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Palladin Regulates Invasion in Glioblastoma Multiforme Through Rho GTPases

By

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Palladin Regulates Invasion in Glioblastoma Multiforme Through Rho GTPases

Mohammad Farran

ABSTRACT

Glioblastoma multiforme (GBM), is one of the most fatal types of human cancers. GBM tumors exhibit significant heterogeneity and can arise through two distinct routes, forming what is known as primary glioblastoma and secondary glioblastoma. Palladin is a fairly recent protein that plays an important role in regulating actin and has been implicated in numerous cellular processes like adhesion, motility, invasion, and metastasis. Changes in palladin expression have also been observed in invasive tumors like breast and pancreatic cancer. In this study, we showed that palladin regulates the invasion of glioblastoma cells by affecting invadopodia formation. We also highlighted cross talks between palladin and Rho GTPases like Cdc42 and Rac1 that are also implicated in GBM invasion. This paper offers interesting insights into the role of palladin and Rho GTPases in the complex networks that drive the formation of invadopodia in glioblastoma multiforme, leading to invasion and subsequent metastasis.

Keywords: Glioblastoma, Palladin, Cell invasion, Invadopodia, Rho GTPases, Cdc42, RhoC, Rac1

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LIST OF ABBREVIATIONS

ATCC: American Type Culture Collection

Arp2/3: Actin-related protein 2/3

BSA: Bovine serum albumin

CAFs: Cancer-Associated Fibroblasts

cAMP: cyclic Adenosine MonoPhosphate

Cdc42: Cell division control protein 42 homolog

CFP: Cyan fluorescent protein

CNS: Central Nervous System

DMEM: Dulbecco's Modified Eagle Medium

EGF: Epidermal Growth Factor

ECL: Enhanced Chemiluminescence

ECM: Extra-cellular matrix

EMT: Epithelial to Mesenchymal Transition

FAK: Focal Adhesion Kinase

FBS: Fetal bovine serum

FMNL-3: Formin-like protein 3

FRET: Fluorescence resonance energy transfer

GAP: GTPase-activating protein

GDI: Guanine nucleotide dissociation inhibitor

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GBM: Glioblastoma multiforme

GTP: Guanosine triposphate

GTPase: Guanosine triphosphatase

HRP: Horse Radish Peroxidase

IDH1: Isocitrate Dehydrogenase 1

LPA: Lysophosphatidic acid

mDia: Mammalian homolog of diaphanous

Mena: Mammalian-enabled protein

MMP: Matrix metalloproteinase

PallD: Palladin

PAK: p21 activated kinases

PDGF: Platelet Derived Growth Factor

PFA: Paraformaldehyde

PI(3,4)P2: Phosphatidylinositol 3,4-bisphosphate

PMA: Phorbol 12-myristate 13-acetate

PVDF: Polyvinylidene fluoride

Rac: Ras-related C3 botulinum toxin substrate

Ras: Rat sarcoma

Rho: Ras homologous

RhoA: Ras homologous member A

RhoB: Ras homologous member B

RhoC: Ras homologous member C

ROCK: Rho-associated coiled-coil kinase

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS: Sodium Dodecyl Sulphate

SHIP2: SH2 domain containing Inositol 5-phosphatase 2

siRNA: Small interfering Ribonucleic Acid

STARD13: Steroidogenic acute regulatory (StAR)-related lipid transfer domain containing 13 protein

TKS4: SH3 and PX domains 2B

TKS5: SH3 and PX domains 2A

WASP: Wiskott-Aldrich Syndrome Protein

WAVE: WASP-Family Verprolin-Homologous

YFP: Yellow Fluorescent Protein

Chapter 1

Literature Review

1.1 Cancer

1.1.1 Cancer overview

Cancer might be the most infamous disease of the 21st century. Cancer incidents have been increasing remarkably with time due to our ever-changing lifestyles and climate. Till present day, cancer is the second leading cause of death worldwide with 1,762,450 estimated new cases in 2019 in the USA alone. (Siegel et al., 2019) But what is this disease and what makes it this deadly?

Cancer, briefly, is uncontrolled cell division and proliferation. It has the potential to develop in almost any organ or structure in the body and fails to stop growing. Cancer cells also have the ability to metastasize from one location to another furthermore making it more dangerous and harder to cure. (Ps & Bj, 2016) The most important aspect of cancer is that it can overcome the body's immune response generated against it and that's why immunocompromised people are at higher risk of developing this disease. (Ps & Bj, 2016)

1.1.2 Development and Classification of Cancer

Our normal body cells regenerate through a tightly controlled cycle called cell division. Cancer arises when cells bypass the regulated cell division leading to uncontrolled cell growth and multiplication eventually forming a lump of tissue, called a tumor. (*What Is Cancer?*, 2007) Note that not all tumors are cancerous though. The

currently accepted distinction between non-cancerous, or benign, tumors and cancerous, or malignant, tumors is that malignant tumors have the ability to spread across the body and form new secondary tumors through a process called metastasis. Benign tumors on the other hand are not metastatic and even cannot regrow upon removal. (*What Is Cancer?*, 2007)

