

**LEBANESE AMERICAN UNIVERSITY**

**Distinctive Roles of RhoA and RhoC in Glioma Cell  
Motility, Invasion, and Invadopodia assembly**

By  
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A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in  
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## **Dedication Page**

*To my supportive and loving parents*

*To my grandmother Majida*

*To my grandmother Imtithal*

*You will always be remembered*

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First and foremost, I thank God for giving me the strength and dedication to complete this body of work.

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# Distinctive Roles of RhoA and RhoC in Glioma Cell Motility, Invasion, and Invadopodia assembly

Rayane Dennaoui

## Abstract

Malignant gliomas remain a medical concern in oncology due to their high morbidity rates and their aggressive behavior in the invasion of normal brain parenchyma. It has been a challenge to cure these types of brain tumors owing to the importance of the brain in regulating a plethora of human physiological functions, chemotherapy resistance, and inefficient surgical resection. Cell motility and invasion are patterns of glioma spreading and are multifaceted processes involving dynamic changes in the actin cytoskeleton. Major regulators that orchestrate actin reorganization and migration are members of the Rho family of GTPases. In this study, we investigate the contribution of two structurally similar Rho proteins, RhoA and RhoC, in glioma migration and invasion. One of our objectives was to explore the effects of both RhoA and RhoC on two-dimensional motility. Our data suggest that both proteins are positive regulators and enhancers of cell migration, adhesion to the extracellular matrix, and protrusion formation through the regulation of actin cytoskeletal dynamics. Another objective for the study was to study the differential roles of RhoA and RhoC in invasion and invadopodia assembly. We have found that RhoC is the main driver of the process in astrocytomas. In addition, we investigated possible cross-talks between RhoA, RhoC, Cdc42, and Rac1. Our data suggest that RhoA and RhoC downregulate both Rac1 and Cdc42 in order to achieve their roles in motility and invasion.

Keywords: RhoA, RhoC, invadopodia, focal adhesions, astrocytoma, motility

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## **LIST OF ABBREVIATIONS**

Arp2/3: Actin-related protein 2/3

BSA: Bovine Serum Albumin

Cdc42: Cell division cycle 42

CFP: Cyan Fluorescent Protein

CNS: Central Nervous System

CRIB: Cdc42/Rac interactive binding

DMEM: Dulbecco's Modified Eagle Medium

ECL: Enhanced Chemiluminescence

ECM: Extracellular matrix

EGF: Epidermal Growth Factor

EMT: Epithelial-mesenchymal transition

FAK: Focal Adhesion Kinase

FBS: Fetal Bovine Serum

FMNL2: Formin-like protein 2

FMNL3: Formin-like protein 3

FRET: Förster Resonance energy transfer

GAP: GTPase Activating Protein

GDI: Guanine Nucleotide Dissociation Inhibitor

GDP: Guanosine diphosphate

GEF: Guanine Nucleotide Exchange Factor

GFAP: Glial Fibrillary Acidic Protein

GST: Glutathione S-transferase

GTP: Guanosine triphosphate

GTPase: Guanosine triphosphatase

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

HGF: Hepatocyte Growth Factor

IDH1: Isocitrate Dehydrogenase 1

LIMK: LIM kinase

MAP2: microtubule-associated protein 2

mDia: Mammalian homolog of diaphanous

MLC: Myosin Light Chain

MLCK: Myosin Light Chain Kinase

MMP: Matrix metalloproteinase

MT-MMP: Membrane-type matrix metalloproteinase

NHE1: Na<sup>+</sup>/H<sup>+</sup> Exchanger

PAK: p21 activated kinases

PBS: Phosphate Buffered Saline

PDGF: Platelet-derived Growth Factor

PI3K: Phosphatidylinositol 3-kinase

PIP2: Phosphatidyl 4,5-bisphosphate

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

PMA: Phorbol 12-myristate 13-acetate

PTEN: Phosphatase and tensin

PVDF: Polyvinylidene fluoride

Rac1: Ras-related C3 botulinum toxin substrate 1

Ras: Rat sarcoma

RBD: Rhotekin binding domain

RhoA: Ras homologous member A

RhoBTB: Rho-related BTB domain-containing protein 1

RhoC: Ras homologous member C

RhoD: Ras homologous member D

RhoF: Ras homologous member F

RhoG: Ras homologous member G

RhoH: Ras homologous member H

RhoJ: Ras homologous member J

RhoQ: Ras homologous member Q

RhoU: Ras homologous member U

RhoV: Ras homologous member V

ROCK: Rho Kinase

ROI: Region of interest

SCAR: Suppressor of Cyclic AMP

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

siRNA: Small interfering Ribonucleic Acid

TGF- $\beta$ : Transforming Growth Factor beta

Tks4: SH3 and PX domains 2B

Tks5: SH3 and PX domains 2A

WASP: Wiskott - Aldrich syndrome Protein

WAVE: WASP-Family Verprolin-Homologous

WAVE1: WASP-family verprolin homologous protein 1

WHO: World Health Organization

YFP: Yellow Fluorescent Protein

# Chapter 1

## Literature Review

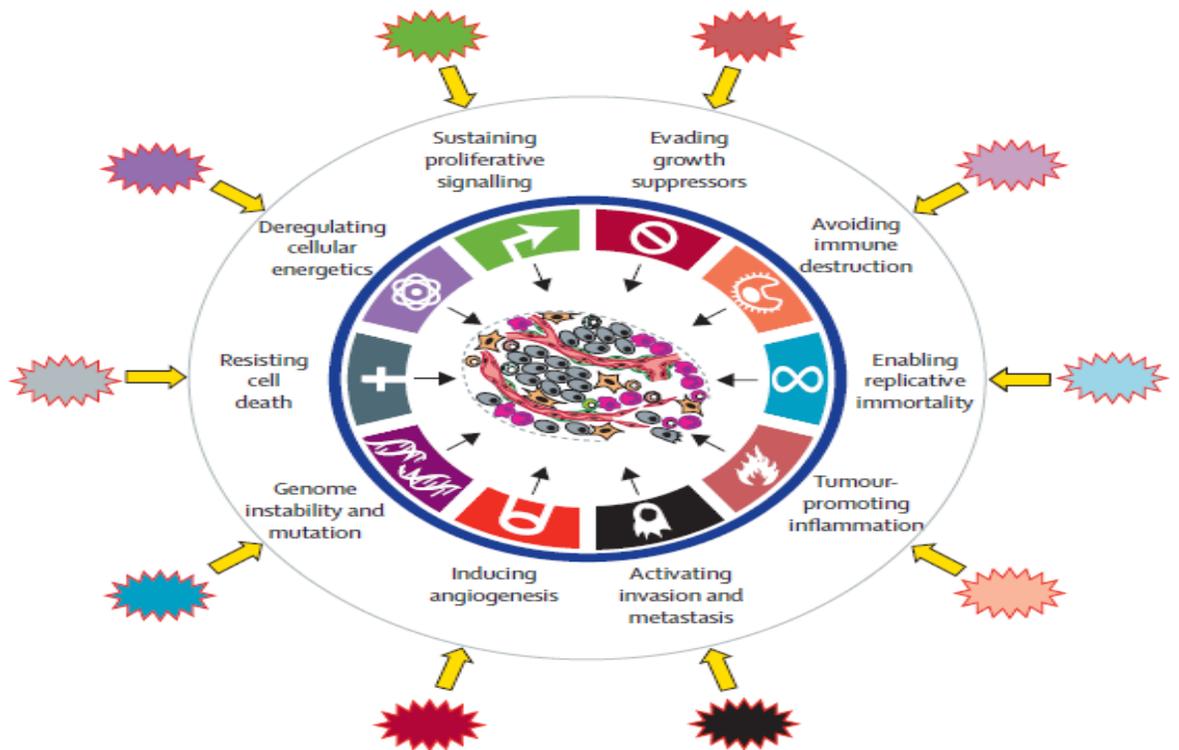
### 1.1 Cancer Overview

The title “cancer” is given to a collective of diseases characterized by sequential genetic aberrations that ultimately lead to the emergence of abnormal cells. These abnormal cells are generally characterized by their defiance to normal constraints on cell proliferation and division, and their acquisition of invasiveness to areas that are usually occupied by other cells (Alberts, 2002).

A mass of abnormal cells is referred to as a tumor. Tumors are further categorized as being benign and usually innocuous, or malignant and cancerous. The distinction between the two types of tumors is that benign tumors are restricted to the original site of formation; whereas malignant tumors possess the capability to invade adjacent normal tissue and spread throughout the body via circulation by a process called metastasis (Cooper, 2000).

Cancer is a complex and multifaceted disease. This can be attributed to the existence of more than 100 types and subtypes of cancer in different body organs with each having distinct properties. This diversity stems from complex mutations and rearrangements in the human genome affecting cells in different organs (Hanahan, 2014). In this context, Hanahan and Weinberg’s model for the hallmarks of cancer provides a logical framework for grasping the similarities and discrepancies between

neoplastic diseases. According to the model shown in Figure 1, most cancer cells, if not all, share a number of essential modifications in cell physiology which are uncontrolled proliferation through reliance on self-sustained growth factors, exhibiting immortality through indefinite replication, unresponsiveness to growth suppressive signals, bypassing apoptosis, inducing angiogenesis, and promoting invasion (Hanahan & Weinberg, 2000). In 2011, the hallmarks of cancer were revisited and four more traits were added, these are: evasion of the immune response, the reprogramming of metabolic energetics, the induction of tumor-associated inflammation, and genomic instability (Hanahan & Weinberg, 2011).



**Figure 1: The ten Hallmarks of cancer.**

Source: (Hanahan & Weinberg, 2011)

## **1.2 Brain Cancer**

### **1.2.1 Brain Tumor Statistics and Epidemiology**

In 2017, the American Brain Tumor Association reported approximately 80,000 cases of primary brain tumors 32.5% of which were malignant primary brain tumors. In addition, it reported that survival post-diagnosis of a primary brain tumor is dependent on the molecular markers, histological features, tumor behavior, and age of the patient (“Brain Tumor Statistics - ABTA,” n.d.).

It is important to note that brain cancers in children vary in location, genetic markers, behavior, and prognosis from their adult counterparts. In fact, the most frequent solid-type tumor observed in children is brain tumor with the highest mortality rate out of all malignant pediatric cancers (Jacques & Cormac, 2013).

According to the American Cancer Society, it is estimated that, in 2018, 23,880 cases of malignant tumors of the brain and spinal cord will be diagnosed in the United States. Moreover, around 16,830 people, of which 9,490 are males and 7,340 are females, are expected to die from brain and spinal cord tumors (“Key Statistics for Brain and Spinal Cord Tumors,” n.d.).

### **1.2.2 Brain Tumors Definition**

Brain tumors are masses of anomalous cells that arise within the brain tissue. These brain neoplasms, like other types of cancers, can be subdivided into one of two categories, benign or malignant, based on their ability of trespassing their borders and on the speed of their growth and proliferation (“Understanding Brain Tumors,” n.d.). Malignant tumors typically acquire aggressive traits, some of which are local invasion of the brain tissue,

neovascularization, necrosis, and cellular atypia. In addition, brain cancers can be grouped broadly as either primary brain cancers or metastatic brain cancers. Primary brain tumors usually arise from any intracranial cell type or structure including the brain tissue, meninges, pituitary gland, pineal gland, or skull bone (DeAngelis & Leoffler, 2005) whereas secondary tumors originate from other body locations and disseminate through blood to the brain and these are referred to as brain metastases. Well documented examples of brain metastases include lung, breast, renal, skin, gastrointestinal tract and testicular carcinomas (DeAngelis & Leoffler, 2005; Hardesty & Nakaji, 2016). Primary brain cancers are usually 10 times less prevalent than intracranial metastases (Lin & DeAngelis, 2015).

### **1.2.3 Brain Tumor Classification**

During the last decade, classification of brain cancer relied heavily on histological parameters visualized by light microscopy to discriminate between over 120 types of brain and central nervous system cancers. In 2007, the World Health Organization enlisted six major types for primary brain cancers, which are as follows: tumors of neuroepithelial origins, tumors of the meninges, hemopoietic tumors and lymphomas, germ cell tumors, and tumors of the sellar region (Louis et al., 2007). Additionally, each of these classes comprises many types, subtypes, and variants which makes the process of identification much more complex. This burden necessitated resorting to a more layered classification of the disease by adding molecular diagnostics to the classification process of brain tumors. Consequently, the WHO reported a novel classification list of the CNS tumors with major adjustments of previously described types, additions of newly recognized cancers, and deletions of some variants with insignificant relevance (Louis et al., 2016).

### **1.2.4 Astrocytomas**

Astrocytomas are well-differentiated neuroepithelial tumors of astrocytic origin that show higher levels of cellularity when compared to normal brain tissue, but low to moderate nuclear pleomorphism. Astrocytic tumors comprise approximately 25% of all types of gliomas. In addition, the location of astrocytic tumor initiation and incidence rates vary with the age group; astrocytomas frequently affect the cerebral hemisphere regions of adults and the brainstem region of children with a peak prevalence in young adults between 30 and 40 years of age (Narayana, Recht, & Gutin, 2010).

#### 1.2.4.1 Astrocytoma grading scheme

The astrocytic tumor grading system has been first published by WHO in 1979. Later revisions included those announced in 1993, 2000, 2007, and recently in 2016 (P. Kleihues, Burger, & Scheithauer, 1993; Paul Kleihues et al., 2002; Tonn, Westphal, & Rutka, 2010). These reports have primarily maintained the same histological sorting assigned by Bailey and Cushing in 1926 (Bailey & Cushing, 1926). Indeed, most of the older reports have utilized immunohistochemical analyses of specific differentiation and proliferation biomarkers in astrocytomas including Glial Fibrillary Acidic Protein (GFAP), microtubule-associated protein 2 (MAP2), and S-100 protein as glial markers to assign the histological class of gliomas (Bajenaru et al., 2002; Lopes, Altermatt, Scheithauer, Shepherd, & VandenBerg, 1996; Tonn et al., 2010). However, the newest version of the WHO report also included molecular markers like IDH mutations, H3 K27M mutations, RELA fusion, WNT and SHH activation and C19MC alteration.

The official WHO grading scheme proceeds as follows with a range from WHO grade I to WHO grade IV in order of ascending malignancy and severity of prognosis (Tonn et al., 2010):

- Grade I: Pilocytic astrocytoma. On a cellular and histological level, this type of tumor is characterized by slow growth and well confinement; it usually affects children and adolescents. Alternatively, on a molecular level, pilocytic astrocytomas have duplications of the oncogenic gene *BRAF* at the 7q34 locus and lack mutations in the *IDH1* and *TP53* genes, and losses of alleles at locus 17p.
- Grade II: diffuse astrocytoma. This tumor type's histological features resemble those of Grade I astrocytomas with the addition of diffuse infiltration and preference in affecting the region above the tentorium cerebelli in young adults. In addition, diffuse astrocytomas frequently exhibit polysomy in chromosome 7 and mutations at the 17p13 locus in *TP53* tumor suppressor gene.
- Grade III: anaplastic astrocytoma. Anaplastic astrocytomas are described as diffusely infiltrative with high levels of cellularity, mitosis and atypia. The thalamus and the brainstem are typical sites of anaplastic astrocytomas. Also, these astrocytoma show gains of chromosome 7, *IDH1* and *TP53* mutations, and an increase in the expression of *PDGF $\alpha$*  and its receptor *PDGFRA*.
- Grade IV: Glioblastoma multiforme. Histologically, glioblastomas represent increased levels of cellularity, cellular pleomorphism, high paced mitosis, clotting of vasculatures with the formation of microvessels, nuclear atypia, and necrosis. These gliomas can emerge at any age, but adults experience this cancer type more frequently.

Glioblastomas of primary and secondary origins share genetic modifications that target the p53, pRb1, Pten/PI3K/Akt and MAPK pathways.

## **1.3 Cell Motility and Invasion**

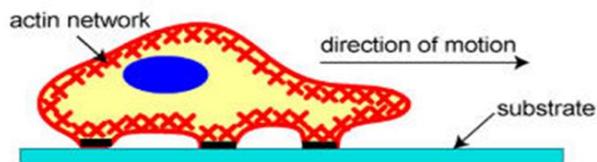
### **1.3.1 Cell Motility**

Cell motility is a pivotal process that takes part during important physiological events, some of which are: the process of embryogenesis, inflammatory response, tissue regeneration and development (Hanna & El-Sibai, 2013; Pollard & Borisy, 2003). In addition, it is an indispensable step undertaken by cancer cells during invasion and metastasis.

