, Henis, Geiger, & Bershadsky, 2009). These will confer anchorage and strength for the cell body to contract and the cell to slide and move forward along the ECM (Wolfenson, Henis, Geiger, & Bershadsky, 2009). Increased RhoA activation will further stabilize focal adhesions, consequently inhibiting cell movement (Cox, Sastry, & Huttenlocher, 2001).

1.7.3. Cell body contraction and tail retraction

After adhesion is acquired, contraction of the cell body and then retraction of the tail are needed (Figure 5).

The first process depends on actomyosin contractility, regulated by Rho. As observed in figure 9, downstream from Rho and upon activation, ROCK induces contractility by phosphorylating MLC. So, sites of adhesion become tense and contractile. Other than the Rho/ROCK pathway, MLC is regulated by MLCK (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999). Other than the ROCK pathway, cell protrusions are inhibited by RhoA, which inactivates cofilin (El-Sibai, et al., 2008; Worthylake & Burridge, 2003). Hence, RhoA is localized to the leading edge to activate ROCK, transmitting tension to the sites of adhesion, and to the rear end to inactivate cofilin and inhibit protrusions.

The final process of cell motility is tail retraction, i.e. adhesions at the rear end must disassemble (Palecek, Huttenlocher, Horwitz, & Lauffenburger, 1998). Reduced RhoA activity inhibits retraction by decreasing actomyosin contractility (Cox, Sastry, & Huttenlocher, 2001; El-Sibai, et al., 2008; Worthylake & Burridge, 2003).

1.7.4. Crosstalk between Rho GTPases

Primary research has shown that each of the different proteins in the family of Rho GTPases has a well-defined unique role in actin regulation and adhesion dynamics during cell migration. Nevertheless, recent research has proved the presence of a prominent crosstalk among all signalling pathways of Rho, Rac, and Cdc42 (Khalil & El-Sibai, 2012). For instance, Rho and Rac

have antagonistic relationships, where the activation of one leads to the inactivation of the other. This is mainly done through stimulating either a GAP or a GEF (Burridge & Wennerberg, 2004; Sander, ten Klooster, van Delft, van der Kammen, & Collard, 1999). Another example is the crosstalk of RhoA with Rac and Cdc42 at the rear end of the cell, which aids in the regulation of the actin cytoskeleton (El-Sibai, et al., 2008).

1.7.5. Altered role in cancer

One of the most important characteristics a cancer can acquire is the ability to move, invade, and metastasize. These processes are tightly regulated by Rho GTPases. Genetic screening studies have conveyed that RhoA and RhoC, two members of the Rho GTPase family, are hyperactive and over expressed in colorectal cancer cells, as well as many other types of tumors (Sahai & Marshall, 2002). Furthermore, the inactivity or the expression of the negatively dominant form of Rho led to the inhibition of motility (Clark, Golub, Lander, & Hynes, 2000).

Mostly important proteins, GAPs and GEFs, have major roles in the dominant inhibition or activation of Rho GTPases. This in turn affects motility, invasion, and metastasis of colorectal cancer cells (Brabletz, et al., 2005; Jaffe & Hall, 2005). Furthermore, it is not necessary for the dysfunction to occur at the level of Rho GTPases only. Any inhibition in the downstream effectors in these pathways will lead to malfunctions in the processes. For instance, inhibiting RhoA by C3T, or inhibiting ROCK by Y27632 will result in less motility, and inhibited maturation of focal complexes into adhesion (El-Sibai, et al., 2008).

Prolonged survival and apoptotic inhibition are both side effects of abnormal Rho activation. This is possible through the pathway involving CDKs, which highly regulate progression of the cell cycle and support tumor growth (Tatsuno, Hirai, & Saito, 2000). In most cancers, CD-1 is highly over expressed. This is correlated to the activation of Rho GTPases and transcription factors, such as ATF-2 and NF-κB. These factors activate CD-1 promoter through direct binding (Guttridge, Albanese, Reuther, Pestell, & Baldwin, 1999). The main Rho GTPase involved is Rac1. When dominantly

active, it stimulates proliferation and transformation through over expressing CD-1. Also, the inhibition of CDK inhibitors, such as p21 which is also a tumor suppressor, is mediated through over expressing a member of the RhoGTPase family, RhoA (Joyce, et al., 1999).

This is why massive research is conducted to use targeted therapies for cancer, especially towards RhoGTPases, such as strongylophorine-26 (McHardy, Warabi, Anderson, Roskelley, & Roberge, 2005) and FTI drugs, or targeting downstream effectors, such as Y-27632 ROCK inhibitor (Du & Prendergast, 1999).

1.8. StarD13

StarD13, or START-GAP2, is also known as the *DLC2* gene. It was first identified by Ching et al. (2003) to be downregulated in hepatocellular carcinoma. It is located on position *13q12.3* (Popescu & Durkin, 2004). StarD13, or steriodogenic acute regulatory protein-related lipid transfer domain-containing protein 13, has a C-terminal START domain and an N-terminal SAM domain. In between, it holds a GAP domain for Rho GTPases. It has four known isoforms: α, β, γ, and δ (Figure 11) (Ching, et al., 2003; Thorsell, et al., 2011; Ullmannova & Popescu, 2006).

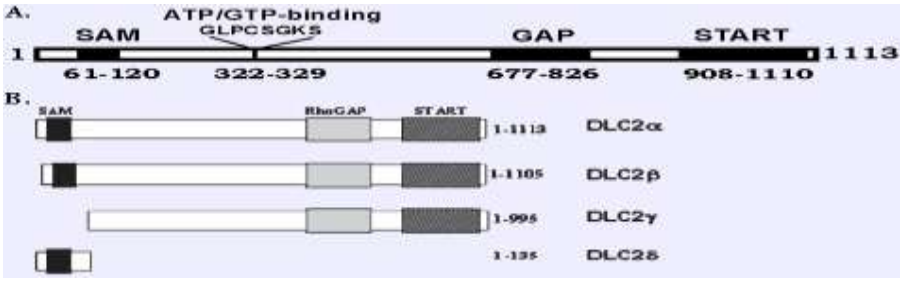

Figure 11: StarD13 structural domains. A: It has N-terminal SAM domain, an ATP/GTP-binding domain, a GAP domain and a C-terminal START domain. B: DLC-2 isomers and their structural domains. Source: (Thorsell, et al., 2011).

1.8.1. Role as a tumor suppressor

StarD13 is a member of the DLC (deleted in liver cancer) family, which is known to be a family of tumor suppressors. It has 64% homology with DLC-1 (Durkin, et al., 2007; Ullmannova & Popescu, 2006). Research

conducted on DLC-1 has shown that it is underexpressed in many types of cancer, such as stomach, uterus, breast, colon, kidney, prostate, and lung (Liao & Lo, 2008). Also, DLC-2 was found to be overexpressed in cells of low rates of growth and proliferation (Ching, et al., 2003). All the above suggest a prominent role of StarD13 as a tumor suppressor (El-Sitt, et al., 2012).

1.8.2. StarD13 activity and localization

Studies showed that StarD13 inhibits Cdc42 and RhoA, which in turn inhibits the formation of actin stress fibers (Ching, et al., 2003). Furthermore, StarD13 is targeted towards the mitochondria through its START domain. This demonstrates its potential role in regulating the permeability of the mitochondrial membranes, activating pathways of apoptosis (Ng, et al., 2006). Another domain that targets StarD13 to focal adhesions is the N-terminal FAT domain, which interacts with a constituent of focal adhesions named tensin2 (Kawai, et al., 2009).