Cancer, essentially, is a genetic disease and different groups of genes play a role in carcinogenesis. First, there are the proto-oncogenes that are key regulators of normal cell division; mutations that affect their function will increase cell growth and multiplication which is a critical step in the development of cancer. (Kontomanolis et al., 2020) Tumor-suppressor genes come in second. These genes, when active, prevent cancer cells from proliferating and thus protect the tissue from abnormal growth, or neoplasia. Similar to proto-oncogenes, when tumor-suppressor genes acquire mutations that affect their activity, they release the suppression on uncontrolled cell division leading to cancer. (Kontomanolis et al., 2020) DNA-repair genes also play a role in carcinogenesis. The role of these genes is to repair DNA damage across our genome which makes them key elements in cancer development since cancer cells are known to exhibit significant chromosomal mutations and aberrations. (Ronen & Glickman, 2001)

Tissues undergo numerous non-cancerous changes before they develop into full-blown cancer. A tissue can develop hyperplasia when its cells divide more than usual causing a build-up, but the normal cell morphology and tissue organization are maintained. (*Evolution of a Cancer*, n.d.) Tissues can also acquire a more serious transformation called dysplasia. In addition to the cellular build-up that is seen in hyperplasia, in dysplasia, the cell morphology and tissues organization also appear to be abnormal. (*What Is Cancer?*, 2007) After dysplasia, tissues could develop into a

carcinoma *in situ* which is arguably considered a cancer but the abnormal cells are still constricted within their layer of origin and have not yet invaded the underlying tissue. (*Evolution of a Cancer*, n.d.; *What Is Cancer?*, 2007)

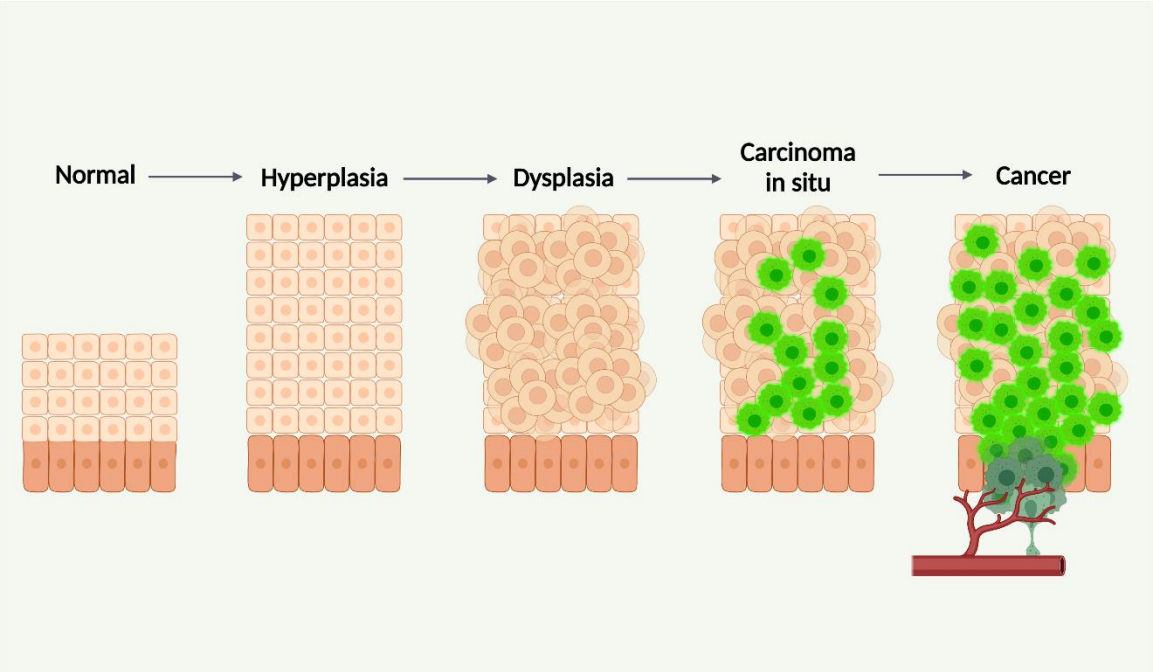

Figure 1. Stages of Cancer Development. (Created with BioRender.com)

1.1.3 Hallmarks of Cancer

In an attempt to describe cancer as a collection of characteristics, Hanahan *et al* developed the famous six hallmarks of cancer that promote the progression of cells from normal to neoplastic states and that are namely: "Sustaining Proliferative Signaling, Evading Growth Suppressors, Resisting Cell Death, Enabling Replicative Immortality, Inducing Angiogenesis, and Activating Invasion and Metastasis." (Hanahan & Weinberg, 2000)

In the following decade, the field of cancer research witnessed significant progress in cancer characterization and conceptualization. This drove Hanahan *et al* to refine their original list of the six hallmarks of cancer by adding two additional global hallmarks

which are: Energy Metabolism Reprogramming and the Evasion of Immune Destruction. (Hanahan & Weinberg, 2011)

Given the complexity and heterogeneity of cancer, the "Hallmarks of Cancer" offer a valuable reference through which we can generalize the definition of cancer to help us better understand its development and to fine-tune the way we therapeutically target it.