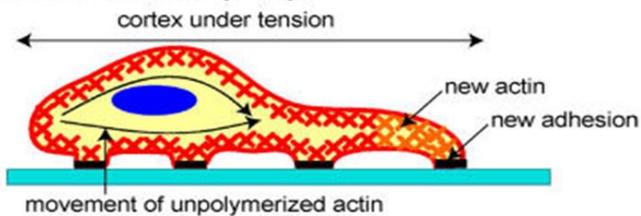
The locomotion of a cell in response to extracellular chemotactic cues, termed as chemotaxis, is the end result of several steps to achieve the cell motility cycle as summarized in figure 2. This cycle first starts when the cell begins to move with directionality depending on the extracellular signal. These signals may vary between having a physical or chemical nature that are usually sensed by receptors on the surface of the cell membrane. Next, the receptors relay these signals to the interior of the cell starting a cascade of signaling that eventually lead to the remodeling of the actin cytoskeleton towards the direction of the cue in the case of a chemoattractant, or opposite to it in the case of a chemorepellant. This results in the formation of a protrusion at the leading edge of the cell. Then, the actin bundles of the protrusion adhere to the underlying extracellular matrix (ECM) at the front via adhesion molecules. As the cell adheres at the

front edge, the cell body and the trailing end de-adhere from the substratum followed by the contraction of the body and rear end of the cell (Ananthakrishnan & Ehrlicher, 2007; Bailly & Condeelis, 2002; DeFea, 2013).

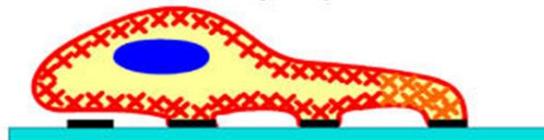
1) Protrusion of the Leading Edge



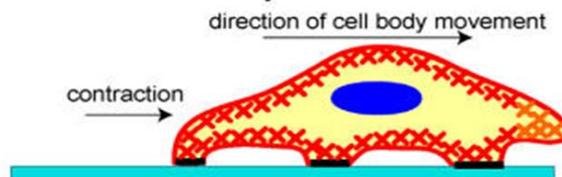
2) Adhesion at the Leading Edge



Deadhesion at the Trailing Edge



3) Movement of the Cell Body



**Figure 2: The multiple steps of the Cell Motility Cycle:** (1) The cell starts with forming a protrusion at its leading edge followed by (2) an adhesion to the substratum as a result of actin rearrangement at that location. Next, (3) deadhesion of old adhesions located at the rear end of the cell occur. Finally, (4) contraction of the cell body and trailing end lead to the forward movement of the cell. This cycle continues until the cell reaches the source of the chemoattractant.

### 1.3.2 Patterns of Glioma Motility and Invasion

Glioblastoma, the most aggressive brain tumor, possesses a high infiltrative capacity. While this cancer rarely metastasizes, migration and invasion constitute the main features of GBM spreading (Johansen et al., 2016). The two main routes used by glioma cells to invade the normal brain tissue are either through blood vessels or axons of white tract myelinated nerve fibers (Armento, Ehlers, Schötterl, & Naumann, 2017; Lefranc, Brotchi, & Kiss, 2005; Rao, 2003). Endothelial cells secrete chemoattractants such as bradykinin to recruit glioma cells to the perivascular areas around the blood vessels (Montana & Sontheimer, 2011).

Integrins, heterodimeric transmembrane proteins, are responsible for linking the extracellular matrix to the cell during adhesion. These proteins mediate ECM-dependent rearrangement of the actin cytoskeleton and activation of intracellular signaling responsible for regulation of cellular migration and adhesion dynamics. Typical integrin ligands include fibronectin, collagen, and laminin and they are overexpressed in gliomas (D'Abaco & Kaye, 2007). When the ligands bind to integrins, they cluster and activate the focal adhesion kinase (FAK) (Mitra & Schlaepfer, 2006). Next, the cytoplasmic domains of integrin cluster links to components of the cytoskeleton to form focal adhesion contacts at the leading edge of the migrating cells giving them the ability to move forward (Ridley et al., 2003). Integrin  $\beta 1$  and FAK are both overexpressed in gliomas (Armento et al., 2017; Gutenberg, Brück, Buchfelder, & Ludwig, 2004). For successful glioma invasion to occur, the ECM must also be remodeled and deteriorated by specific enzymes such as metalloproteases (MMPs), Disintegrin and Metalloproteinase with Thrombospondin

Motifs (ADAMTS), 6-O-sulfatases, the serine protease plasmin, heparanases, cathepsins, and urokinase (uPa) (Lu, Weaver, & Werb, 2012).

### **1.3.3 Cell Invasion and Invadopodia assembly**

In order for cancer cells to migrate and invade local or distant tissues, they must form actin protrusive structures which include lamellipodia, filopodia, and invadopodia. Typically, lamellipodia and filopodia are apparent at the leading edge of a migrating cell and their functions are mainly restricted to giving directionality orders for 2-dimensional migration. However, a distinctive characteristic of the third class of protrusive structures, the invadopodia, is that they are mainly involved in ECM degradation and eventually invading the matrix in a 3D manner (Alblazi & Siar, 2015). Invadopodia are defined as irregular sized F-actin rich basal membrane projections formed by invasive cancer cells (Eddy, Weidmann, Sharma, & Condeelis, 2017; Linder, Wiesner, & Himmel, 2011; Yamaguchi, 2012). These structures accumulate in them a large number of cytoskeletal modulators such as Tks4, Tks5, N-WASP, Arp2/3 complex, cofilin, cortactin, Mena, formin, talin, and fascin (Beaty et al., 2014; Blouw et al., 2015; Eddy et al., 2017; Hagedorn et al., 2014; Iizuka, Abdullah, Buschman, Diaz, & Courtneidge, 2016; Ren et al., 2018; Siar et al., 2016; Yamaguchi, 2012).

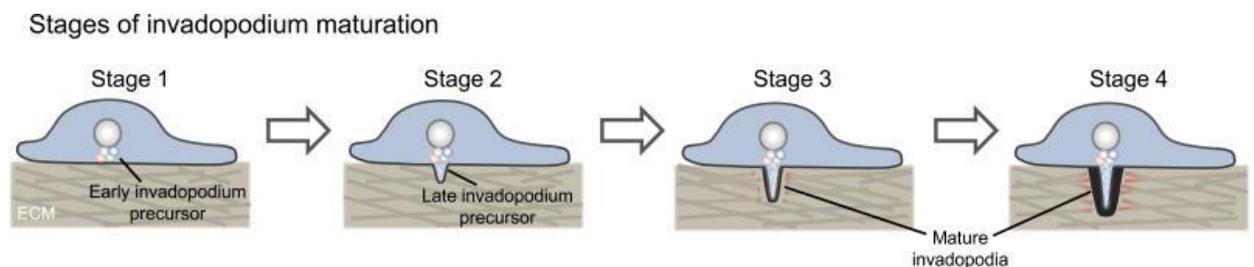
#### **1.3.3.1 Signals for Invadopodia precursor initiation**

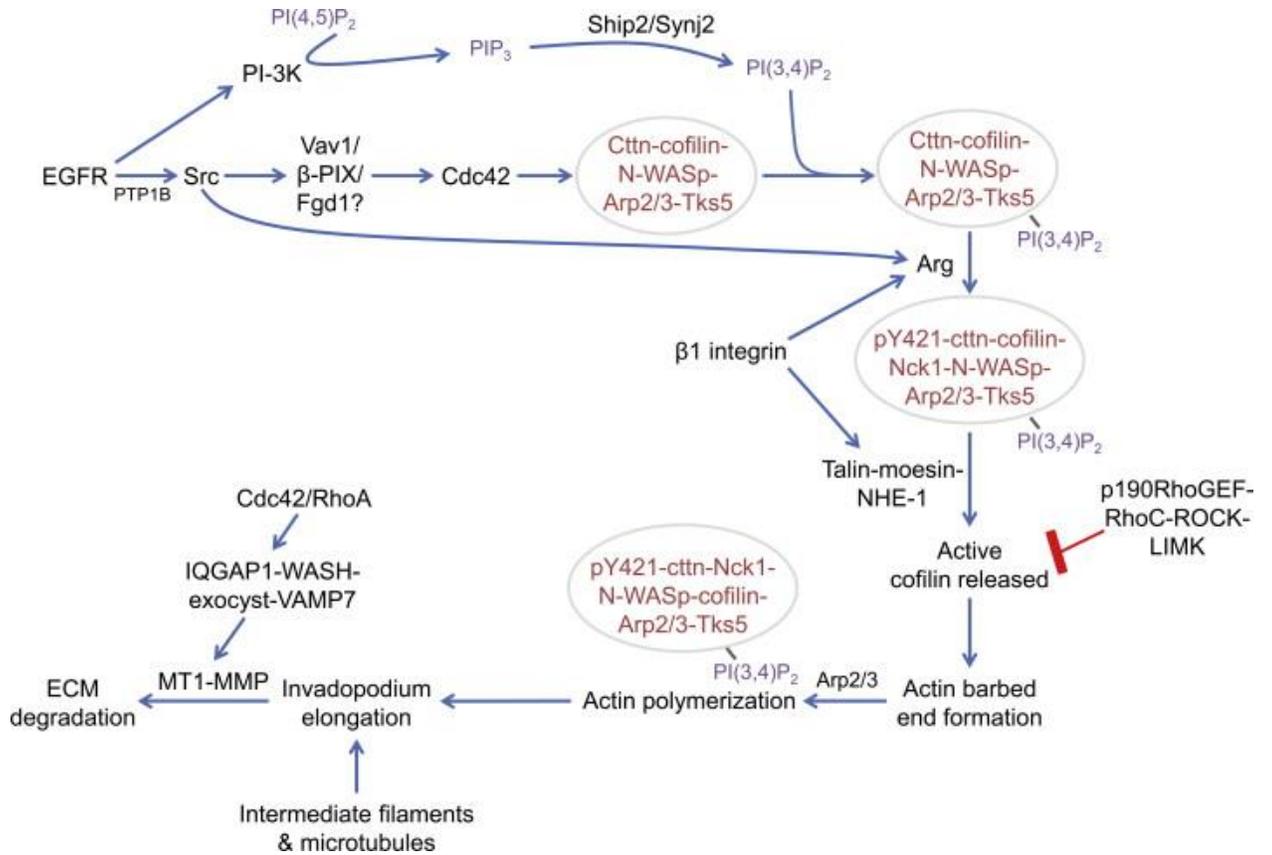
Several types of stimuli have been discovered to participate in initiating precursors of invadopodia formation including growth factors (EGF, PDGF, TGF- $\beta$ , HGF), epithelial-mesenchymal transition (EMT), hypoxia, MMP activities, and oncogenic

transformation (Clark, Whigham, Yarbrough, & Weaver, 2007; Eckert et al., 2011; Eddy et al., 2017; Pignatelli, Tumbarello, Schmidt, & Turner, 2012; Yamaguchi et al., 2005).

### 1.3.3.2 The 4 Steps of Invadopodia formation

The first step towards the formation of invadopodia is the initiation of the invadopodium precursor core or nucleus. This step involves the recruitment of N-Wasp, Arp2/3 complex, and cofilin around the actin–cortactin complex. Then, the core of the invadopodium precursor is stabilized by Tks5 that tethers the precursor core to PI(3,4)P2 in the plasma membrane. This anchorage to the cell membrane will subsequently recruit the adhesion receptor  $\beta 1$  integrin to the core, activate it, and links to further ECM ligands such as talin offering more stability to the core structure. The third step of invadopodia formation involves actin polymerization as a result of the activation of the NHE-1-cofilin pathway, and further actin polymerization contributes to more elongation and stabilization of the invadopodia. Finally, microtubule in addition to filament recruitment leads to maximum elongation of the protrusion, and degradation of the ECM occurs when MMP2 and MMP9 are recruited (Beatty & Condeelis, 2014; Eddy et al., 2017; Sharma et al., 2013). The different stages of invadopodia assembly are portrayed in figure 3.





**Figure 3: The different stages showing invadopodium core initiation, stabilization, and maturity and the signaling pathways regulating the process.**

Source: (Beaty et al., 2014)

## 1.4 The Rho family of GTPases

### 1.4.1 General characteristics of Rho GTPases

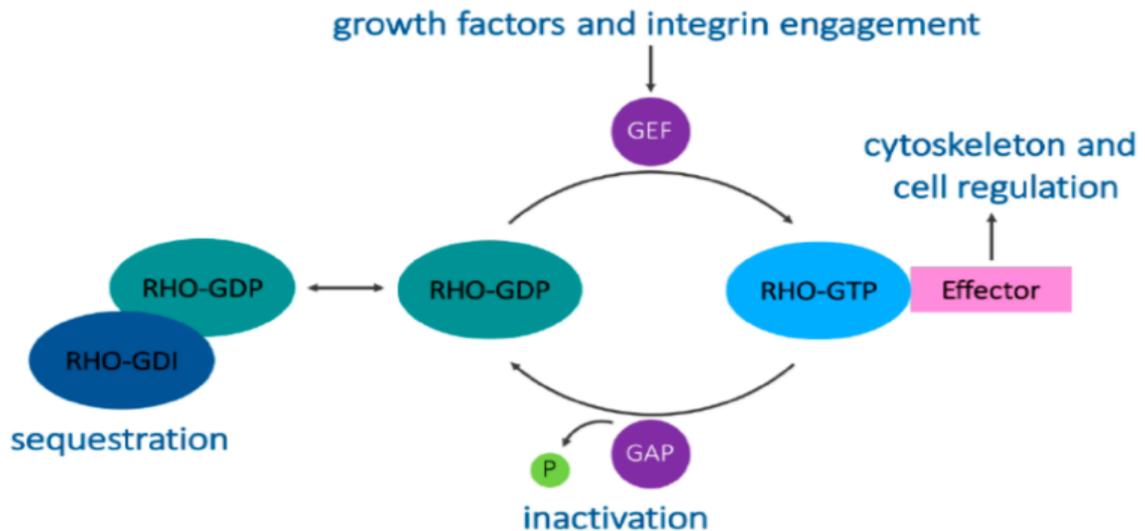
The Rho family of GTPases comprises of 20 members of small GTP binding proteins in humans with molecular sizes ranging from 20 to 40 KDa. Rho GTPases are implicated in a plethora of physiological processes including organization of the actin cytoskeletal dynamics, regulation of gene expression, and processes that involve actin rearrangement such as: vesicular trafficking, cell cycle progression, cell

morphogenesis and cell polarity and migration (Etienne-Manneville & Hall, 2002; Ji & Rivero, 2016; Ju & Gilkes, 2018). This family is further divided into eight subfamilies, based on their sequence identity and similarity, which are: the Rac/RhoG subfamily, Rho subfamily, the Cdc42/RhoQ/RhoJ subfamily, the RhoF/RhoD subfamily, the Rnd subfamily, the RhoBTB subfamily, the RhoH subfamily and the RhoU/RhoV subfamily (Lawson & Ridley, 2018). Rho, Cdc42, and Rac1 remain the best studied among all Rho GTPases. Members of the Rho GTPases family are either categorized as typical (classic) or atypical depending on their ability of GTP hydrolysis (Haga & Ridley, 2016; Lawson & Ridley, 2018).

#### **1.4.2 The regulation of the Rho GTPases**

- Regulation of typical members of the Rho Family

Rac, Rho, Cdc42 and RhoF/RhoD typical subfamilies are considered to be molecular switches due to their interchangeability between GDP/GTP forms as seen in Figure 4. The members of these subfamilies are typically activated by Guanine nucleotide exchange factors (GEFs) through the exchange of bound GDP with GTP. Conversely, typical Rho GTPases are inactivated by GTPase-activating proteins (GAPs) through hydrolyzing the bound GTP. In addition, a third class of regulators of the Rho family members is Guanine-nucleotide dissociation inhibitors (GDIs) that are responsible for sequestering the GTPases in the cytoplasm away from the plasma membrane by binding to their C-terminal prenyl groups on Rho proteins and preventing them from interactions with their downstream effectors and relaying their functions (Haga & Ridley, 2016; Hanna & El-Sibai, 2013; Ju & Gilkes, 2018).