Moreover, research proved the RhoGAP activity of StarD13 on RhoA, i.e. RhoA is inhibited by StarD13. This is through the inhibition of actin stress fibers assembly, mediated by RhoA. Successively, and through this Rho-mediated pathway, cell transformation is inhibited, as well as the modulation of cell attachment, cell migration, and cell differentiation (Leung, et al., 2005; Lin, et al., 2010; Xiaorong, Wei, Liyuan, & Kaiyan, 2008).

1.9. Purpose of the study

Throughout this study, our aim was to investigate the role of StarD13 on the proliferation and motility of colorectal cancer cell lines. First, we studied its effect on cellular proliferation and viability upon knocking down and over expressing StarD13. Then, we examined its RhoGAP activity as well as its possible interaction with Rac1 and Cdc42, and their effect on the migration, invasion, and adhesion of colon cancer cells. The experiments include Trypan Blue Exclusion method, MTT, WST proliferation kits, wound healing assay, immunostaining, invasion, adhesion...

Chapter II

MATERIALS & METHODS

2.1. Cell culture

Human colorectal cancer cell lines (Caco-2 and HT-29) obtained from ATCC, were cultured in Dulbecco's Modified Eagle Medium, DMEM, (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum, FBS, (Sigma-Aldrich) and 100U or 1% (v/v) penicillin/streptomycin (Sigma-Aldrich) in a humidified chamber at 37°C and 5% CO_2. Cells were cultured in T-75 flasks (Corning).

2.2. Antibodies and reagents

Goat polyclonal anti-StarD13 antibody was obtained from Santa Cruz Biotechnology. Rabbit monoclonal anti-RhoA, mouse monoclonal anti-Rac1, mouse monoclonal anti-Cdc42 and mouse monoclonal anti-vinculin antibodies were purchased from Upstate Biotechnology, Lake Placid, NY. Anti-goat, anti-rabbit 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).

2.3. Cell transfection with siRNA

Goat FlexiTube siRNA (5nmol) for StarD13, RhoA, Rac1 and Cdc42 were obtained from Qiagen. Consecutively, their target sequences are:
Hs_StarD13_3: 5'-CCCGCAATACGCTCAGTTATA-3',
Hs_StarD13_8: 5'-ATGGCTACATCCCTACTAATA-3',
Hs_RhoA_6: 5'-TTCGGAATGATGAGCACACAA-3',
Hs_Rac1_6: 5'-ATGCATTTCCTGGAGAATATA-3',
and Hs_Cdc42_7: 5'-CATCAGATTTGAAATATTTAA-3'.

Cells were transfected with the siRNA at a final concentration of 10nM 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 pulled down and/or analyzed by western blotting using the appropriate antibodies. Also, the effect of the corresponding knockdown was studied.

2.4. Cell transfection with vectors

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

The GFP-StarD13 construct was a generous gift from Dr. Hitoshi Yagisawa from the University of Hyogo, Japan.

The constructs were transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen), after which they were grown on selective media containing the appropriate antibiotic. The vectors were then extracted using MaxiPrep plasmid extraction kit (Qiagen).

2.5. Western blotting

Cell lysates were prepared by scraping the cells in a sample buffer consisting of 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125M 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 1:1000 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 1:2000 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 plates at density of $1x10^6$cells/mL and were transfected by either control or StarD13 siRNA for 72hrs. Total RNA was extracted using the RNeasy extraction kit (Qiagen) according to the 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^{o}C for 30min, initial PCR activation step at 95^{o}C for 15min, followed by 25 cycles of denaturation at 94^{o}C for 1min, annealing for StarD13 at 50^{o}C, for actin 50°C, for TGFα 48°C, and for TGFβ 50°C for 1min and extension at 72^{o}C for 1min followed by a final extension step at 72^{o}C for 10min.

10µl of the PCR products were run on 1% 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. 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 thus appearing bright. The percentage of dead cells was reported.

2.8. Cell proliferation reagent (WST-1)

Cells were seeded in 96-well plates (growth area: $0.6cm^2$) 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, 10µl 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 450nm. 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.

2.9. Cell proliferation kit I (MTT)

Cells were cultured in 96-well flat bottom microplates (100µL/well) in a humidified incubator for 72h at 37°C after treatment. 10µL of the MTT labelling

reagent was added to each well and then incubated for 4h. Afterwards, 100μL of the Solubilization solution is added per well and the plate incubated overnight. MTT is a tetrazolium salt that forms formazan when in contact with metabolically active cells. Quantitation of formazan is done colorimetrically, using ELISA, at 595nm. 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

Cells were plated on cover slips, and the appropriate treatment was applied. They were then 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 0.1M glycine then incubated with 0.1M 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 lysed and incubated with GST-CRIB or GST-RBD and the pull-down assay performed using the RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell BioLabs) following the manufacturer's instructions. Lysates were incubated with GST-RBD (for RhoA) or GST-PAK (for Rac1/Cdc42) for 1 hour at 4 °C. GTP-RhoA, GTP-Rac1 or GTP-Cdc42 was detected by western blotting using the anti-RhoA, anti-Rac1 or 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. Cells were then 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. 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 CO_2 incubator for 1 hour. Plates were then washed and put on ice. Meanwhile, the cells were trypsinized and counted to $4x10^5$cell/mL. 50μL of cells were added in each well and incubated at 37°C in a 5% CO2 incubator for 30 minutes. The plates were shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 minutes, washed, and stained with crystal violet (5mg/mL in 2% ethanol) for 10 minutes. After staining, 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 550μm using ELISA.

2.14. 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^oC 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). 100μL 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.

2.15. 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/). All results showed stasitical significance with a p-value ≤ 0.05.

Chapter III

RESULTS

3.1. StarD13 knockdown increases colorectal cancer cell viability

We first wanted to study the role of StarD13 on colorectal cancer cell viability. We knocked down StarD13 using two different siRNA oligos. The inhibition results were shown by western blot and RT-PCR. The loading control was β-actin (Figure 12A). Due to knockdown, around 38% decrease in dead cells was observed, using the exclusion method (Figure 12B). Correspondingly, WST-1 reagent and MTT kit showed around 40% increase in cellular proliferation of transfected cells with StarD13 siRNA (Figures 12C-D).