**Figure 4: Regulation of typical Rho GTPases by Rho-GAPs, GEFs, and GDIs.**

Source: (Ju & Gilkes, 2018)

- Regulation of atypical members of the Rho Family

As opposed to typical Rho GTPases, atypical members of the Rho family have dismal GTP/GDP cycling. Consequently, most of these proteins are trapped in a GTP bound conformation. Little is known about their regulation; however, other mechanisms have been proposed to play a role in the regulation of their activity including post-translational modifications, expression, ubiquitination followed by degradation in the proteasome (Blom, 2017; Dickover et al., 2014; Haga & Ridley, 2016; Ji & Rivero, 2016).

### 1.4.3 Downstream effectors of Rho GTPases

#### 1.4.3.1 Downstream Effectors of Rho

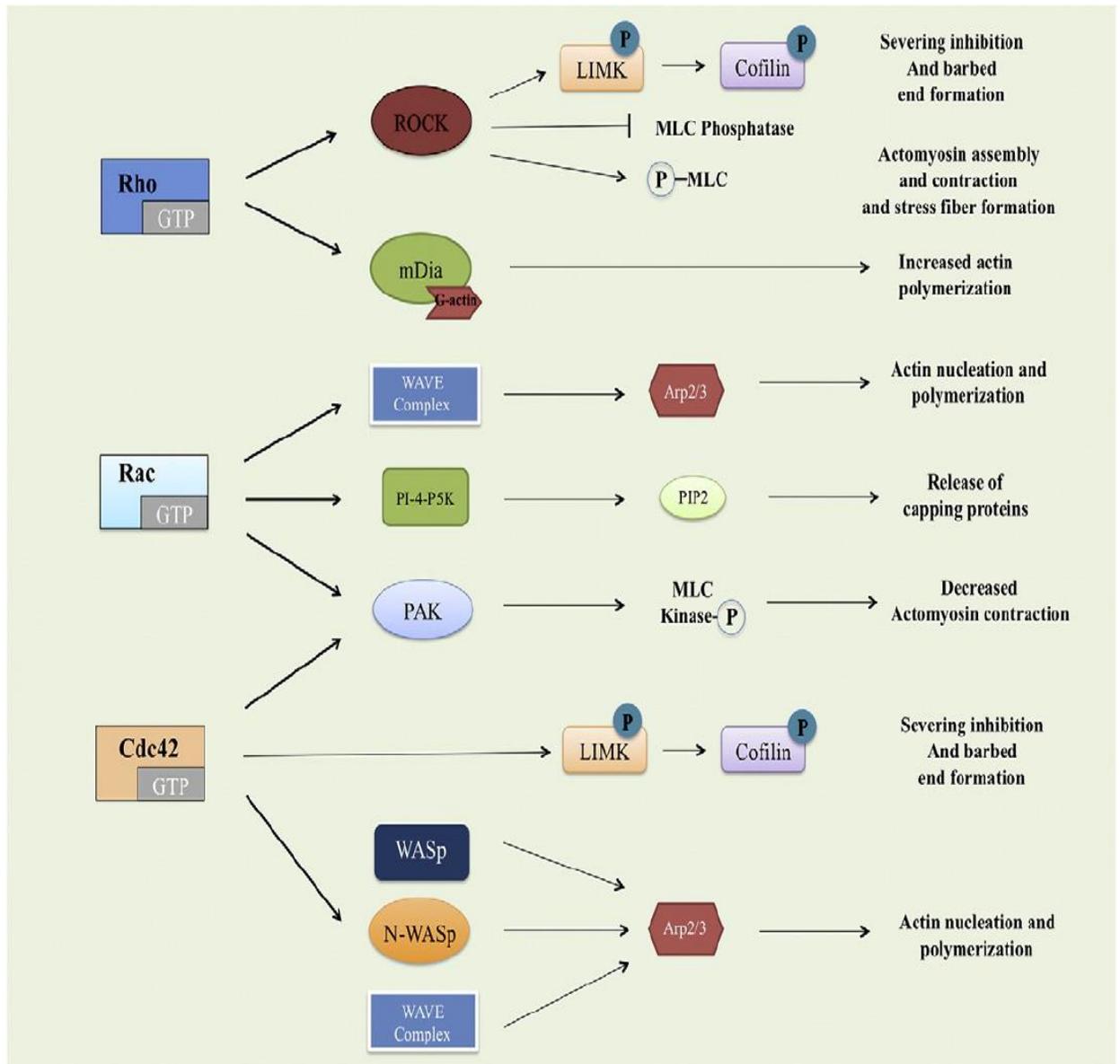
Rho is known to mediate actomyosin contractility, focal adhesions dynamics, and the formation of stress fibers. In order to perform these actin modulating functions, Rho proteins are required to activate their downstream effectors to relay the message dictated by the initial signal. Some of the well-known Rho effectors include the Rho-associated

protein kinase (ROCK), the mammalian homolog of diaphanous (mDia), the LIM kinase (LIMK), Rhotekin, and RhoGDI (Amano, Nakayama, & Kaibuchi, 2010; Costa & Parsons, 2010; Shi et al., 2013). ROCK, also known as p160 ROCK, is a serine/threonine kinase that belongs to the AGC family of protein kinases and constitutes two members ROCK1 and ROCK2 with 64% amino acid sequence similarity and 92% kinase domain similarity (Amano et al., 2010). ROCK induces actomyosin contractility via the phosphorylation of downstream myosin light chain (MLC). In an opposite manner, ROCK inhibits MLC phosphatases and consequently reduces MLC dephosphorylation (Hanna & El-Sibai, 2013). LIMK is a dual specific serine/ threonine and tyrosine kinase which is also a downstream target of Rho; it inactivates cofilin by phosphorylation. The end result is the inhibition of actin severing, barbed ends production, and upregulation of actin turnover (Hanna & El-Sibai, 2013; Wang & Townes-Anderson, 2016). In addition, mDia mediates stress fiber formation and actin nucleation downstream of Rho (Jaffe & Hall, 2005).

#### 1.4.3.2 Downstream effectors of Rac1 and cdc42

It is believed that cdc42 and Rac1 activation lead to the formation of filopodia and lamellipodia respectively (Hall, 1998). Filopodia are described as protruding actin rich bundles resembling microspikes and emanating from the plasma membrane, whereas lamellipodia are flat cellular protrusions at the leading edge of the cell (Chang et al., 2016; Svitkina, 2018). These actin structures are formed by actin nucleation and polymerization by downstream effectors of Rac1 and cdc42. These downstream targets include scaffolding proteins such as WASP, N-WASP, and WAVE that belong to the Wiskott-

Alrich Syndrome Protein (WASP) family. These proteins stimulate the actin nucleation protein Arp2/3 leading to the polymerization of actin at the leading edge (Lane, Martin, Weeks, & Jiang, 2014). In addition, a shared effector of Rac and Cdc42 called p21 activating kinase (PAK) inhibits MLCK by phosphorylation and thus inhibits MLC phosphorylation and actomyosin contractility (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999). PAK also phosphorylates LIMK and consequently activates it (Edwards, Sanders, Bokoch, & Gill, 1999). Moreover, Rac activates PIP-5 kinase leading to PIP<sub>2</sub> formation. Eventually, this leads to the inhibition of actin capping proteins (Hall, 1998).



**Figure 5: Downstream effectors and cellular functions of the Rho GTPases Rho, Rac, and cdc42.**

Source: (Hanna & El-Sibai, 2013)

## 1.5 RhoA and RhoC

RhoA and RhoC were among the first three members of Rho GTPases to be discovered back in 1985 (Madaule & Axel, 1985). The two proteins share more than 88 % of their amino acid sequence identity and both affect immensely the actin cytoskeleton

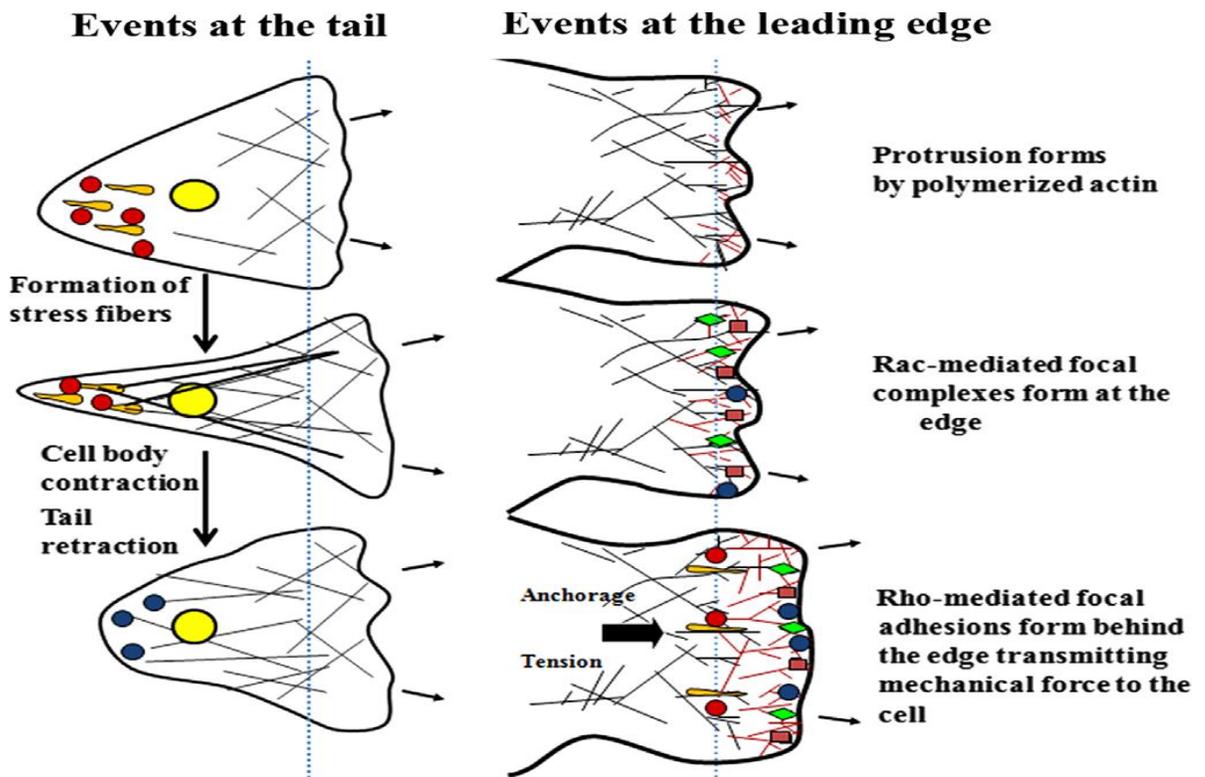
reorganization (Ridley A.j., 2013; Vega, Fruhwirth, Ng, & Ridley, 2011). RhoA was shown to be upregulated in different human tumor types especially those of epithelial origin such as gastric, testicular, colon, pancreatic, ovarian and breast cancer (Dreissigacker et al., 2006; Fritz, Just, & Kaina, 1999; Hirsch & Wu, 2007, p. 42; Horiuchi et al., 2003; Kamai et al., 2004; Liu et al., 2004; Orgaz, Herraiz, & Sanz-Moreno, 2014). Similarly, RhoC upregulation and activation was evident in metastatic melanomas, breast and ovarian cancers among many other types (Horiuchi et al., 2003; Kawata et al., 2014; Orgaz et al., 2014; Ridley, 2004).

Despite the high resemblance between the two isoforms, they appear to have distinct patterns and functions in the process of tumorigenesis. ROCK1 and ROCK2 are downstream targets for both RhoA and RhoC; however, their distinct roles in cell migration and invasion necessitates interaction with different effectors to execute their differential functions (Vega et al., 2011). For instance, it has been reported that the formin proteins FMNL2 and FMNL3 interact and activate RhoC, and not Rho A, in the MDA-MB-231 breast cancer cell line and PC3 prostate cancer cell line respectively.

### **1.5.1 Distinctive roles of RhoA in cancer cell motility and invasion**

RhoA has been proved to be implicated in the regulation and turnover of stress fiber generation, focal adhesion formation, and actomyosin contractility. In this context, it was originally thought that RhoA's related functions in motility are restricted to the rear edge of the cell mediating retraction. However, later findings showed that RhoA can also partake in processes such as membrane ruffling, lamellipodia formation, and membrane blebbing (El-Sibai et al., 2008; O'Connor & Chen, 2013). In addition, membrane ruffling and stress fiber formation require two of the Rho effectors ROCK and mDia. These

findings challenge the classical understanding in a way that RhoA appears to be a central mediator in the formation of actin-based membrane protrusions at the leading edge. The role of RhoA at the leading edge of a moving cell can be validated by the necessity of the maturation of focal contacts, primarily driven by Rac1, into focal adhesions, hence RhoA and Rac1 must work antagonistically in order to achieve a successful motility cycle as seen in Figure 6. Previous work from our lab add support to this notion by which state-of-the-art FRET for RhoA biosensor showed cycles of activation and inactivation of RhoA at both the leading edge and tail of astrocytoma cells SF-268. Also, a decrease in Rac1 activation was detected when a specific RhoA GAP, StarD13, was inhibited, which is in accordance with the antagonistic relationship between RhoA and Rac1 activity (El-Sibai et al., 2008; Khalil et al., 2014)



**Figure 6: Model of StarD13 regulation of RhoA activation at the tail and at the leading edge of astrocytoma cells undergoing cell motility.**

Source: (El-Sibai, unpublished)

### **1.5.2 Distinctive Roles of RhoC in cancer cell motility and invasion**

Apart from RhoA, RhoC was shown to participate with a distinctive role in cell migration. Indeed, it was reported that the expression of RhoC increased during the epithelial-mesenchymal transition of colon carcinoma mesenchymal transition (EMT) and that RhoC plays a role in regulating EMT-induced malignancy (Bellovin et al., 2006). In addition, RhoC assists in polarizing cell migration by controlling the activity of Rac1 and the breadth of lamellipodia at the leading edge (Vega et al., 2011). Most importantly, RhoC has gained a lot of attention in the past years because of its upregulation relevance with metastasis of several types of cancers. This upregulation in expression and activity has been correlated with poor prognosis and with tumor progression (Ridley A.j., 2013). John Condeelis and his group have investigated the role of RhoC in invadopodia formation in adenocarcinoma cell line MTLn3 and found that RhoC can control the process by the local regulation of cofilin phosphorylation at the invadopodium core (Bravo-Cordero et al., 2011). Their data suggest that RhoC is implicated in invasion, more specifically invadopodia formation and regulation; hence, this would explain why RhoC is associated with poor prognosis and can serve as a marker for metastatic tumors (Islam et al., 2009).

## 1.6 Purpose of the Study

The overall objective for this study is to elucidate the distinctive roles of the closely related isoforms RhoA and RhoC in astrocytoma cell line SF-268 and glioblastoma SNB-19 motility and invasion, while taking into consideration cross-talks with other Rho GTPases. This topic is still considered to be poorly investigated with respect to advances in other tumor types' progression. The first aim in our study was to look at the localization and activation of RhoA and RhoC by means of immunostaining and using RhoA and RhoC FRET biosensors. The second aim was to investigate the effect of RhoA and RhoC depletion on motility, adhesion to collagen, membrane ruffling, and on the expression levels of the 2 proteins Arp2 and Arp3; all of which being aspects that correlate with 2 dimensional migration and cytoskeleton rearrangements governing migration. Then, we wanted to elucidate the distinct roles of RhoA and RhoC on invasion broadly and on invadopodia formation specifically. Finally, we deciphered the cross-talk between RhoA, RhoC, and two other Rho GTPases, Rac1 and Cdc42, by assessing their expression and activation levels to proceed with looking at the expression of their well-known effectors, WAVE and WASP, respectively.

# **Chapter 2**

## **Materials and methods**

### **2.1 Cell culture**

Human malignant glioblastoma cell line (SNB-19) and human astrocytoma cell line (SF-268) were obtained from ATCC, and were cultured in DMEM medium supplemented with 10% FBS and 100 U penicillin/streptomycin at 37°C and 5% CO<sub>2</sub> in a humidified chamber.

### **2.2 Antibodies and reagents**

The following primary antibodies were used in this study: mouse monoclonal anti-RhoA antibody, rabbit polyclonal anti-RhoC, rabbit polyclonal anti-cdc42, rabbit polyclonal anti-vinculin, rabbit polyclonal anti-Tks4, rabbit polyclonal anti-cortactin, rabbit monoclonal anti-WASP, rabbit polyclonal

WAVE-1, rabbit polyclonal Arp2, mouse monoclonal Arp3, rabbit polyclonal anti-beta actin were purchased from abcam (Abcam Inc., Cambridge, UK). Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were obtained from promega (Promega Co., Wisconsin). Fluorescent secondary antibodies (Alexa Fluor 488) was obtained from Invitrogen. For F-actin cytoskeleton visualization, cells were stained with Rhodamine-phalloidin (Invitrogen).

### **2.3 Cell transfection with siRNA**

Flexi Tubes siRNA for RhoA oligo 1 and 6 and for RhoC oligo 1 and 5 were obtained from Qiagen (Qiagen, USA). The cells were transfected with the siRNA at final concentration of 10 nM using Hiperfect (Qiagen, USA) according to the manufacturer's specifications. Control cells were transfected with siRNA sequences targeting GL2 Luciferase (Qiagen, USA). After 72 hours, protein levels in total cell lysates were analyzed by western blotting using the appropriate antibodies or the effect of the corresponding knockdown was assessed.

### **2.4 Western immunoblotting**

Whole cell lysates were prepared by scraping the cells with laemmli sample buffer containing 4% SDS, 20% glycerol, 10%  $\beta$  mercaptoethanol, 0.004% bromophenol blue and 0.125M Tris HCL (pH 6.8). SDS-PAGE was carried out under standard conditions and proteins were blotted onto a PVDF membrane. The membranes were then blocked with 5% bovine serum albumin for 1 hour and then incubated overnight at 4°C with either primary antibody against RhoA (abcam, 1:500 dilution), RhoC (abcam, 1:200 dilution) ,or actin (abcam, 1:2500). After the incubation with the primary antibody, the membranes were washed and incubated

with secondary antibody at a concentration of 1:1000 for 1 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with western blotting chemiluminescent reagent ECL (GE Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

## **2.5 Motility assay**

Cells were plated on a 35mm petri dish and transfected with si-Luciferase, si-RhoA, si-RhoC, or both. The assay was performed 72 hours after transfection. For motility analysis, cells were imaged in DMEM (10% FBS) media, buffered using HEPES and overlaid with mineral oil on a 37°C stage. Images were collected every 60 seconds for 2 hours using a 20X objective lens on Zeiss Observer Z1 microscope. The speed of cell movement was quantified using the ROI tracker plugin in the ImageJ software, which was used to calculate the total distance travelled by individual cells. The speed is then calculated by dividing this distance by the time (120 minutes) and reported in  $\mu\text{m}/\text{min}$ . The speed of at least 10 cells for each condition was calculated. The net distance travelled by the cell was calculated by measuring the distance travelled between the first and the last frame.

## **2.6 Immunostaining**

Cells were plated on glass coverslips on collagen with a final concentration of 50 $\mu\text{g}/\text{mL}$ , starved for 3 hrs and treated with Phorbol 12-Myristate 13-Acetate ester (PMA) an hour before starting the experiment with a final concentration of 500 nM. Cells were fixed with 4% paraformaldehyde for 10 minutes at 37°, and permeabilized with 0.5% Triton-X 100 for 15 minutes on ice. For blocking, cells

were incubated with 1% filtered BSA in PBS for 1 hour. Samples were then stained with primary antibodies overnight at 4° and with fluorophore-conjugated secondary antibodies for 1 hour. Fluorescent images were taken using a 63X objective lens on Zeiss Observer Z1 microscope.

## **2.7 Pull-down assay**

Cells were plated on a 100 mm petri. Again after 24 hrs, the cells were starved for 3 hrs and treated with Y27632, a well-established ROCK inhibitor, half an hour before starting the experiment with a final concentration of 10 mM. Following treatment period, cells were lysed, and the pull-down assay performed using the RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell BioLabs) following the manufacturer's instructions. Briefly, cell lysates were incubated with GST-PAK (for Cdc42) for 1 hour at 4 °C with gentle agitation. Then, the samples were centrifuged, and the pellet washed for several times. After the last wash, the pellets were resuspended with sample buffer and boiled for 5 minutes. GTP-Cdc42 were detected by western blotting using anti-Cdc42 antibodies provided in the kit. Total protein extracts were collected prior to the incubation with GST beads and used as a loading control.

## **2.8 Adhesion assay**

96-well plates were coated with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37 °C then washed with washing buffer (0.1% BSA in DMEM). The plates were then blocked with 0.5% BSA in DMEM at 37 °C in a CO2 incubator for 1 hour. This was followed by washing the plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to  $4 \times 10^5$

cell/ml. 50  $\mu$ l of cells were added in each well and incubated at 37°C in a CO<sub>2</sub> incubator for 30 minutes. The plates were then shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 minutes, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 minutes. Following the staining with crystal violet, the plates were washed extensively with water, and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550 nm using an ELISA reader.

## **2.9 Invasion Assay**

Cells were transfected with either control, RhoA, RhoC, or both 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<sup>6</sup> cells/ml. In the meantime, inserts were pre-warmed with 300 $\mu$ l of serum free medium for 30min at room temperature. After rehydration, 250 $\mu$ l of media was removed from inserts and 250 $\mu$ l of cell suspension was added. Inserts were then placed in a 24-well plate, and 500 $\mu$ l of complete media (with 10% serum) was added to the lower wells. Plates were incubated for 24hrs at 37°C in a CO<sub>2</sub> incubator. Following 48 hours of incubation period, inserts were stained for 20min at room temperature with 400 $\mu$ l of cell stain provided with the kit. Stain was then extracted with extraction buffer (also provided with the kit).100ul of extracted

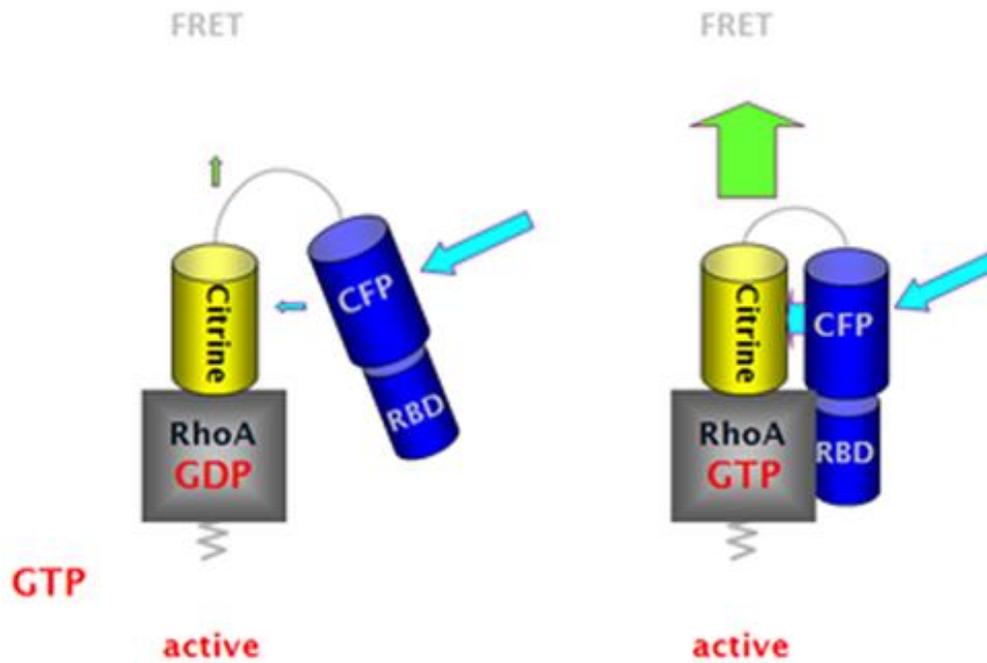
stain was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical Density was then measured at 560 $\mu$ m.

## **2.10 Förster Resonance energy transfer (FRET)**

SNB-19 cells were transfected with 2.5  $\mu$ g of the RhoA fluorescence resonance energy transfer (FRET)-based biosensor plasmid, cdc42 Fluorescence resonance energy transfer (FRET)-based Biosensor plasmid, or Rac1 fluorescence resonance energy transfer (FRET)-based biosensor plasmid. The RhoA biosensor consists of (from the N-terminus) the Rho binding domain (RBD) of the effector Rhotekin, a cyan fluorescent protein (CFP), a protease resistant 17-mer unstructured linker, a yellow fluorescent protein (YFP) domain, and a full length RhoA (Pertz, Hodgson, Klemke, & Hahn, 2006). The biosensor for Cdc42 incorporates monomeric Cerulean (mCer) at the N-terminus, followed by two tandem p21 binding domains (PBD) derived from PAK1 with a structurally optimized linker in between the two PBDs and monomeric Venus (mVenus) followed by full-length, wild-type Cdc42 at the C-terminus (Hanna, Miskolci, Cox, & Hodgson, 2014). The RhoC biosensor RhoC FLARE was created by linking ROCK1 residues 905-1046 to monomeric Cerulean, an unstructured linker of optimized length, monomeric Venus, and full-length RhoC (Zawistowski, et al., 2013).

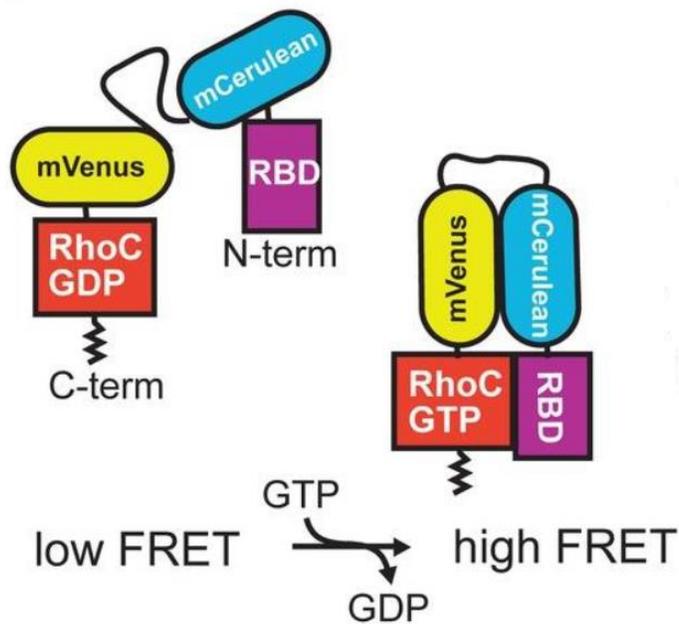
The Rac1 biosensor corresponds to a wild-type 3- linker single chained biosensor having a CFP and YFP domain. This biosensor is unpublished and was obtained from Louis Hodgson's lab. FRET image sequences were obtained with

an automated Zeiss Observer Z1 microscope equipped with filter wheels in the excitation and emission light path.



**Figure 7: The RhoA FRET biosensor.** The biosensor consists of (from the N-terminus) the Rho binding domain (RBD) of the effector Rhotekin, a cyan fluorescent protein (CFP), a protease resistant 17mer unstructured linker, a yellow fluorescent protein (YFP) domain, and a full length RhoA.

Source: (Pertz, et al., 2006)



**Figure 8: The RhoC FRET biosensor.** The biosensor was created by linking ROCK1 residues 905-1046 to monomeric Cerulean, an unstructured linker of optimized length, monomeric Venus, and full-length RhoC.

Source: (Zawistowski, et al., 2013)

## 2.11 Quantitation of focal adhesions

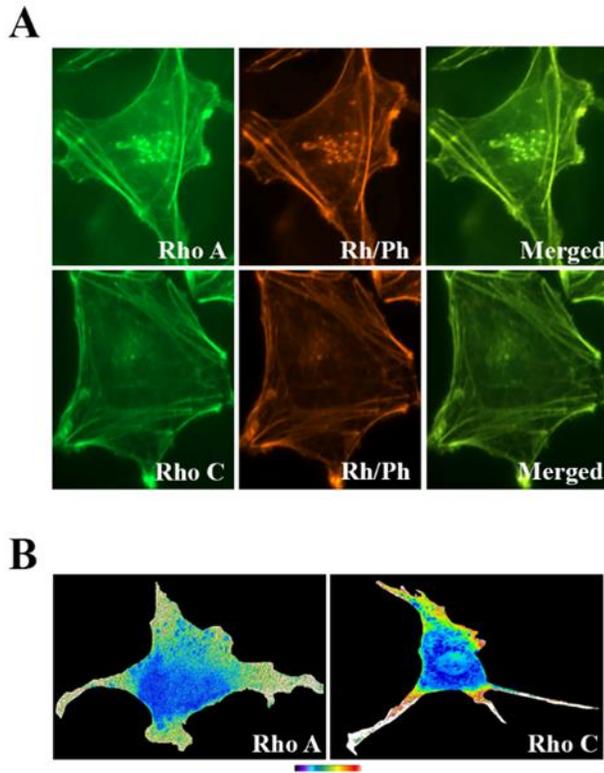
Image J was used to quantitate focal adhesions. Briefly, two main plugins were used to quantitate focal adhesions, these two plugins are CLAHE and Log3D. CLAHE enhances the local contrast of the image and Log3D filters the image based on user predefined parameters which will allow us to detect and analyze focal adhesions (Horzum, Ozdil, & Pesen-Okvur, 2014).

# Chapter 3

## Results

### **3.1. RhoA and RhoC colocalize at invadopodia with F-actin and both RhoA and RhoC have increased levels of activation at cell border peripheries in human malignant glioblastoma cell line SNB-19**

Before exploring the motility and invasion aspects of human glioblastoma cell line SNB-19, we were interested in looking at the subcellular localization of RhoA and RhoC. This was achieved by immunofluorescence. The cell samples were stained using anti-RhoA and anti-RhoC. Figure 10A represents the corresponding immunofluorescence images. Then we were interested in retrieving localization and levels of activation data about RhoA and RhoC by the advent of RhoA and RhoC biosensors after transfecting SNB-19 cells with a single chain CFP/YFP RhoA FRET biosensor and a single chain CFP/YFP RhoC FRET biosensor respectively (Pertz, et al., 2006; Zawistowski, et al., 2013). Representative images are shown in figure 9B.