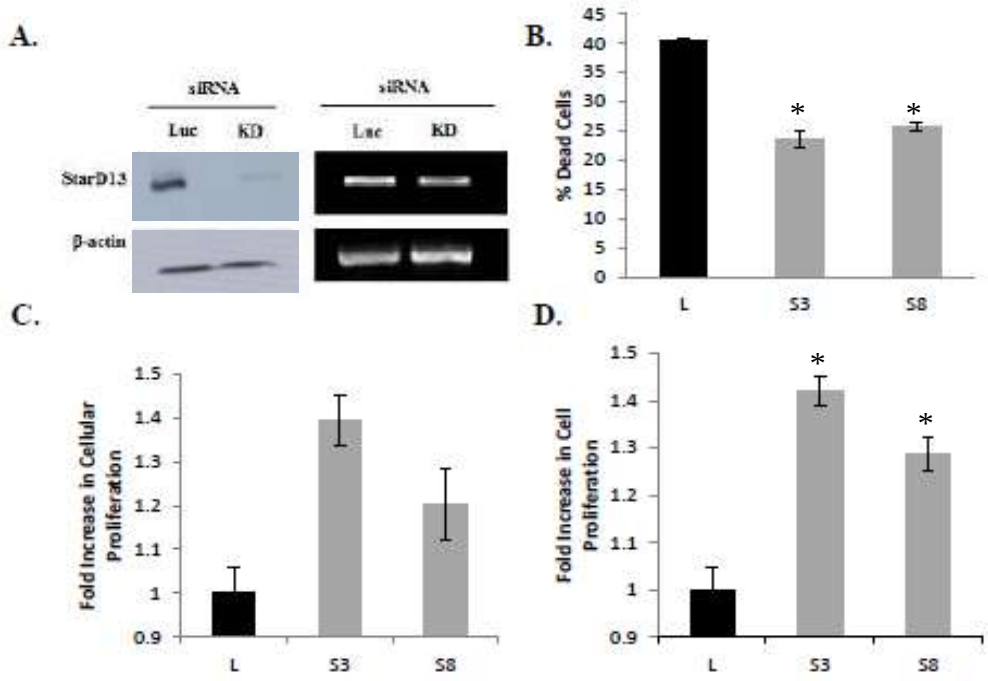

Figure 12: StarD13 knockdown increases cell viability. Control cells were transfected with Luciferase, while treated cells were transfected with two oligos of StarD13 siRNA: oligo 3 and 8. Experiments were done after 72h from transfection. **A.** After cell lysis, western blot analysis was performed for StarD13 (upper left gel) and for β-actin as a loading control (lower left gel). Also, StarD13 level was assessed through RT-PCR (upper right gel), and β-actin used as a loading control (lower right gel). **B.** Trypan blue exclusion method was done and the % dead cells was determined. **C.** WST-1 was used to determine cell proliferation. Results are expressed as fold increase from the control. **D.** Also, MTT proliferation kit was used, and the results are shown as fold increase from the control. (n = 3; mean ± SEM)

3.2. StarD13 overexpression decreases colorectal cancer cell viability

Control cells were transfected with the GFP vector alone, while treated cells were transfected with the GFP-StarD13 vector. Cell viability was then compared between samples. The over-expression of StarD13 in cells led to a significant increase in the percentage of dead cells, using the Trypan blue exclusion method (Figure 13A). Consistently, a decrease of 20% and 45% in cell proliferation, using WST-1 reagent and MTT kit respectively, was shown (Figures 13B-C).

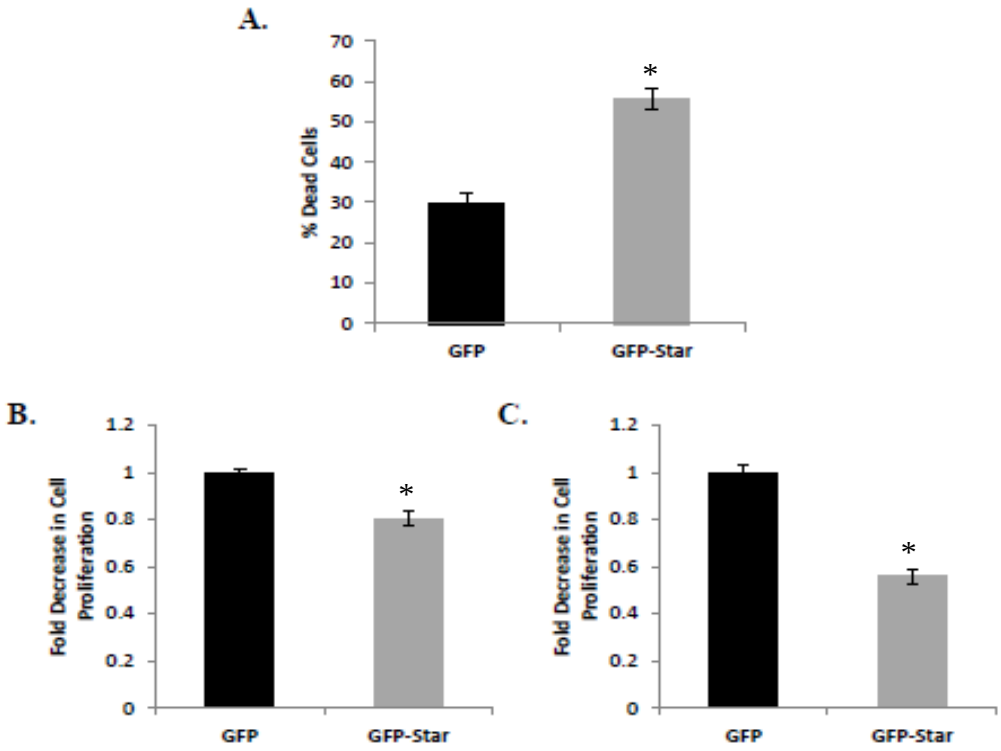

Figure 13: Overexpression of StarD13 decreases cell viability. Cells were either transfected with GFP alone as a control, or with GFP-StarD13. Duration of the treatment was 24h. **A.** By Trypan blue exclusion method, dead cells increased by 50%. **B.** Using the WST-1, a 20% decrease in cell proliferation was observed. **C.** A dramatic decrease in cell proliferation of around 45% was seen by the MTT kit. (n = 3; mean ± SEM)

3.3. Role of StarD13 in cell proliferation

After studying the phenotype of cells affected by StarD13 knockdown, at the level of proliferation and viability, we wanted to check what is happening at the molecular level. We started by investigating the potential role of StarD13 in the TGF-α and TGF-β1 pathways. However, no significant effect was conferred (Figure 14A-B).

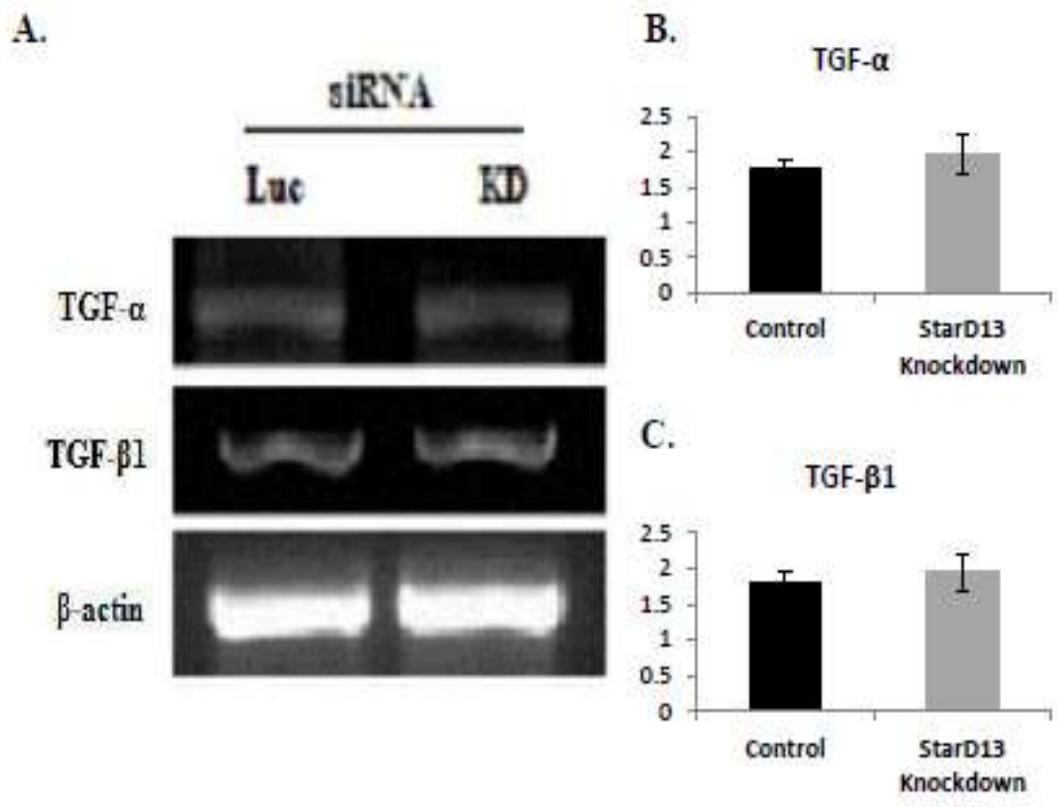

Figure 14: Effect of StarD13 knock down on the expression of TGF-α and TGF-β1 mRNAs. Control cells were transfected with Luciferase, while treated cells were transfected with StarD13 siRNA. 72 hours after transfection, RT-PCR was performed with specific primers. **A.** β-actin was used as a loading control (lower gel). In both, the upper and middle gels, TGF-α and TGF-β1 expression respectively was not affected. **B.** The bands from the gels were quantified using the ImageJ software, and then normalized to the control. (n = 3; mean ± SEM)