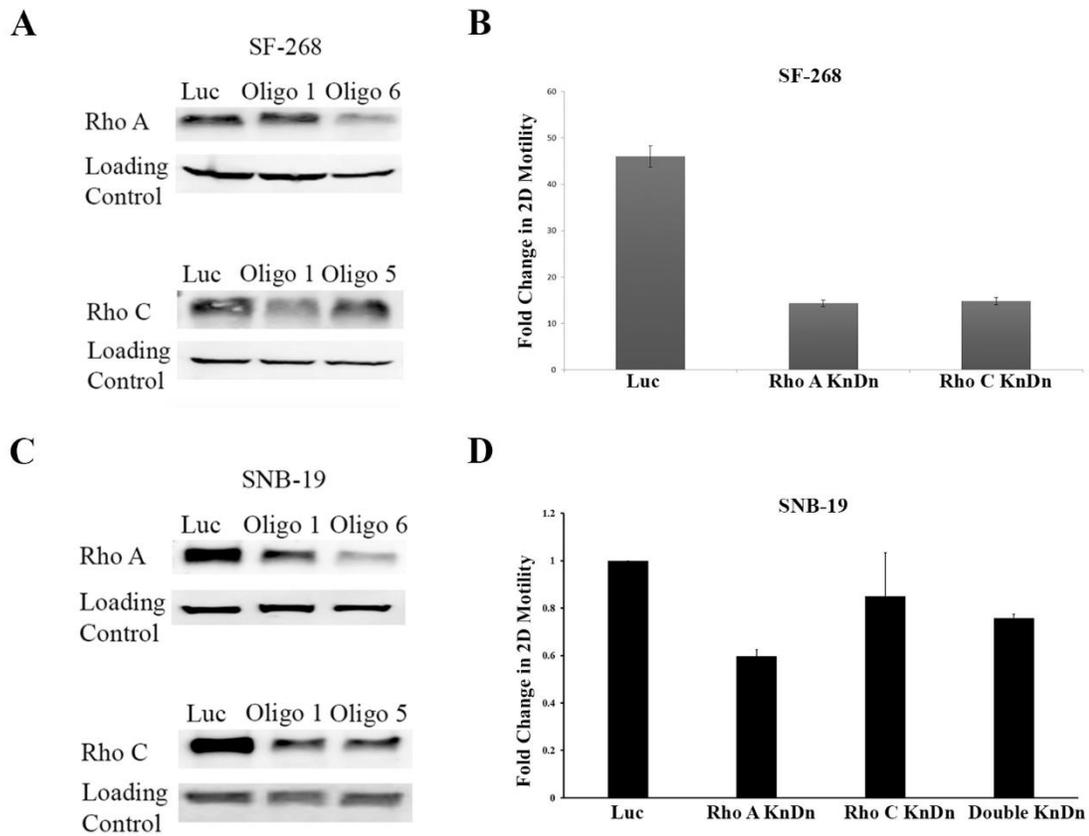
**Figure 9: RhoA and RhoC localization and activation in human malignant glioblastoma cell line SNB-19.** A) Representative micrographs of (SNB19) cells that were fixed and stained by immunofluorescence for RhoA (left), F-actin (middle), and a merge is also represented (right) (Upper lane); and for RhoC (left), F-actin (middle), and a merge is represented (right) (Lower Lane). Images of the cells were taken using a 60x objective. B) Representative micrographs of (SNB19) cells that were transfected with the FRET RhoA biosensor (upper lane) and FRET RhoC biosensor (lower lane). The cells were imaged using the FRET channel and the raw FRET images were normalized to the CFP images. The images reflect the FRET ratio = Raw FRET image/CFP.

## 3.2. RhoA and RhoC are positive regulators of 2D motility in astrocytoma and glioblastoma

### 3.2.1. RhoA and RhoC depletion decreases human astrocytoma SF-268 and human glioblastoma SNB-19 cell migration

We first looked for the levels of expression of both RhoA and RhoC proteins using two siRhoA and two siRhoC oligos in SF-268 and SNB-19 to choose the better ones for enhanced knockdown ability, so that we can proceed with those for the rest of our

experiments. Western immunoblotting results show knockdown in each oligo of both RhoA and RhoC, but we chose to proceed with siRhoA oligo 6 and siRhoC oligo 5 for yielding better knockdown results (Figure 10A and 10C). After performing 2-D time-lapse microscopy and quantitating cell speed on ImageJ, results revealed that RhoA and RhoC knockdown in SF-268 reduce cell motility speed by roughly 69 % and 67 % respectively. On the other hand, RhoA, RhoC, and RhoA/RhoC double knockdown in SNB-19 cells decrease cell motility speed by approximately 40 %, 15 %, and 24 % respectively.

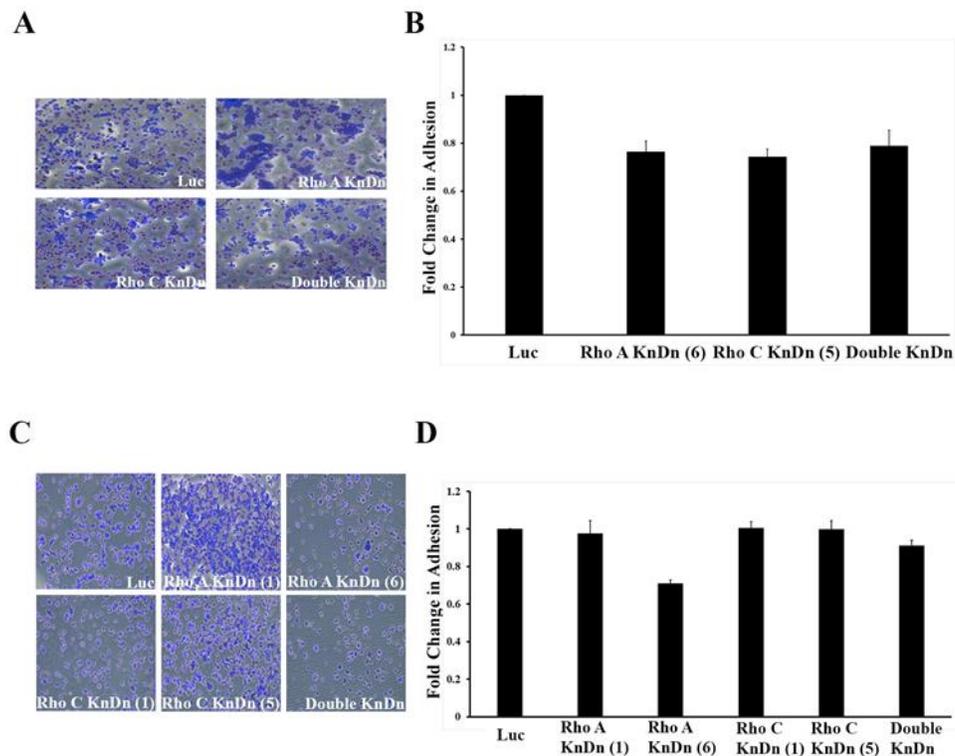


**Figure 10: Both RhoA and RhoC are required for astrocytoma and glioblastoma cancers cell motility.** A) SF268 cells were transfected with control siRNA, RhoA siRNA, RhoC siRNA, or both for 72 hours. B) Quantitation of the net speed migration fold in SF-268, normalized to the control, of projected 120 frames from time lapse movies of cells with an overall duration of 2 hours displaying random cell motility in serum. C) SNB19 cells were transfected with control siRNA, RhoA siRNA, RhoC siRNA, or both for 72 hours. D) Quantitation of the net speed migration fold in SF-268, normalized to the control, of

projected 120 frames from time lapse movies of cells with an overall duration of 2 hours displaying random cell motility in serum. Data are the mean  $\pm$  SEM taken from 15 randomly selected cells.

### 3.2.2. RhoA and RhoC knockdown decreases adhesion to collagen in human astrocytoma cell line SF-268, but only RhoA knockdown decreases adhesion in human glioblastoma cell line SNB-19

Given the importance of the formation of adhesions in promoting a successful cycle of cell motility, we next wanted to assess the ability of both cell lines SF-268 and SNB-19 to adhere to collagen after knocking down RhoA, RhoC, or both proteins. The results showed approximately 25 % decrease in the three cases in SF-268 (Figure 11A-B). However, the knockdown of RhoA, RhoC, or both proteins decreased adhesion by 30 %; had no change in adhesion; and decreased adhesion slightly by 9 % respective of knockdowns (Figures 11C-D).

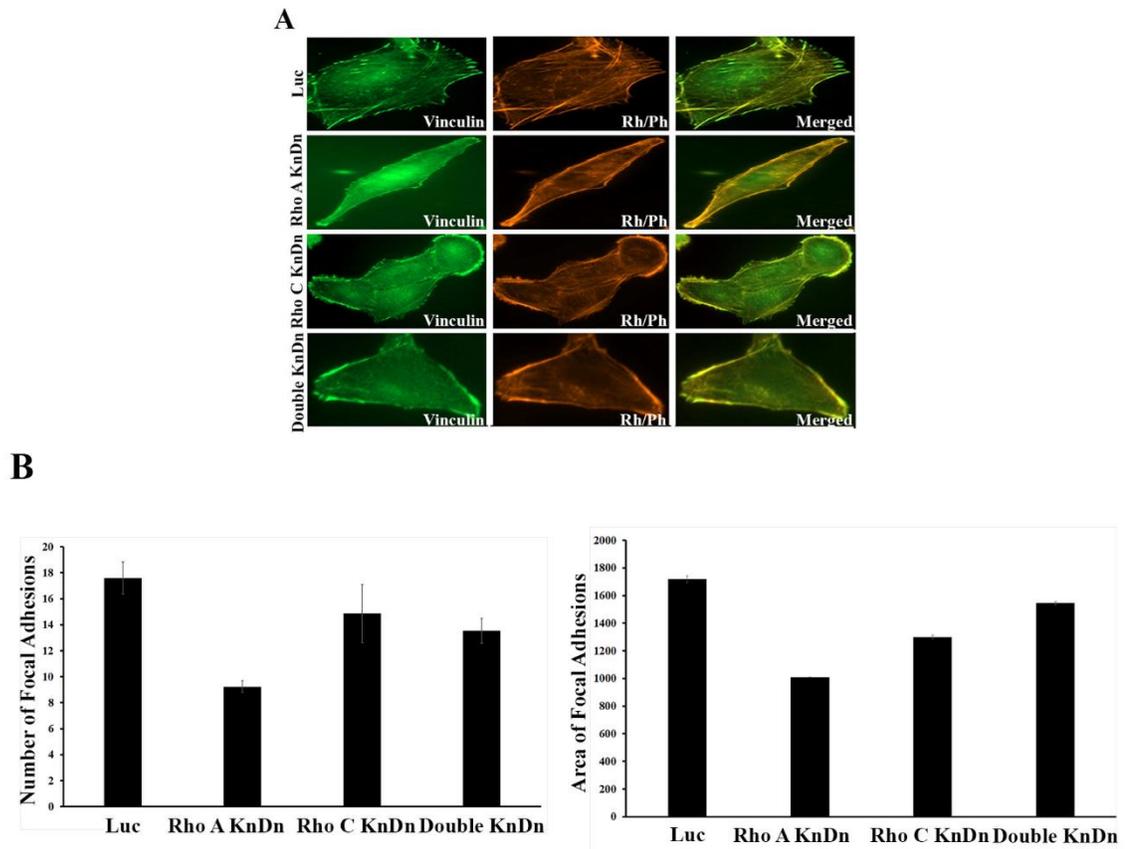


**Figure 11: RhoA and RhoC promote cell adhesion to collagen in SF-268 cells, whereas only RhoA promotes the process in SNB-19 cells.** A) Representative micrographs of SF-268 cells transfected with si-

Luciferase (Upper lane left), si-RhoA (Upper lane right), siRhoC (lower lane left), or both si-Rho/RhoC (Lower lane right), fixed and stained with crystal violet (as described in methods). B) Quantitation of solubilized Crystal violet from SF268 plates and the absorption of the plates were measured at 550 nm using an ELISA plate reader. Data are measured in fold change of adhesion normalized to the control. C) Representative micrographs of SNB-19 cells transfected with si-Luciferase (Upper lane left), si-RhoA oligo 1 (Upper lane middle), si-RhoA oligo 6 (Upper lane right), siRhoC oligo 1 (Lower lane left), siRhoC oligo 1 (Lower lane middle), or both si-Rho/RhoC (Lower lane right), fixed and stained with crystal violet (as described in methods). D) Quantitation of solubilized Crystal violet from SNB19 plates and the absorption of the plates at 550 nm using an ELISA plate reader. Data are measured in fold change of adhesion normalized to the control. Data are the mean  $\pm$  SEM from 3 independent experiments.

### **3.2.3 RhoA and RhoC knockdown decrease focal adhesion formation**

In order to confirm the cell adhesion assay results in human astrocytoma cell line SF-268, we wanted to look at the formation of focal adhesions, mediators of the adhesion process. We proceeded with knocking down RhoA, RhoC, or both proteins and stained for vinculin, a major component of focal adhesion structures (Humphries et al., 2007). The knockdown of RhoA, RhoC, and both proteins depleted the formation of focal adhesions by 48 %, 16 %, and 23 % respectively (Figures 13A-B). The cells were imaged using 60x objective. The focal adhesions were then quantitated with a specific macro according to the manufacturer's instructions using ImageJ (Horzum et al., 2014).

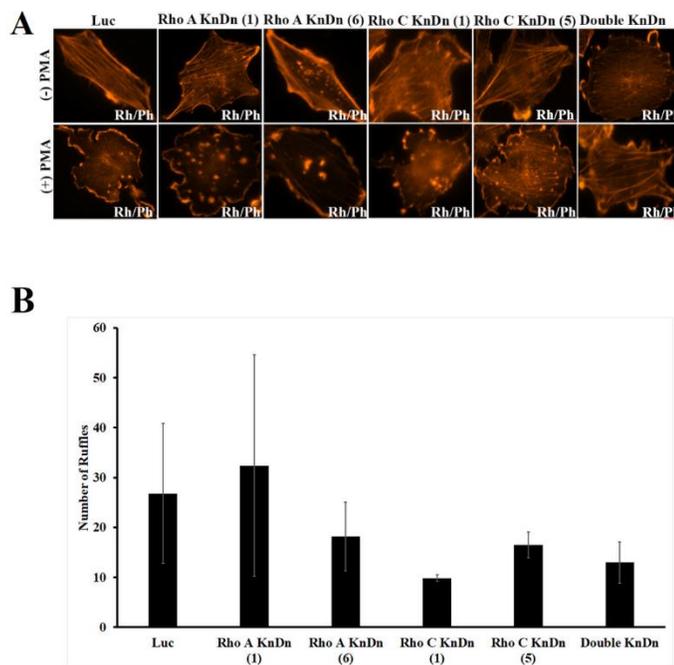


**Figure 12: RhoA, with the help of RhoC, promote the formation of focal adhesions.** A) Representative micrographs of (SF-268) transfected with si-luciferase (upper line), si-RhoA (second lane), siRhoC (third lane), and siRhoA/RhoC (Lower lane) that were fixed and stained with anti-vinculin. Cells were imaged using a 60x objective. B) Quantitation of number of focal adhesions (Left figure) and areas of focal adhesions (Right figure) upon RhoA, RhoC, and double RhoA/RhoC knockdowns. Data are the mean  $\pm$  SEM from 3 independent experiments.

### 3.2.4 RhoA and RhoC knockdown decreases the formation of membrane ruffles and actin protrusions

Membrane ruffling was reported to be an indicative sign of cancer cell motility, this event usually occurs due to rapid reorganization of the cell plasma membrane and occurs right before the formation of lamellipodia (Jiang, 1995; Mahankali, Peng, Cox, &

Gomez-Cambronero, 2011). So it was of interest to investigate the degree of protrusions formation in human glioblastoma cell line SNB-19 to validate our 2D motility results. For that purpose, we starved the cells with serum free media for 3 hrs and then treated the cells with a potent PKC activator and ruffling promoter, PMA, for 30 minutes. Afterwards, the cells were fixed and stained for F-actin. Quantitation of the ruffles on ImageJ software was done by subtracting the background followed by thresholding and measuring ruffles according to their size. The results show that upon knockdown of RhoA, RhoC, or both proteins, membrane ruffling decreases by 32%, 39 %, or 52 % respectively (Figure 13A and B).

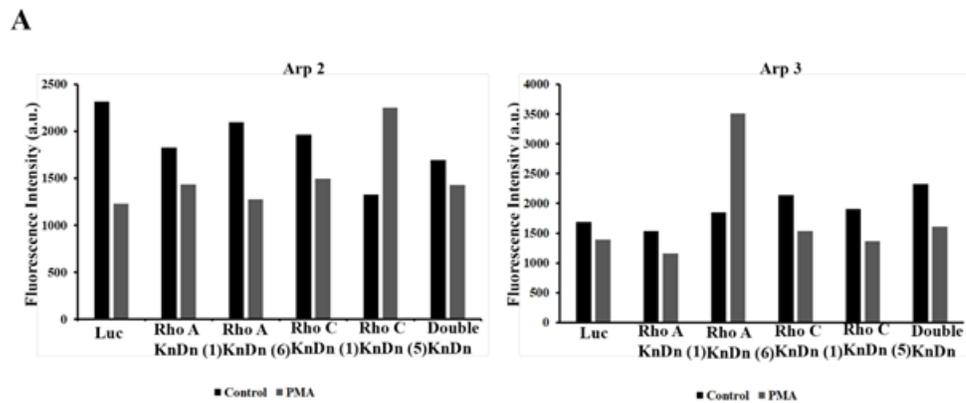


**Figure 13: The formation of membrane ruffles in SNB-19 are both RhoA and RhoC dependent. A)** Representative micrograph of (SNB-19) cells that were transfected with si-Luciferase, si-RhoA, si-RhoC, or both siRhoA/RhoC (Order from Left to right), treated with PMA (Lower lane), fixed, and stained with Rhodamine phalloidin. **B)** Quantitation of the number of membrane protrusions coupled with their fluorescence intensity upon knockdown RhoA, RhoC, or both proteins. Data are the mean  $\pm$  SEM from 3 independent experiments

### 3.2.5 Arp2 and Arp3 expression changes upon knockdowns of RhoA, RhoC, and both proteins.

Because the formation of protrusive structures involved in motility is correlated to changes in the actin cytoskeleton reorganization, it was important to examine Arp2 and Arp3 fluorescence intensities upon knocking down RhoA, RhoC, or both proteins and treatment with PMA in SNB-19 cells. Our results show that the levels of fluorescence intensity of Arp2 barely change, increase by 83%, and increase by 15% respective with the knockdown of RhoA, RhoC, and both proteins.