3.4. StarD13 knockdown down regulates tumor suppressor p53

Another attempt to study the mechanism by which StarD13 knockdown is increasing cellular proliferation was by checking the protein level of the tumor suppressor p53. Cells were transfected with a StarD13 siRNA for 72 hours, and then lysed and the total proteins extracted. Immunoblotting was performed using anti-p53 antibody. Results show a 25% decrease in the expression level of the p53 protein upon StarD13 knockdown (Figure 15A-B)

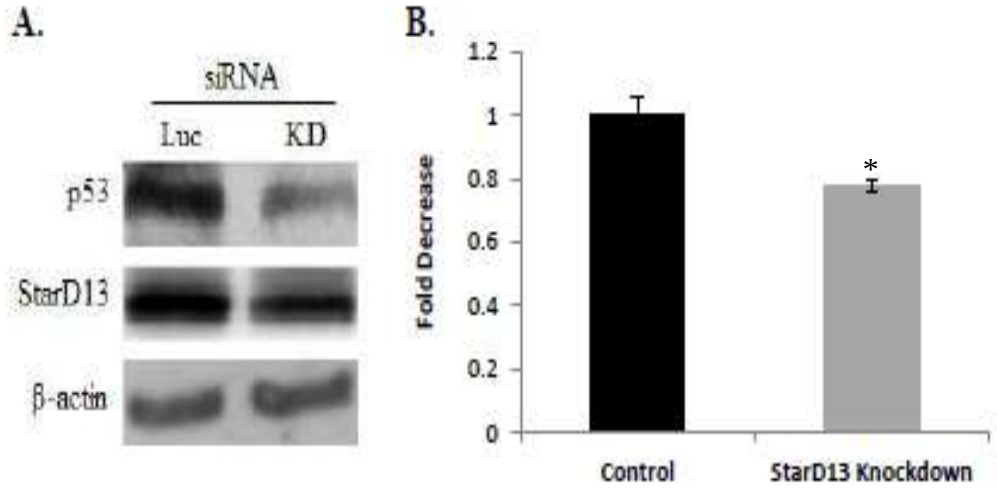

Figure 15: StarD13 knockdown down regulates tumor suppressor p53. Cells were transfected with either luciferase control siRNA or StarD13 siRNA for 72 hours. **A.** Cells were then lysed and immunoblotted by western blot analysis using anti-p53 antibody (upper gel). The knockdown of StarD13 was successful as seen in the middle gel, and the loading control used was β-actin (lower gel). **B.** The bands from the gels were quantified using the ImageJ software, and then normalized to the control. (n = 3; mean ± SEM)

3.5. StarD13 knockdown up-regulates anti-apoptotic Bcl-2 and down regulates pro-apoptotic protein Bax

Furthermore, an important aspect was to study the effect of StarD13 knockdown on cellular proliferation through its effect on the expression levels of the anti-apoptotic protein Bcl-2 and pro-apoptotic protein BAX. Cells were transfected with a StarD13 siRNA for 72 hours, and then lysed and the total proteins extracted. Immunoblotting was performed using anti-Bcl-2 and anti-BAX antibodies. Results show a 30% increase in the expression level of the Bcl-2 protein upon StarD13 knockdown (upper gel), while consistently showing a 20% decrease in the level of BAX protein expression (second gel) (Figure 16A-B).

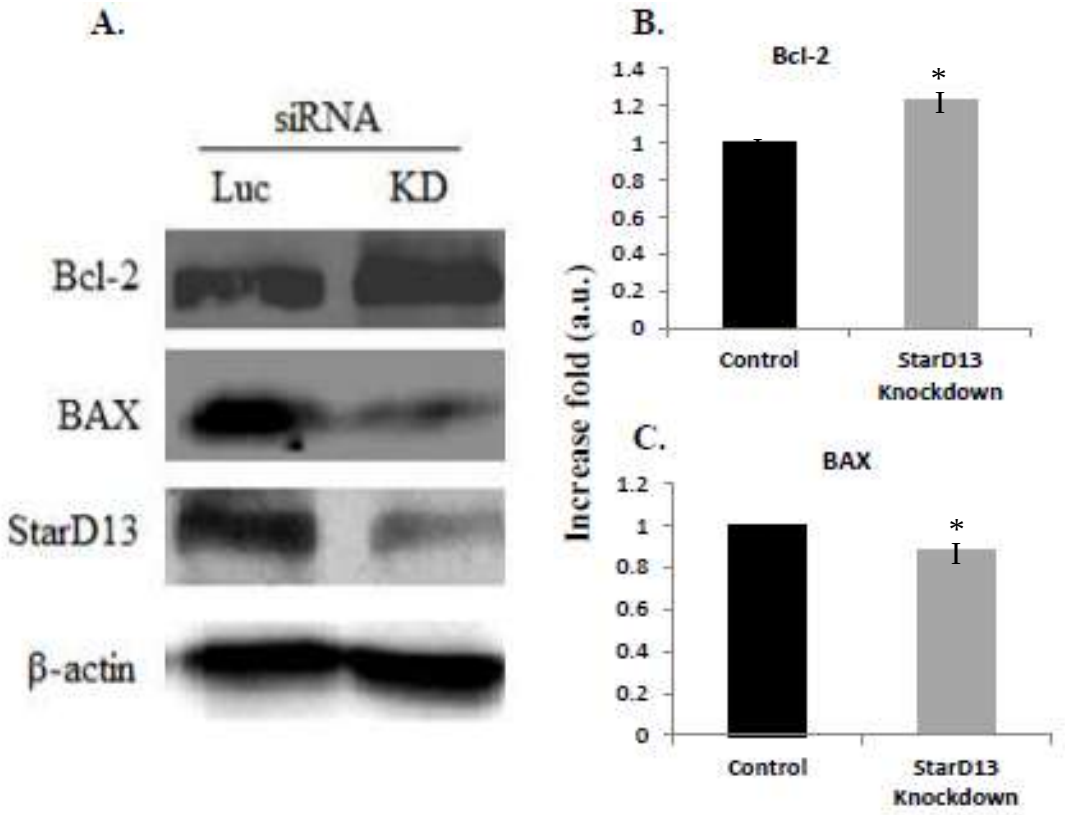

Figure 16: StarD13 knockdown up-regulates anti-apoptotic protein Bcl-2 and down-regulates the pro-apoptotic protein BAX. Cells were transfected with either luciferase control siRNA or StarD13 siRNA for 72 hours. **A.** Cells were then lysed and immunoblotted by western blot analysis using anti-Bcl-2 antibody (upper gel). The knockdown of StarD13 was successful as seen in the middle gel, and the loading control used was β-actin (lower gel). **B.** Results were quantified using the ImageJ software, and then normalized to the control. (n = 3; mean ± SEM)

3.6. StarD13 is a GAP for RhoA and Cdc42

We were interested in determining the effect of StarD13 over expression on the activation of members of the family of RhoGTPases. For this reason, we performed a pull-down assay to detect the levels of active RhoA, Cdc42, and Rac1 in cells transfected with the siRNA as compared to the activation levels in cells transfected with control vectors. Results showed an 80% decrease in RhoA activation, a 1.5-fold decrease in Cdc42 activation, and a mild increase in Rac1 activation (Figures 17A-B).

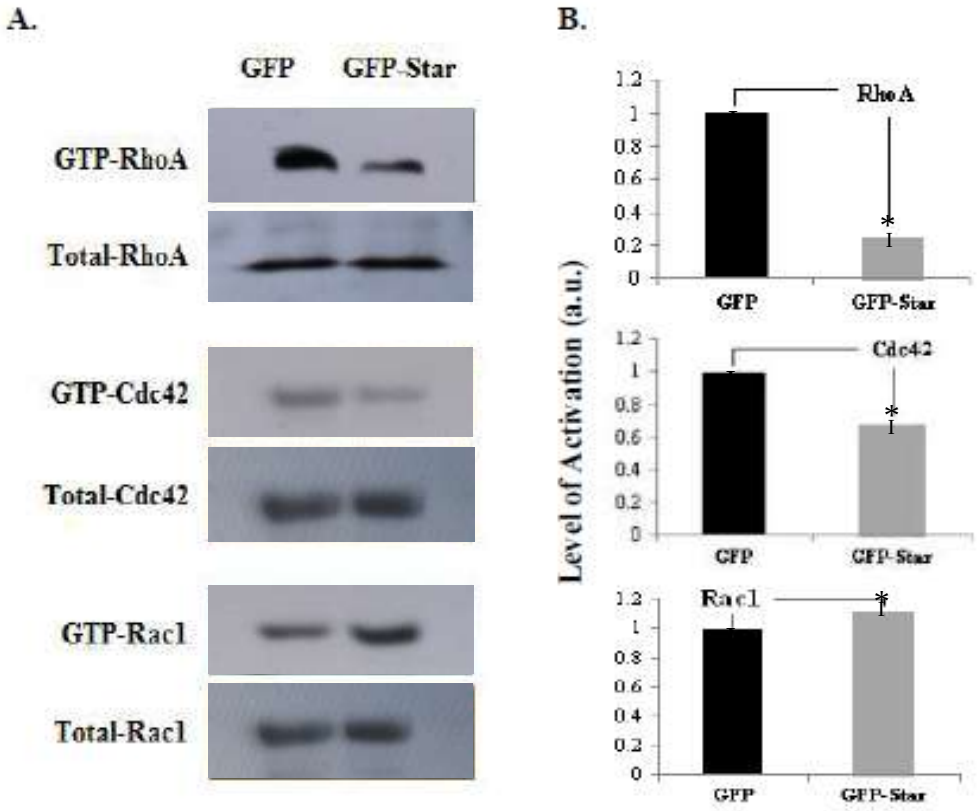

Figure 17: StarD13 is a specific GAP for RhoA and Cdc42. A. Cells were transfected with either luciferase as control (right lanes), or StarD13 siRNA (left lanes). After 72 hours, cells were lysed and incubated with GST-RBD (Rhotekin binding domain) (upper panels), or with GST-CRIB (Cdc42 and Rac interactive binding domain) (middle and lower panels) to pull down active RhoA and active Cdc42 and Rac1 respectively. Samples were then blotted with RhoA, Cdc42, and Rac1 antibodies. The lower gels in each panel are western blots for the total cell lysates, used as loading controls. **B.** The bands from the active RhoA, active Cdc42, and active Rac1 gels were quantified using the ImageJ software, and then normalized to the amount of total proteins. (n = 3; mean ± SEM)

3.7. Role of StarD13 in 2D cell motility

After proving that StarD13 has a major role in cancer cell proliferation, and establishing that it is a Rho GAP, we then wanted to further assess its role in 2D motility. Thus, we knocked down StarD13 and its effect was assayed using the wound healing assay. Results showed a relative decrease in motility of around 1.5 folds in terms of wound closure in cells with StarD13 knock down (4.28μm/hr) with respect to control cells (6.1μm/hr) (Figure 18A-B).

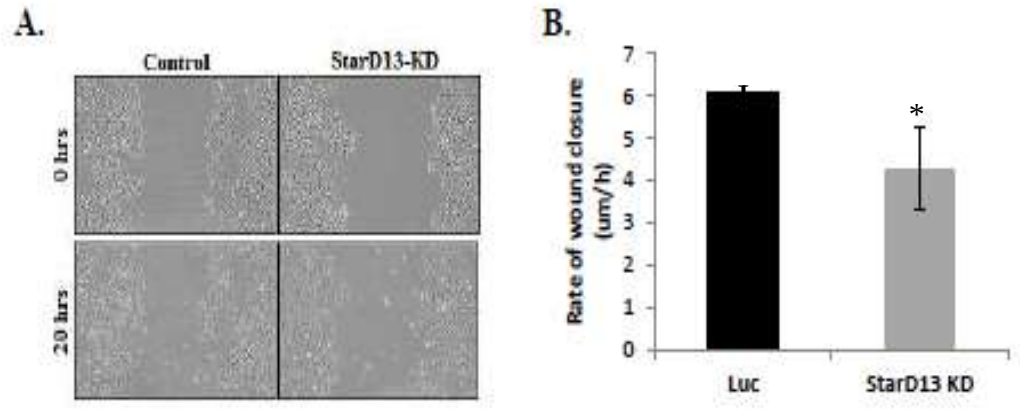

Figure 18: Wound Healing experiment upon StarD13 knockdown. Control cells were transfected with Luciferase, while treated cells were transfected with StarD13 siRNA. Wounds were done after 72hr from transfection. **A.** Cells from the control and KD were grown in plates forming a monolayer, then wounded and directly pictured (upper micrographs). After 20 hours, the pictures were taken at the same frame (lower micrographs). **B.** Quantitation of the wound healing experiments was done as following: width of each wound was measured at 11 dissimilar points, and their average rate of the wound closure was calculated as μm/hr. (n = 3; mean ± SEM)

3.8. StarD13 knockdown promotes actin stress fiber formation

To further investigate the phenotypic nature of cells upon StarD13 knock down, we immunostained control and treated cells with Rhodamin phalloidin to stain actin stress fibers. Results show that the knock down of StarD13 promotes the formation and stabilization of actin stress fibers (Figure 19).

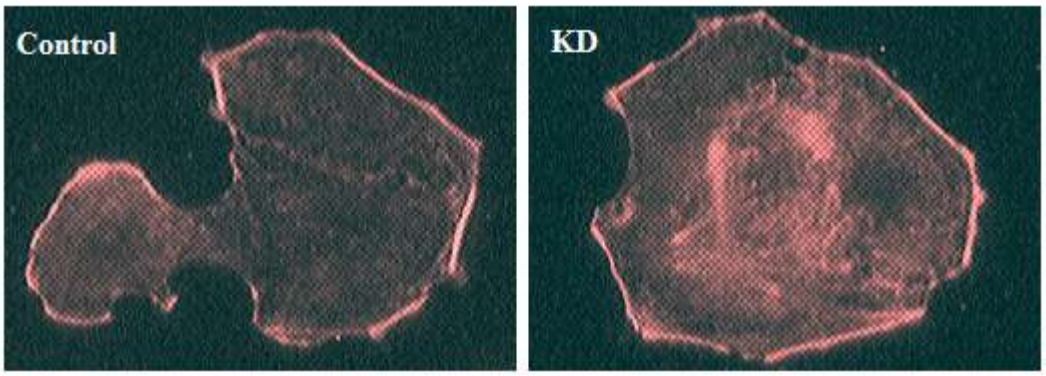

Figure 19: Role of StarD13 in actin stress fiber formation. Cells were either transfected with the control luciferase (left panel), or with StarD13 siRNA (right lane). After 72 hours, cells were fixed and immunostained using Rhodamin phalloidin to stain actin fibers. Micrographs were imaged using the 60x objective.