In contrast, only RhoA knockdown show drastic change in Arp3 fluorescence intensity by which it shows more than 2 folds increase than the control (Figure 14).

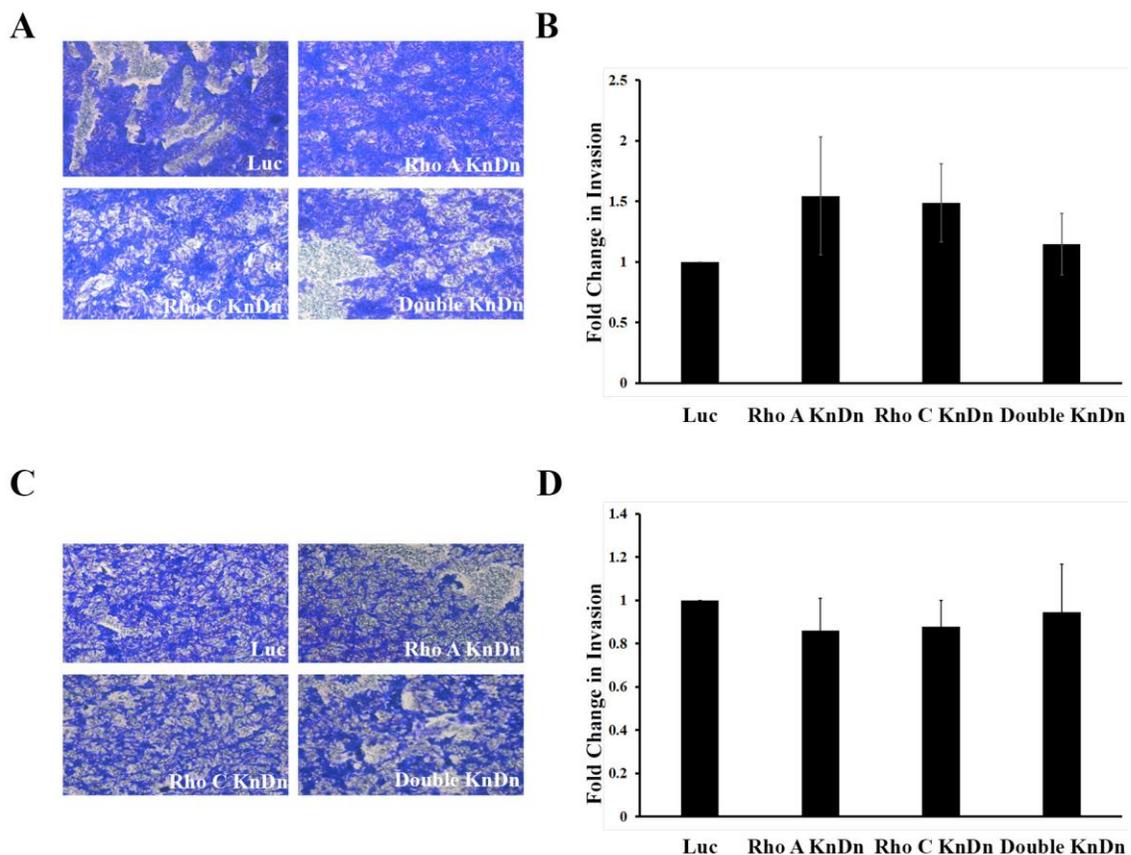


**Figure 14: RhoC affects actin rearrangement by downregulating Arp2 expression whereas RhoA affects actin rearrangement by downregulating Arp3.** A) Representative micrographs of (SNB19) cells showing fluorescence intensity of Arp2 (Left Graph) or Arp3 (Right Graph) following the knockdowns of RhoA, RhoC, or both.

### 3.3. RhoA and RhoC are antagonists in invasion

#### 3.3.1. RhoA and RhoC knockdown increases cellular invasion in SF-268 cell line, but decreases invasion in SNB-19 cell line.

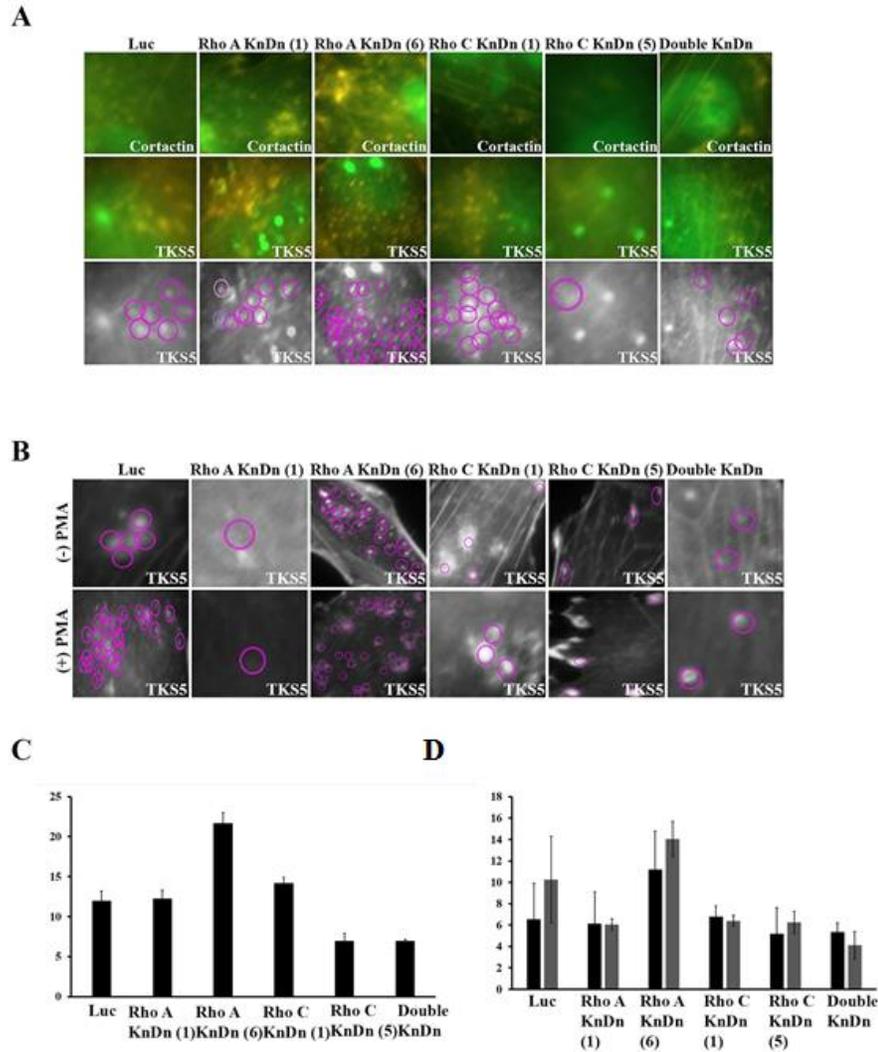
After establishing the role of RhoA and RhoC in 2D cell motility and actin reorganization, we were interested in estimating their role in 3D invasion. For this reason, we carried out an in-vitro collagen-based invasion assay with FBS as a chemoattractant. The trans-well chambers were filled with serum free media and used as negative controls. The results show an increase in cellular invasion by 54 %, 49%, and 15% for the knockdown of RhoA, RhoC, and RhoA/RhoC double knockdown respectively in SF-268 (Figure 15A-B). On the other hand, for the same knockdowns, a decrease in cellular invasion was shown by 14%, 12% and 7% respectively in SNB-19 (Figure 15C-D).



**Figure 15: RhoA and RhoC regulate 3D invasion in SF-268 and SNB-19 cell lines.** SF-268 cells and SNB-19 cells were transfected with si-Luciferase, siRhoA, siRhoC, and both siRhoA/RhoC. Cells that invaded to the basement side of the collagen basement membrane towards FBS for 48 hours were stained with cell stain according to manufacturer's specifications. After that, the cell stain was extracted and the absorbance was measured at 560 nm. A) Representative micrographs of SF268 invading cells. B) Quantitation of Optical Density for SF-268 measured in arbitrary units. C) Representative micrographs of SNB-19 invading cells. D) Quantitation of Optical Density for SNB-19 measured in arbitrary units. Data are the mean  $\pm$  SEM from 3 experiments.

### **3.3.2. RhoA knockdown increases invadopodia formation, whereas RhoC knockdown decreases invadopodia formation in SF-268 and SNB-19.**

Since the formation of invadopodia is a prominent feature of cancer cell invasion, we were interested in looking at the effect of RhoA, RhoC, and RhoA/C double knockdowns on the number of invadopodia formed in both cell lines. SNB-19 cells were starved for 3 hours and stimulated with PMA for one hour before our experiment whereas SF-268 cells weren't stimulated due to their inherent ability of formation of invadopodia even when untreated. TKS4 and cortactin are 2 molecular markers used for the determination of mature invadopodia presence. Accordingly, the cells were fixed and stained with anti-Tks4 and anti-cortactin separately in the case of SF268 coupled with rhodamine phalloidin or stained with anti-Tks4 alone with rhodamine phalloidin for SNB-19. Our results indicate that RhoA, RhoC, and double knockdown respectively lead in SF-268 to an 83% increase, 42% decrease, and approximately 42 % decrease in invadopodia number. On the other hand, the same knockdowns led to a 37% increase, a 39% decrease, and a 60% decrease in invadopodia number respectively (Figure 16A-D).

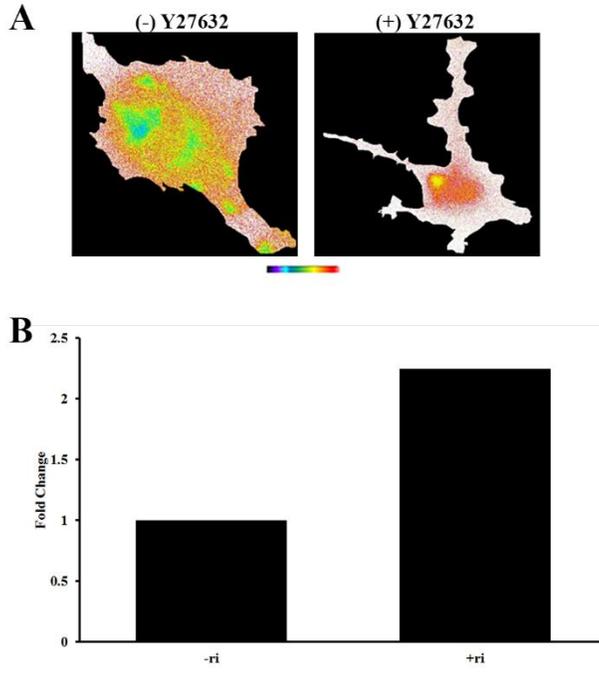


**Figure 16: Invadopodia formation is RhoC dependent in SF268 and SNB19, whereas RhoA negatively regulates invadopodia formation.** A) SF-268 cells were stained with anti-cortactin merged with rhodamine phalloidin (upper lane) or anti-Tks4 with Rhodamine phalloidin (middle lane) after being transfected with si-luciferase, si-RhoA oligo 1, si-Rho A oligo 6, si-RhoC oligo 1, si-RhoC oligo 5, and si-RhoA/RhoC represented in order from left to right in the representative figures. Lower lane shows the colocalization macro used to uniquely identify mature invadopodia developed by Ved Sharma. B) SNB-19 cells were left untreated (upper lane) or treated with PMA (lower lane) and stained with anti-Tks4 and rhodamine phalloidin after being transfected with si-luciferase, si-RhoA oligo 1, si-RhoA oligo 6, si-RhoC oligo 1, si-RhoC oligo 5, and si-RhoA/RhoC represented in order from left to right in the representative figures. C) Quantitation of invadopodia number in SF-268 after the previously mentioned transfections. D) Quantitation of invadopodia number in SNB-19 after transfection and treatment with PMA. Data are the mean of +/- SEM of 3 independent experiments for each cell line.

### **3.4 Cross-talk between RhoA, RhoC, and Rac1**

#### **3.4.1 ROCK inhibition leads an increase in Rac1 activation in human glioblastoma cell line SNB-19**

After revealing several aspects of 2D and 3D motility in glioma cell lines, we were interested in studying the cross-talks and signaling pathways between the GTPases in our study and Rac1, a major Rho-GTPase widely implicated in 2D motility. Rac1 has been shown to drive the formation of lamellipodia and is required to complete the motility cycle (Hanna & El-Sibai, 2013; Steffen et al., 2013). Thus, we were interested in looking at the activation levels of Rac1 by using FRET. SNB-19 cells were starved for 3 hours with serum-free media, stimulated with Y27632, and transfected with Rac1 biosensor. Our results show an increase in the activation of Rac1 by more than 2 folds in Y-27632 stimulated cells (Figure 17A and B).

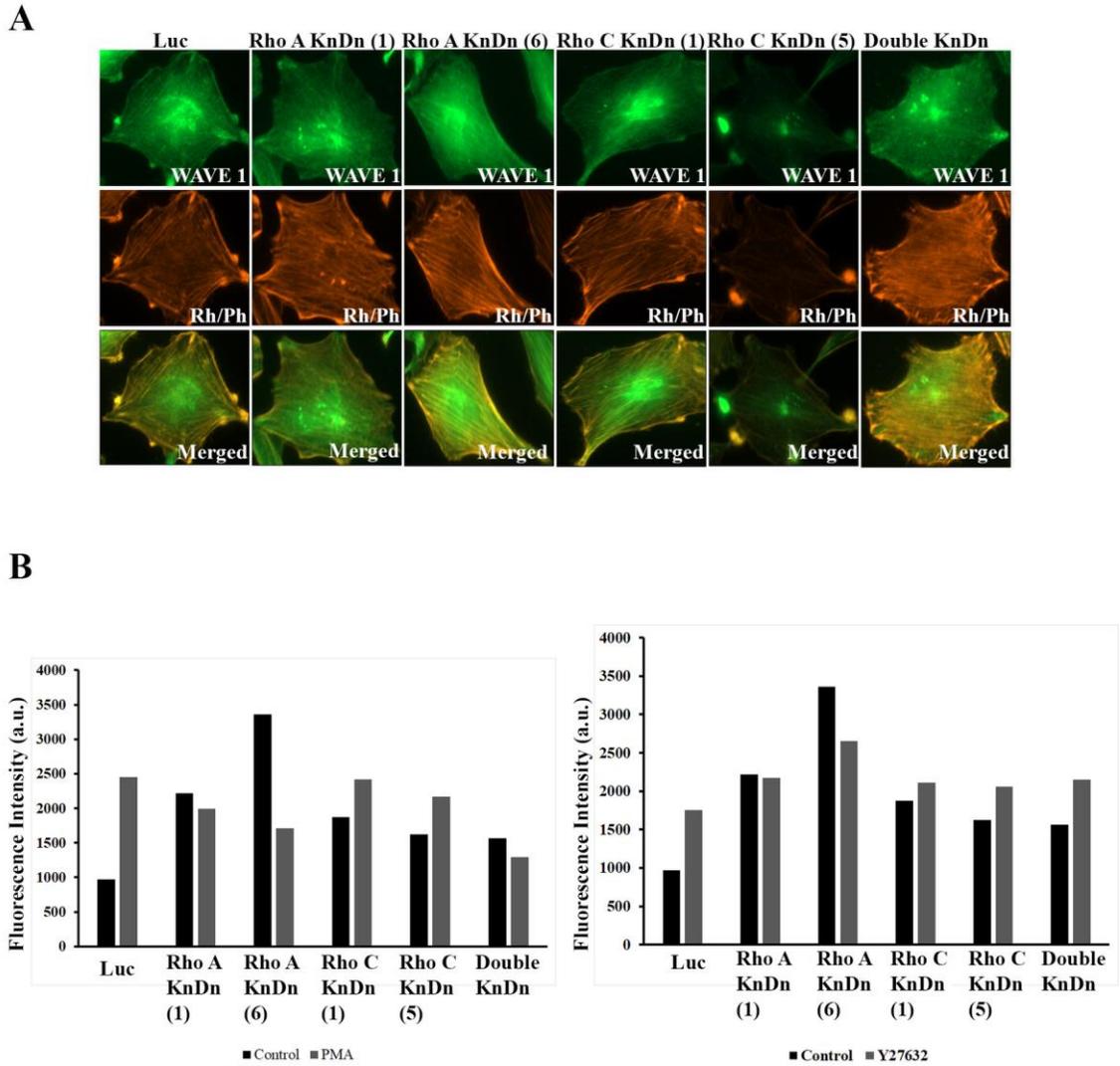


**Figure 17: The RhoA/RhoC/ROCK pathway downregulates the activation of Rac1.** A) Representative micrographs of (SNB-19) cells that were left untreated (Left image) or Y-27632 treated (right image), fixed, and transfected with the FRET Rac1 biosensor. The cells were imaged using the FRET channel and the raw FRET images were normalized to the CFP images. B) Quantitation graphs reflecting the FRET ratio = Raw FRET image/CFP.