3.9. StarD13 knockdown increases cell adhesion to collagen

Since StarD13 knockdown has showed the increase in actin stress fiber formation and stabilization, we next wanted to study the effect of this same knock down treatment on the adhesion of these colorectal cancer cells to collagen, which is a main component of the ECM. The results showed around a three-fold increase in the adhesion of these cells with knock down as compared to the control cells (Figures 20A-B).

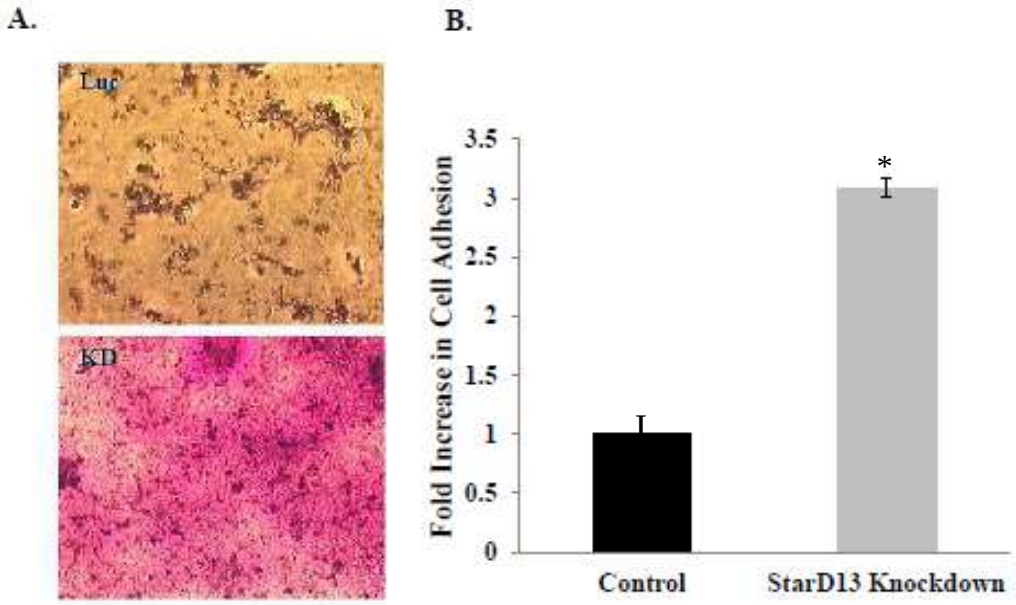

Figure 20: StarD13 knock down increases cellular adhesion to collagen. A. Representative micrographs of cells fixed and stained with crystal violet to detect adhesion. **B.** Quantitation of the micrographs. Crystal violet was solubilised and the absorption was taken at 550μm using ELISA. Data is measured in arbitrary units and normalized to the control. (n = 3; mean ± SEM).

3.10. Effect of RhoGTPases on cellular adhesion to collagen

Given that StarD13 is a RhoGAP and has been shown to be involved in regulation of cellular adhesion, we studied the direct effect of three members of the Rho GTPase family, i.e. RhoA, Cdc42, and Rac1. The results showed a 20% decrease in cellular adhesion upon RhoA knockdown (Figure 21A) and around a 25% decrease upon Cdc42 Knockdown (Figure 21B). However, the knockdown of Rac1 showed an antagonistic result, with a 10% increase in cellular adhesion to collagen (Figure 21C).

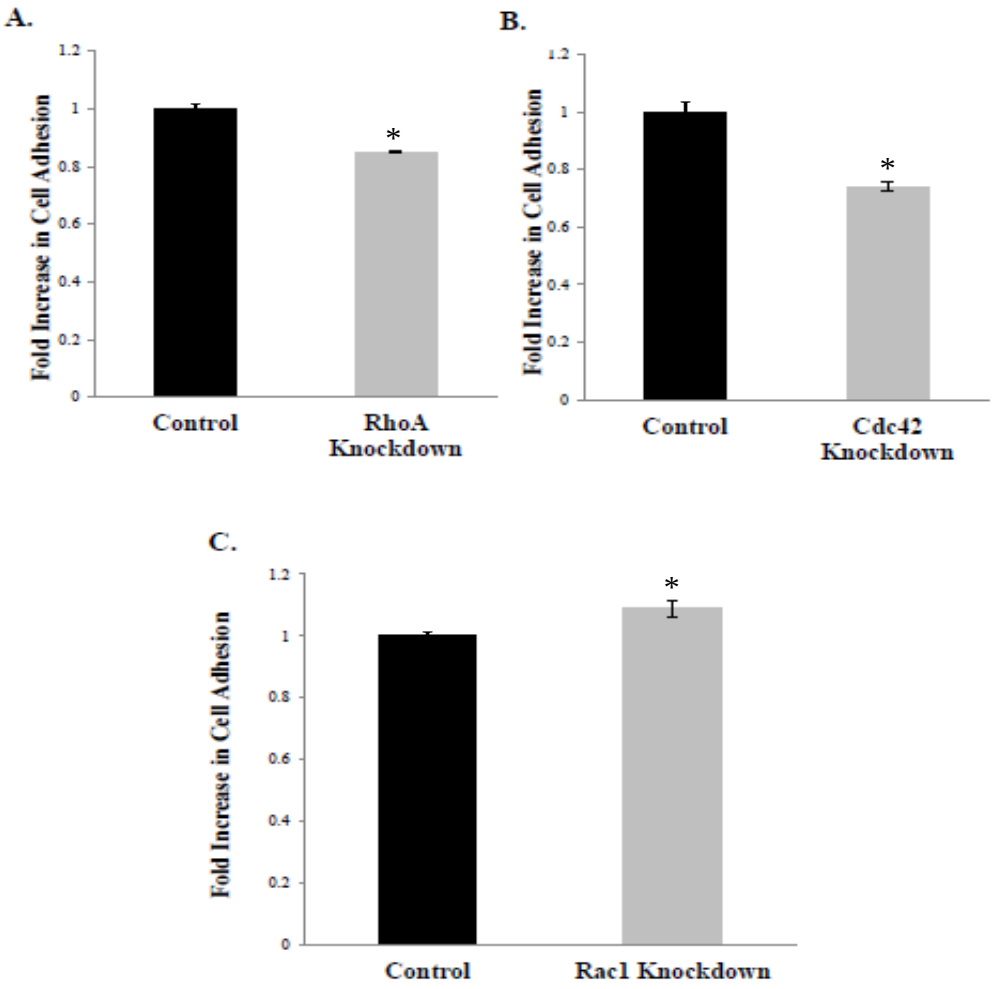

Figure 21: Effect of RhoA, Cdc42, and Rac1 knockdowns on cell adhesion. A. Quantitation of the adhesion assay with RhoA knock down. **B.** Quantitation of the adhesion assay with Cdc42 knock down. **C.** Quantitation of the adhesion assay with Rac1 knock down. (n = 3; mean ± SEM)

3.11. StarD13 knockdown increases cellular invasion

After studying the effect on adhesion, we then assayed the effect of StarD13 knock down on cellular invasion. This was an in vitro collagen-based invasion assay using FBS as a chemoattractant. Unlike in the results we got with the 2D motility, there was around a two-fold increase in cell invasion in cells with StarD13 knock down as compared to the control cells (Figures 22A-B).

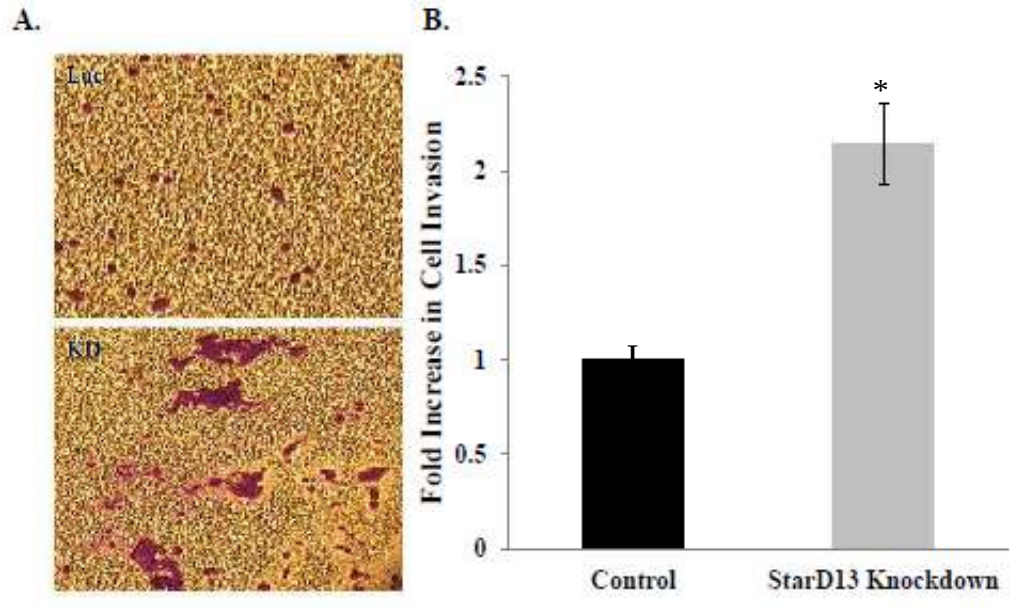

Figure 22: StarD13 knock down increases cellular invasion. A. Representative micrographs of invaded cells on the bottom side of the membrane stained with cell stain according to assay instructions. **B.** Cell stain was extracted and colorimetric measurements were taken at 560μm, using ELISA. Data is measured in arbitrary units and normalized to the control. (n = 3; mean ± SEM).

3.12. Effect of RhoA, Cdc42, and Rac1 knockdowns on cell invasion

Furthermore, we studied the direct effect of three members of the Rho GTPase family, i.e. RhoA, Cdc42, and Rac1, on cellular invasion, with collagen as a chemoattractant. The results showed around a 40% decrease in cellular invasion upon RhoA knockdown and Cdc42 Knockdown (Figures 21A-B). However, the knockdown of Rac1 showed an antagonistic result, with a 10% increase in cellular invasion (Figure 21C).

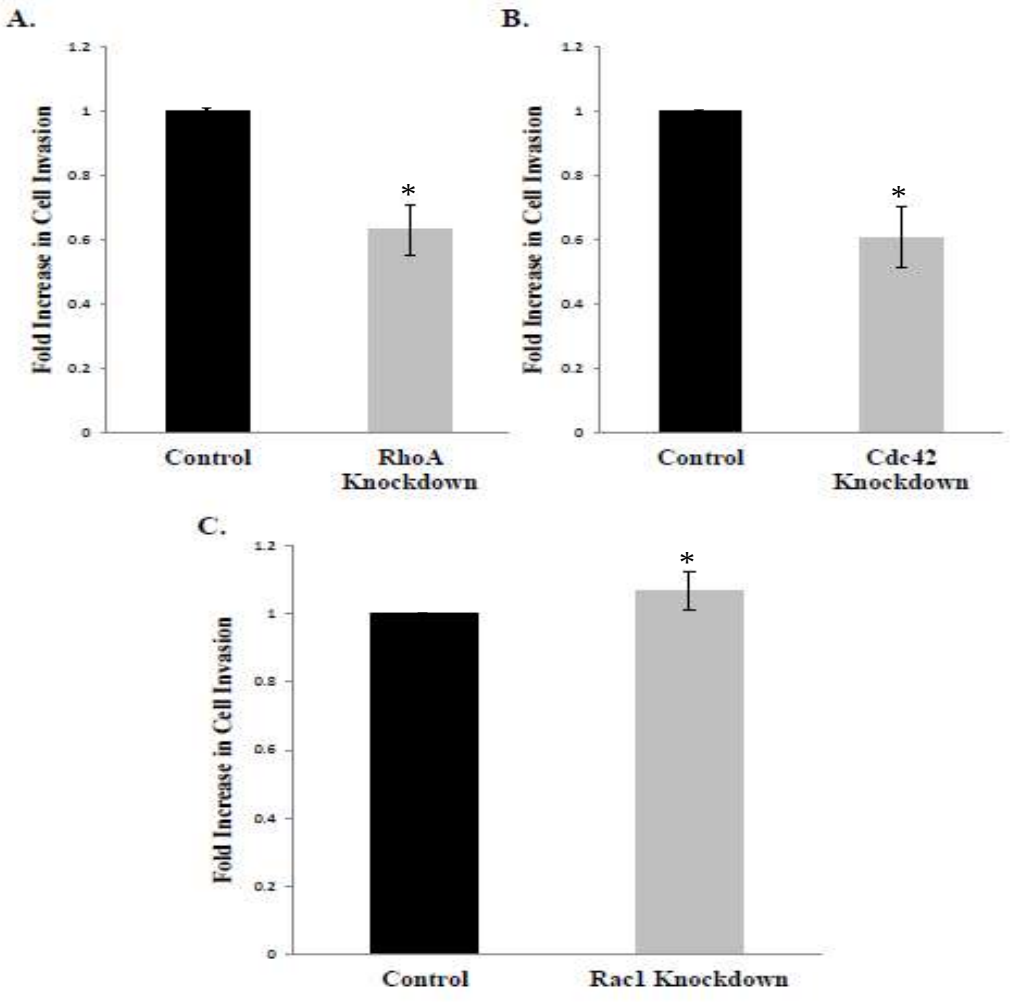

Figure 23: Effect of RhoA, Cdc42, and Rac1 knockdowns on cell invasion. A. Quantitation of the invasion assay with RhoA knock down. **B.** Quantitation of the invasion assay with Cdc42 knock down. **C.** Quantitation of the invasion assay with Rac1 knock down. (n = 3; mean ± SEM)

Chapter IV

DISCUSSION

StarD13 was previously identified to be a tumor suppressor gene in hepatocellular carcinoma (Ching, et al., 2003). Later studies showed that it localizes to focal adhesions in HeLa cells (Kawai, et al., 2009). In this study, an overall characterization of StarD13 in colorectal cancer is provided, in terms of effect on cellular viability and proliferation, sub-cellular localization, GAP activity, and motility and invasion.