### 3.4.2 RhoA and RhoC knockdowns increase the expression of WAVE, the downstream effector of Rac1

To verify our previous result that the Rho/ROCK pathway downregulates Rac1, we attempted to correlate this with the expression of a known downstream effector of Rac1, WAVE1. We knocked down RhoA, RhoC, or both proteins in SNB-19; starved the cells for 3 hrs; stimulated with either PMA (for one hour) or Y27632 ROCK inhibitor (for 30 minutes), fixed the cells, and stained using an anti-WAVE antibody with F-actin. Our results indicate that the knockdown of RhoA, RhoC, or both proteins

increase expression of WAVE by 51%, 17%, and approximately 23% respectively upon treatment with Y27632 (Figure 18B).

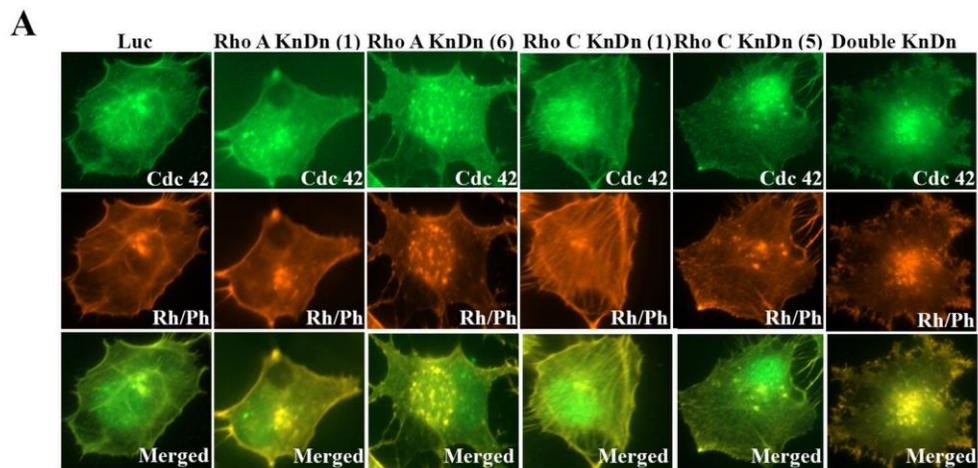


**Figure 18: RhoA and RhoC decrease the expression of WAVE1, by downregulating its upstream activator Rac1.** A) Representative micrographs of (SNB19) cells with control cells in addition to RhoA, RhoC, and double protein knock downs (Order from Left to right). These cells were fixed and stained with anti-WAVE1 and rhodamine phalloidin. B) Quantitation of the fluorescence intensity of WAVE1 with PMA (Left Graph) or Y27632 (Right Graph) treatments with their corresponding knockdowns.

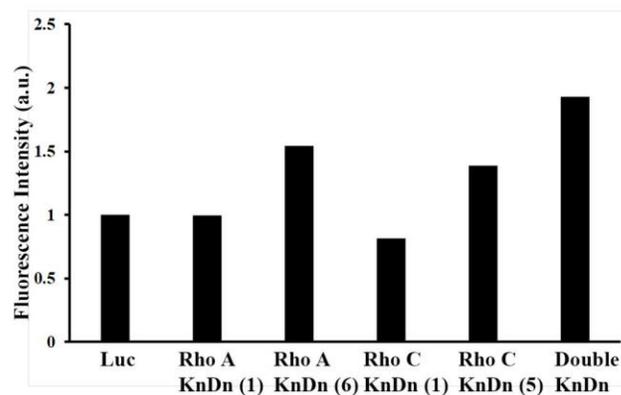
### 3.5 Cross-talk between RhoA, RhoC, and Cdc42

### 3.5.1. RhoA and RhoC knockdowns increase the expression Cdc42

Since *cdc42* is an important regulator of cancer cell motility and invasiveness, we were interested in investigating regulation by and cross-talks with RhoA and RhoC. We started with looking at the expression of *cdc42* upon knocking down RhoA, RhoC, or both proteins, fixing the cells, and staining for *cdc42* with F-actin in SF-268 cells. As a result, *cdc42* fluorescence intensity was shown to increase upon RhoA, RhoC, and double knockdowns by 54%, 39%, and 93 % respectively (Figure 19A and B).



**B**

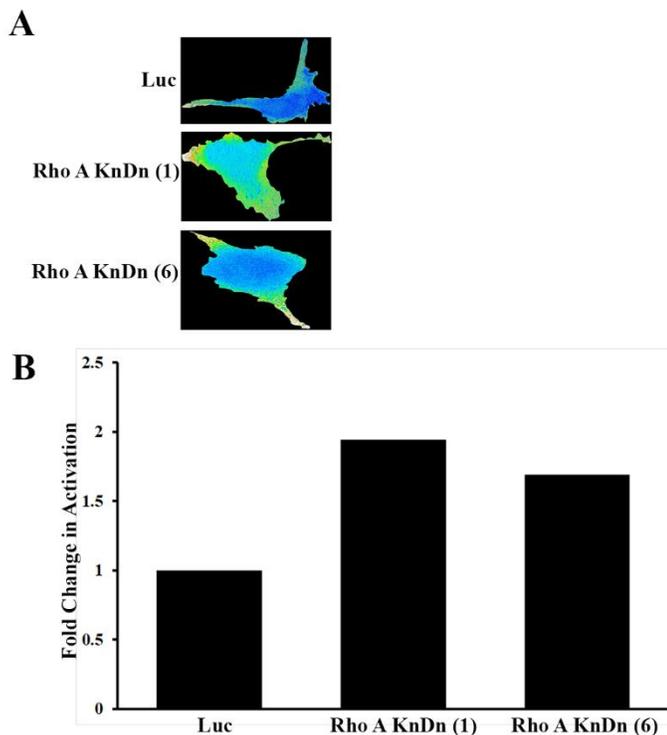


N=1

**Figure 19: RhoA and RhoC downregulate the expression of Cdc42.** A) Representative micrographs of (SF-268) cells with control, RhoA, RhoC, and double protein knock downs. (Order from Left to right). These cells were fixed and stained with anti-Cdc42 and rhodamine phalloidin. B) Quantitation of the fluorescence intensity of Cdc42 treatments with their corresponding knockdowns.

### 3.5.2 RhoA knockdown increases expression and activation of Cdc42 shown by FRET analysis

Next, we wanted to check the activation levels of Cdc42 as a response to RhoA depletion. So, we knocked down RhoA with the 2 different oligos 1 and 6 in SNB-19 cells, transfected with the Cdc42 biosensor, fixed, and imaged. Our results show that RhoA knockdown with oligo 1 and 6 led to an increase in Cdc42 activation by 94% and 69% respectively when compared to the control (Figure 20A and B).



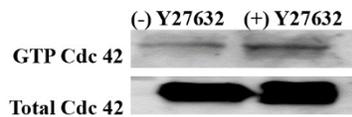
**Figure 20: RhoA negatively regulates the activation of Cdc42.** A) Representative micrographs of (SNB-19) cells that were transfected with si-luciferase (Upper image), siRhoA oligo 1 (middle image), and siRhoA oligo 6 (lower image), fixed, and transfected with the FRET Cdc42 biosensor. B) The cells

were imaged via using the FRET channel and the raw FRET images were normalized to the CFP images. The images reflect the FRET ratio = Raw FRET image/CFP.

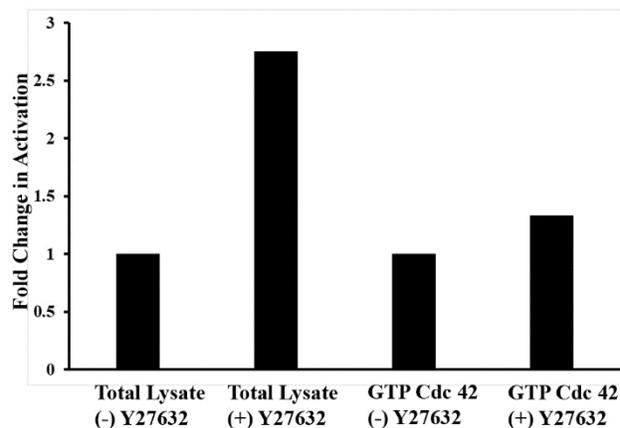
### 3.5.3 The inhibition of ROCK by Y27632 increases the activation of Cdc42

To validate our findings from the FRET activation data concerning RhoA downregulation of cdc42 activation, we treated SF-268 cells with Y27632, the pharmacological inhibitor of ROCK and measured the level of activation of RhoA by pull down assay using GST-PAK beads. Our results represent an increase in expression of Cdc42 by 175% and an increase in activation by approximately 33% (Figure 21A and B).

**A**



**B**



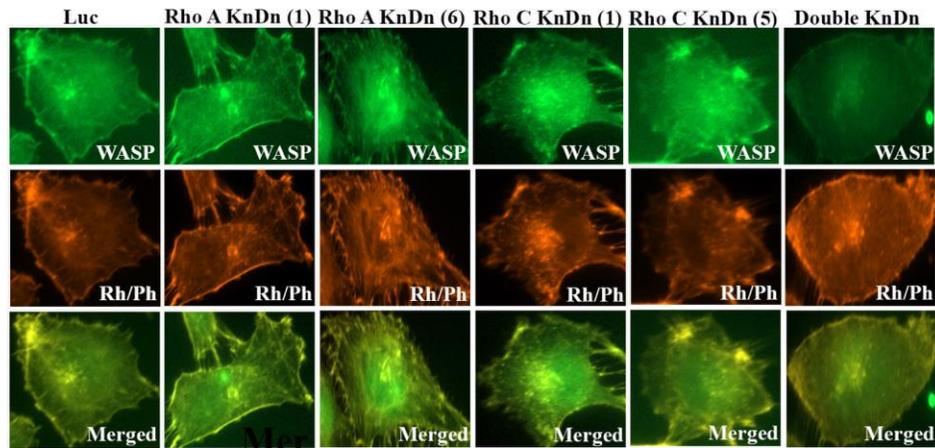
**Figure 21: RhoA negatively regulates the activation of cdc42.** A) Representative micrographs of (SNB19) cells untreated or treated with Y27632. The cells were lysed and incubated with GST-PAK beads to pull down active cdc42. GTP-cdc42 activation levels were isolated and detected by western blotting

using anti-cdc42 antibody (upper panel). Total protein lysates were also collected prior to the incubation and were used as a loading control (lower panel).B) Quantitation of active cdc42 using ImageJ and normalized to the corresponding total lysate band.

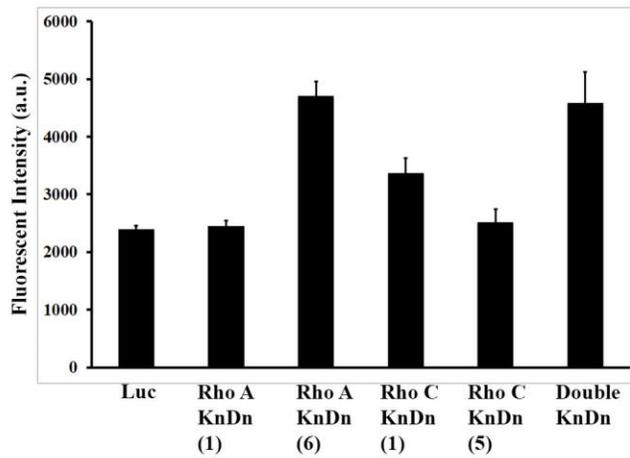
### **3.5.4 The silencing of RhoA and RhoC leads to increased expression of WASp, a cdc42 downstream effector**

As we did in the case of WAVE and Rac1, we wanted to see if the downregulation of RhoA and RhoC of cdc42 was extrapolated to its downstream effector WASp. To achieve our aim, we looked at the fluorescence intensity of WASp in both SF-268 and SNB-19 cells upon knocking down RhoA, RhoC, and both proteins followed by starving the cells in serum free media and stimulating with PMA for SNB-19. The cells were then fixed and stained with an anti-Wasp and rhodamine phalloidin for F-actin visualization. Our results reveal that the knockdown of RhoA alone or double knockdown of RhoA and RhoC increase the intensity of fluorescence by 96%, whereas the knockdown of RhoC increases intensity by around 10% in SF-268 (Figure 22 A and B). On the other hand, the knockdown of RhoA and double knockdown of RhoA and RhoC together lead to an increase of fluorescence intensity by 75% and 57% respectively. In contrast, RhoC knockdown did not have a significant effect on the fluorescence intensity of Wasp in SNB-19 (Figure 23A and B).

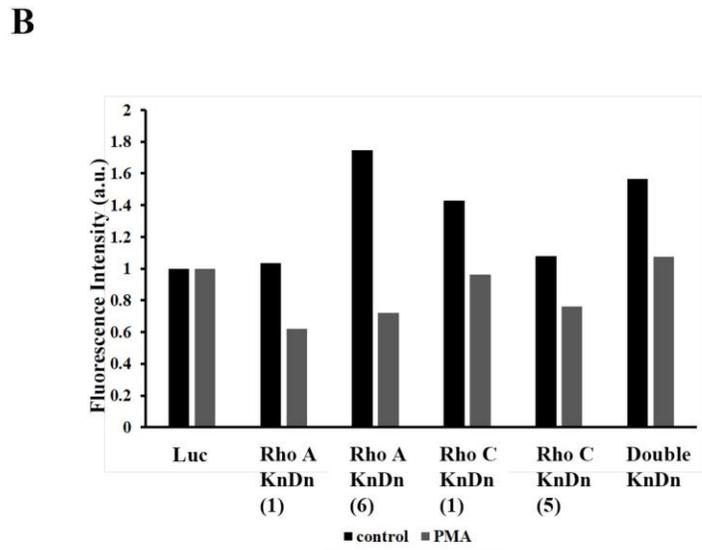
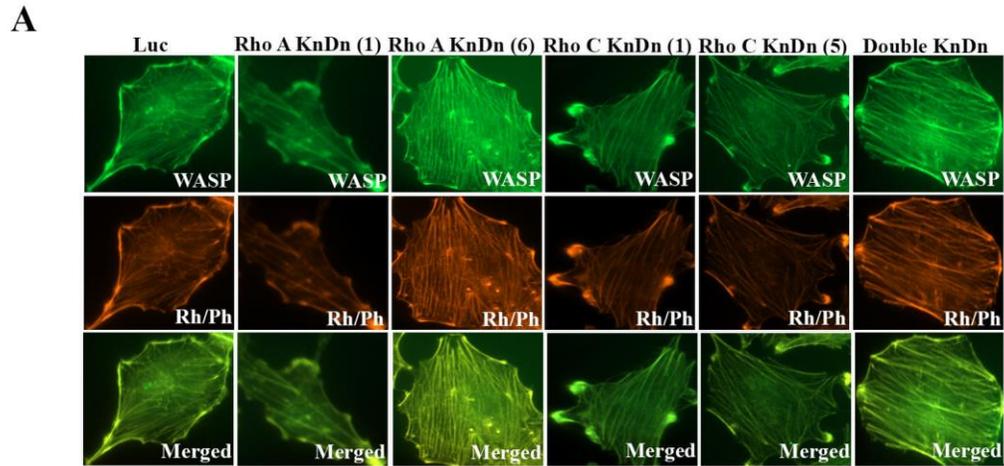
A



B



**Figure 22: RhoA and RhoC downregulate the expression of Wasp, cdc42 effector, in SF268.** A) Representative micrographs of (SF-268) cells with control, RhoA, RhoC, and double protein knock downs. (Order from Left to right). These cells were fixed and stained with anti-Wasp and rhodamine phalloidin. B) Quantitation of the fluorescence intensity of wasp with their corresponding knockdowns.