We considered an in vitro model of colorectal cancer cell line, i.e. Caco-2 and HT-29, to first look at the effect of StarD13 on cell proliferation and viability. The silencing of StarD13 in both cell lines led to a decrease in cell death, as by the Trypan blue exclusion method, and an increase in cellular viability, as by the WST-1 and MTT proliferation assays. Consistently, StarD13 overexpression using a GFP vector led to an increase in cell death, as by the exclusion method, and a decrease in cell proliferation by the WST-1 and MTT assays. This was consistent with several previous studies done on astrocytoma and breast cancer cell lines in our lab (El-Sitt, et al., 2012). Accordingly, StarD13 seems to play a role of a tumor suppressor in different cancers especially colorectal cancer cells, consistent with the literature.

In order to explain how the silencing and over-expression of StarD13 is affecting cell viability and proliferation on the molecular level, we performed RT-PCR runs, using primers specific for the mitogen TGF-α and the tumor suppressor TGF-β1. Contrary to our expectations, there was no effect on mRNA levels of both proteins. This suggests that StarD13 knockdown affects cellular viability and proliferation through a different pathway. Thus, to further investigate the molecular pathway, we evaluated the protein expression of other tumor suppressors, through western blot analysis, where StarD13 silencing down regulated p53 tumor suppressor genes. We also studied the expression of anti-apoptotic Bcl-2 protein and pro-apoptotic BAX protein. As expected, Bcl-2 increased expression and BAX decreased

expression upon StarD13 knock down. These results prove the effect of StarD13 on colorectal cancer cellular viability, proliferation, through the regulation of tumor suppressor, anti-apoptotic, and pro-apoptotic proteins.

Earlier studies showed that StarD13 has a GAP domain (Ullmannova & Popescu, 2006) and localizes to focal adhesions (Kawai, et al., 2009). In our system, we further confirmed its function as a Rho GAP, as detected by the increase in RhoA and Cdc42 activation, but the decrease in Rac1 activation upon under expression of StarD13. Previous studies, done in hepatocellular carcinoma, showed consistent results, thus supporting our findings (Xiaorong, Wei, Liyuan, & Kaiyan, 2008). These data suggest that StarD13 might have a role in regulating RhoA, thus might affect cellular motility.

After performing a series of experiments regarding proliferation and viability of colorectal cancer cells, we then wanted to study the StarD13 on 2D cell motility. Indeed, knockdown of StarD13 in these cell lines inhibited cell motility. Thus, although it is known to be tumor suppressor, StarD13 is needed for 2D cell motility. This was in accordance with previous studies in our lab, where StarD13 silencing inhibited migration of astrocytoma and breast cancer cells (data not shown). Moreover, our immunostaining results showed StarD13 to promote actin stress fiber formation and stabilization. Nevertheless, contradictory to our results, a preceding study on normal endothelial cells reported that StarD13 inhibition led to an increase in cell migration (Lin, et al., 2010). This inconsistency can be explained by the fact that normal cells are exceedingly different than colorectal cancer cell systems. These cancer cells used in our study typically display distinct cell morphology and altered signaling pathways.

To further investigate the inhibition of 2D cell motility due to StarD13 knock down, we performed an adhesion assay, which showed a major increase in the stabilization and adhesion of cells to collagen upon silencing StarD13. Knowing that StarD13 is a RhoGAP, and since RhoA has been widely proven to be indispensible for the formation of focal adhesions (Arjonen, Kaukonen, & Ivaska, 2011; Wolfenson, Henis, Geiger, & Bershadsky, 2009), and that increasing Rho activation stabilizes focal adhesions inhibiting cell motility (Cox, Sastry, & Huttenlocher, 2001;

Sander, ten Klooster, van Delft, van der Kammen, & Collard, 1999), we formulated a hypothesis that StarD13 knockdown is keeping RhoA active in focal adhesions. Thus, we were interested in looking at the dynamics of cellular adhesion after RhoA, Cdc42, and Rac1 knockdowns. RhoA and Cdc42 knockdowns resulted in decreased adhesion, while Rac1 knockdown resulted in an increase in cellular adhesion to collagen. Consistent to our work, studies done on breast cancer cell lines showed that silencing RhoA decreased cellular adhesion to collagen I (Wu, Wu, Rosenthal, Rhee, & Merajver, 2010).

Another recent study done on normal prostate cells showed that silencing of DLC1 reduces migration (Shih, Takada, & Lo, 2012). In fact, recent studies done on DLC1 showed that DLC1 plays differential roles in regulating cell migration and transformation depending on its interaction with tensins (Cao, Voss, Zhao, Kaneko, & Li, 2012). This highlights the differential role of the DLC family of proteins as tumor suppressors yet needed for cell motility. A comparable dilemma is illustrated in a recent review on TGF-β that is known to exert tumor-suppressive effects in normal cells yet paradoxically, in protumorigenic cells its role is reversed (Massagué, 2008).

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, i.e. 3D motility. 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. We then perforemed RhoA, Cdc42, and Rac1 knockdowns. These also resulted in opposing data as compared to what we got in the adhesion assay. RhoA and Cdc42 knockdowns led to decreased invasion, while Rac1 knockdown led to an increase in cellular invasion.

This can be explained by focal adhesions that might play an alternative role in cellular invasion. In fact, a modern report studied the involvement of focal adhesions in the degradation of the surrounding matrix. Results revealed that specifically at

focal adhesion sites, several cell lines degraded underlying ECM. This process was proved to have occurred through the proteolytic activity of MMPs and not due to physical tension exerted by FAs onto the matrix (Wang & Mc Niven, 2012). This solidifies our data with starD13 knockdown, where we typically have an increase in RhoA activity, thus promoting cellular invasion. Furthermore, it was formerly discovered that in 3D matrices, cancer cells can switch between diverse means of movement (Sahai & Marshall, 2002). This pertains to the interaction between dissimilar signaling conditions. Hence, cells can change between an elongated protrusive and a more rounded blebbing movement fashion. Thus in our study, the diminution of StarD13 amplified cellular adhesion to the ECM, obstructing 2D cellular migration of mesenchymal cells. Nonetheless, this was reflected in an increase in 3D movement, suggesting that cells tend to switch to a more amoeboid-like motility when they cannot move in an adhesion-dependent manner. Therefore, the ability of tumor cells to switch between modes of motility may limit the effectiveness of prospective inhibitory strategies targeting particular cell morphology, thus promoting the selection of a different mode to escape inhibition.

Chapter V

CONCLUSION

Even though it is previously known as a tumor suppressor gene product, in our current study, we have described the role of StarD13 in colorectal cancer cell proliferation and motility, for the first time. Our results showed that StarD13 negatively affects cellular viability and proliferation. Having a RhoGAP activity, StarD13 is highly involved in cellular motility. To our surprise, StarD13 has opposing roles in 2D and 3D motility. StarD13 knock down inhibited 2D cell movement by further stabilizing and forming actin stress fibers. However, this down regulation of StarD13 has increased both, cellular adhesion to collagen as well as cellular invasion, i.e. 3D movement.

Supplementary experiments are still to be done to further study the role of StarD13 on proliferation and viability by flow cytometry. Other further studies include finding the difference of StarD13 levels in normal versus diseases colorectal tissues, as well as performing immunohistochemistry on those exact tissues targeting the different Rho GTPases (RhoA, RhoC, Rac1, Cdc42), StarD13, and others such as ROCK1/2, etc. Moreover, future work consists doing wound healing assays upon the knockdown of the different Rho GTPases. Finally, studies performed on the molecular level of the action of StarD13 on cell motility will be done by looking at the protein levels of integrins and MMPs, as well as performing matrigel experiments.

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