**Figure 23: RhoA and RhoC downregulate the expression of Wasp, cdc42 effector, in SNB-19.** A) Representative micrographs of (SNB-19) cells with control, RhoA, RhoC, and double protein knock downs. (Order from Left to right). These cells were fixed and stained with anti-Wasp and rhodamine phalloidin. B) Quantitation of the fluorescence intensity of wasp with their corresponding knockdowns.

## **Chapter 4**

### **Discussion**

The high infiltrative ability of malignant astrocytoma and glioblastoma imposes severe threats to brain cancer patients. The low average survival rate of patients, poor prognosis, and resistance to chemotherapy are all reasons that call forth for further investigations of molecular mechanisms and determinants regulating the highly invasive nature of these tumors. Unfortunately, the role of genes that promote invasion and motility in glioblastomas progression remain relatively understudied (Fortin Ensign, Mathews, Symons, Berens, & Tran, 2013). Rho GTPases were proposed to have a considerable contribution to glioma cell invasion given the overexpression of multiple members of the family and their roles in driving cell motility and invasion through actin reorganization (Kwiatkowska & Symons, 2013; Nakada et al., 2007; O'Connor & Chen, 2013). Indeed, the overexpression of RhoA and RhoC particularly have been widely implicated in various tumor types. For example, RhoA activation and upregulation were shown to improve invasiveness of ovarian cancer cells when bound to S100A4 protein (Horiuchi et al., 2008). Also, RhoC activation was shown to foster cervical squamous cell carcinoma progression through its downstream effector, ROCK (Chen, Cheng, Zhang, Li, & Geng,

2014). On that account, the aim of our study was to determine the differential effects of the 2 Rho isoforms, RhoA and RhoC, on motility and invasion in human astrocytoma SF-268 and glioblastoma SNB-19 cell lines.

First of all, we were interested in looking at the subcellular localization and activation levels of both RhoA and RhoC in glioblastoma. Our results showed that both RhoA and RhoC colocalize at invadopodia sites within the cells and at cell peripheries suggesting that they might participate in controlling motility and invasion in gliomas. This comes as no surprise knowing that the ability of Rho GTPases in cell migration regulation is mediated by the formation of specific actin-structures, such as lamellipodia, filopodia, and invadopodia (Parri & Chiarugi, 2010). Accordingly, cancer cells must form protruding invadopodia in order to invade the underlying ECM. In addition, these cancer cells must localize to cell edges to regulate the formation of protrusions at the leading edge for an active cell motility cycle to occur.

Next, we wanted to explore the effect of siRNA mediated transfection of RhoA alone, RhoC alone, or both on the two-dimensional migration of astrocytomas and glioblastomas. Our results revealed that the knockdown of RhoA and RhoC decreased cell speed in both cell lines SF268 and SNB19. This is in accordance with previous findings done on Human Cytomegalovirus (HCMV) Infected Glioblastoma stating that RhoA and RhoC positively regulate glioblastoma cell migration (Tseliou et al., 2016).

To validate our hypothesis that RhoA and RhoC are positive contributors in 2D motility of gliomas, we carried on with studying aspects of glioma cell adhesions after knocking down our proteins of interest both qualitatively, by collagen adhesion assay, and quantitatively, by measuring the numbers and areas of focal adhesions by vinculin staining

in both cell lines SF-268 and SNB-19. Our results showed that adhesion to collagen decreased in the 3 knockdowns in SF-268, whereas adhesion only decreased upon knockdown of RhoA in SNB-19. This supports the necessity of RhoA for the formation of focal adhesions. Moreover, vinculin immunostaining results show the reduction in focal adhesion numbers upon RhoA knockdown. On the other hand, RhoC knockdown leads to a decrease in focal adhesions, but with the prominence of punctate focal contacts at the cell periphery increasing instead. It is well-established now that focal contacts that delineate early adhesion and initiation of the motility cycle are Rac1-activation dependent and concomitant with the suppression of RhoA. In contrast, maturation of focal adhesions is dependent on RhoA activity and Rac1 inhibition (El-Sibai et al., 2008; Lawson & Burridge, 2014). This validated our results with the knockdown of RhoA by which mature focal adhesions were much reduced. In addition, a study conducted with the knockdown of RhoC in prostate cancer cell lines yielded the same result as ours suggesting a role for RhoC, collaborating with RhoA, in changing the localization and expression of focal adhesion-associated proteins. Also, the formation of focal complexes while knocking down RhoC might depict a switch from mature focal adhesions to nascent focal contacts by upregulating either Rac1 or Cdc42.

Due to the importance of membrane ruffling and protrusion formation, we also assessed the prevalence of membrane ruffling upon knocking down our proteins of interest. Our results showed prominent decrease in ruffling and added more reliability to our previous findings.

Since membrane ruffling is reliant on the actin polymerization, we were then interested in looking at arp2 and arp3 levels of fluorescence intensity, somehow reflecting

expression, upon knocking down the proteins, stimulating with PMA, and staining with the respective antibodies. The results were variant and did not correspond well to our previous results probably because the intensity was measured for the entire cell surface area and not the areas of membrane ruffles.

After establishing the positive regulation of RhoA and RhoC on various features of 2D motility, we wanted to gain more insight on the regulation of 3D invasion by the two proteins. Therefore, we started off with performing in-vitro collagen-based invasion assays on both cell lines SF-268 and SNB-19 upon once again in parallel with knocking down the proteins of interest. Our results indicate that invasion either increased upon the knockdowns of RhoA and RhoC, in the case of SF-268, or decreased, in the case of SNB-19. Previous studies investigating the roles of RhoA and RhoC on 3D invasion in prostate cancer cell line PC3 suggested that the depletion of RhoA led to increased invasiveness; whereas in the case of RhoC depletion, invasion was reduced (Vega et al., 2011). Therefore, a more specific approach was needed to verify if the same pattern occurs in our glioma cell lines. Next, we resorted to staining for cortactin and TKS4, both of which are considered to be molecular markers for invadopodia formation coupled with F-actin staining to screen for invadopodia upon the knockdown of RhoA and RhoC. Our results show approximately a double fold increase in the number of invadopodia upon knockdown of RhoA. Conversely, RhoC knockdown showed a significant decrease in the formation of invadopodia. These results correlate with the findings of the Ridley group (Vega et al., 2011). In addition, John Condeelis and his group proposed a model in breast adenocarcinoma cells MTLn3 highlighting the roles of RhoA and RhoC in invadopodia initiation and maturation. They revealed that RhoA activity was low at invadopodia core

precursors and that RhoA drives invadopodia maturation, and not precursor formation, by delivering MT-MMP1 for ECM degradation. According to their model, RhoC silencing results in inefficient tumor invasion with shorter invadopodia morphology (Beatty & Condeelis, 2014). In this context, our results reveal for the first time in glioma cell lines that RhoC drives invadopodia assembly and formation and invadopodia are RhoC dependent; whereas RhoA negatively regulates invadopodia formation and invadopodia are RhoA independent.

Our last aim for the study was to investigate cross-talks between RhoA, RhoC, and other important RhoGTPases that also drive cancer cell motility and invasion: Rac1 and Cdc42 to possibly further explain our previous results. First, we inhibited common downstream effectors of RhoA and RhoC, ROCK1 and ROCK2, by treating SNB-19 cells with Y27632 and looked for the activation of Rac1. Our results show that Y27632-treated cells have increased activation of Rac1 suggesting that the RhoA/RhoC/ROCK pathway downregulates Rac1. To validate this result, we then transfected our proteins of interest in SNB-19 cells, treated with Y27632 or PMA and stained for WAVE, a downstream effector of Rac1 involved in actin polymerization (Fortin Ensign et al., 2013), with F-actin in order to look at the fluorescence intensity of WAVE. Indeed, we noticed an increase of WAVE expression upon the knockdown of both RhoA and RhoC. The collective of these data further confirm our previous result concerning RhoC knockdown on the formation of focal complexes in that RhoC depletion may have upregulated Rac1 and hence focal contacts were seen instead of mature focal adhesions. Next, we wanted to verify the effect of RhoA and RhoC knockdowns in SNB-19 on Cdc42 expression, reflected by fluorescence intensity while staining for Cdc42 with F-actin. The results show an increase in Cdc42 expression upon knocking down RhoA, RhoC, or both. In addition, we notice

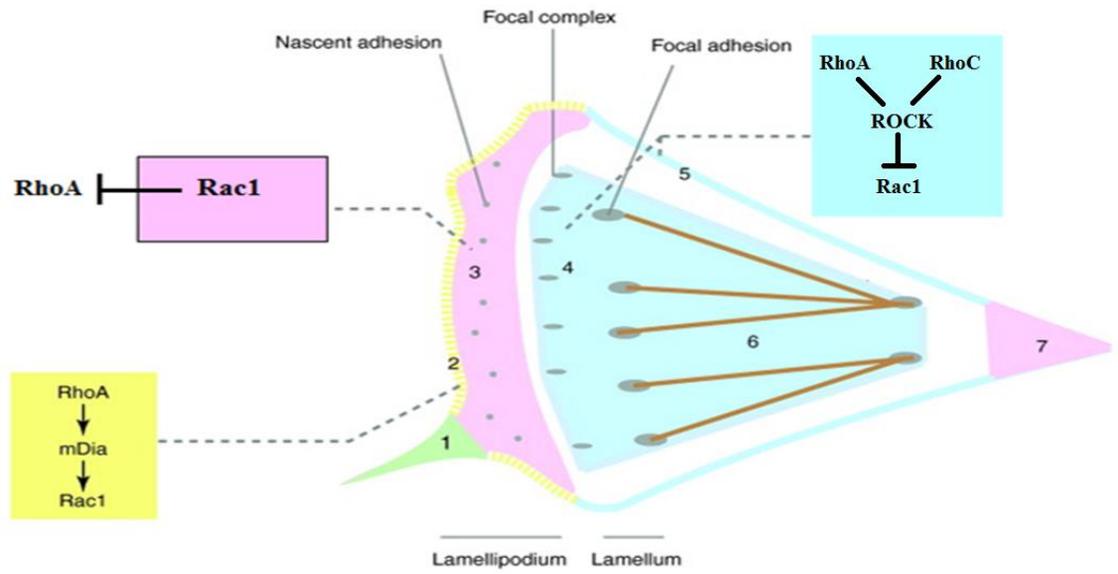
the prominence of invadopodia structures with cdc42 and F-actin colocalization in the case of knocking down RhoA RhoC, or both RhoA and RhoC. Consequently, we expected that the increase in invadopodia formation while knocking down RhoA was due to the upregulation of Cdc42 and that invadopodia formation was also Cdc42 dependent. Then, we wanted to make sure whether knockdown of RhoA led to also to the activation of Cdc42 and not only to its increased expression by FRET analysis. FRET results showed an increase in the activation of Cdc42 with RhoA depletion. This was also supported by the pull down experiment results by which ROCK inhibition led to increased activation of Cdc42. Lastly, we looked at the expression of WASp, a downstream effector of Cdc42 (Fortin Ensign et al., 2013), by measuring fluorescence intensity when staining with F-actin. Unsurprisingly, we saw an increase in WASp fluorescence in all knockdown cases further supporting our results that RhoA and RhoC downregulate Cdc42 in addition to Rac1. A current study in our lab investigates the effect of Cdc42 on motility and invasion in SNB-19 and has revealed that invadopodia are Cdc42 dependent adding support to our hypothesis. In addition, FRET studies performed on rat glioblastoma cells that were invading the brain parenchyma revealed high activation levels of Rac1 and Cdc42, whereas the activation of RhoA activity was higher in the perivascular region having reduced activities of Rac1 and Cdc42 (Fortin Ensign et al., 2013; Hirata et al., 2012).

## **Chapter 5**

### **Conclusions**

In the current study, the distinction between RhoA and RhoC's impacts on cell motility and invasion was our primary goal. We found that both RhoA and RhoC are indispensable for migration, adhesion and the formation of membrane ruffling preceding lamellipodial formation. Indeed, the data fit perfectly together knowing that RhoA is the driver of maturation of focal adhesions and actomyosin contractility, both features being essential for executing a proper and a spatiotemporally regulated migration cycle. Our data adds that RhoC assists this 2D migration by downregulating Rac1, an antagonist for Rho's role in adhesion, that otherwise remains stuck in the stage of focal contacts without proceeding to complete focal adhesion maturity. In addition, we suggest that RhoC constitutes one of the main forces behind invasion and invadopodia formation in glioma cells, whereas RhoA negatively regulates invasion and invadopodia assembly. Thus, we suggest that RhoA and RhoC's collaborative pattern in cell migration and adhesion shifts to an antagonistic relationship upon the formation of 3D protrusive structures. To the best

of our knowledge, this is the first time anyone established this finding in glioma invasion patterns. It also appears that RhoA antagonizes other 2 Rho GTPases, Rac1 and cdc42 which raise further questions of the feedback loops connecting all four Rho GTPases.



**Figure 24: Model for the antagonistic relationship between Rac1 and RhoA/RhoC in 2D motility in astrocytomas.**

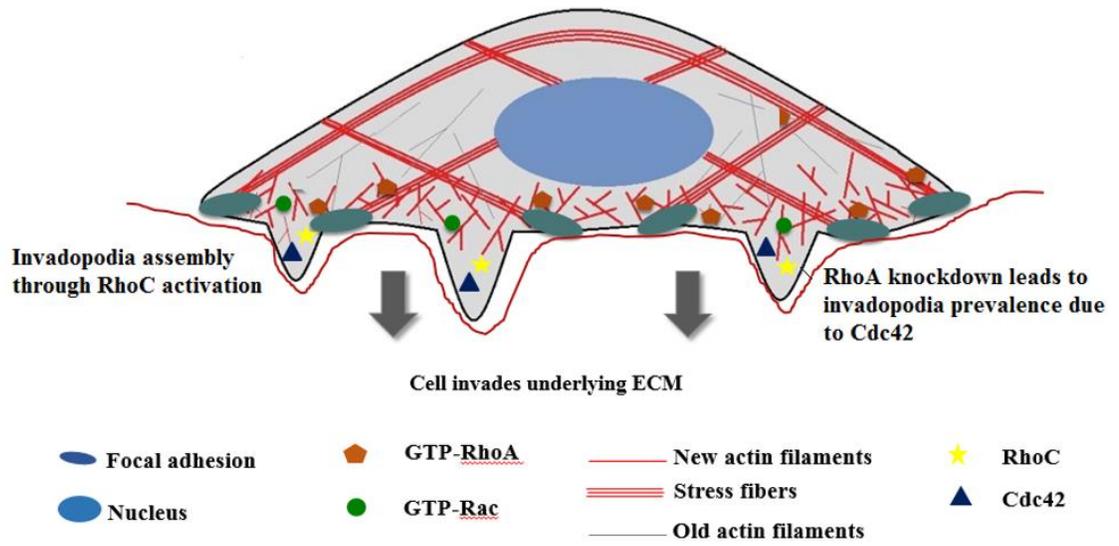


Figure 25: Model for Rho GTPases regulation of 3D invasion of astrocytomas.

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