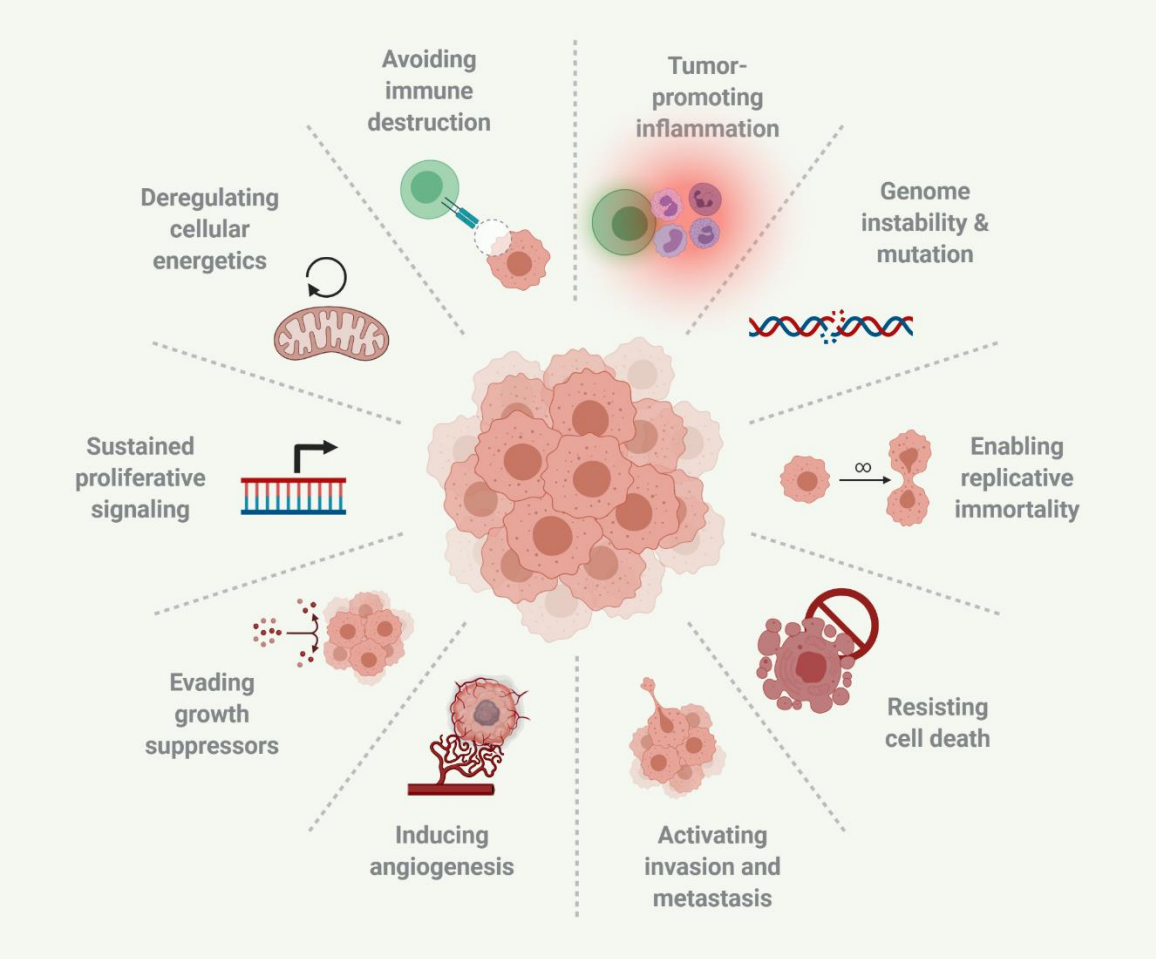

Figure 2. The Hallmarks of Cancer. (Created with BioRender.com)

1.2 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most frequent tumor of the central nervous system and the most malignant. First recorded back in 1800, it is now graded by the World Health Organization as a grade IV CNS tumor and described as one of the

most fatal cancers as it has a very poor prognosis and a bad overall survival rate. (Stoyanov et al., 2018; Tykocki & Eltayeb, 2018)

After extensive research concerning Glioblastoma, the "multiforme" aspect of it became very clear as GBM tumors exhibit significant heterogeneity involving diverse unique subclones, each with its own genetic and epigenetic profile that can affect the overall survival of the patient and how the tumor responds to therapy. (Stoyanov et al., 2018)

Not only that, but it has also been shown that GBM can arise through two distinct routes, forming what is known as primary glioblastoma and secondary glioblastoma. Primary glioblastoma is the most common type of GBM, and it arises when a specific triggering mutation affects a neural stem cell precursor forming a GBM stem cell that gives rise and sustains a GBM tumor without a prior established neoplasia. (Ohgaki & Kleihues, 2013) Secondary glioblastoma, on the other hand, forms as a series of aggregated mutations affecting initial less malignant astrocytomas, leading to the formation of GBM tumors with distinctive isocitrate dehydrogenase 1 (IDH1) mutations. Genetic screening for these IDH1 mutations allows the early distinction between primary and secondary glioblastomas, which is important since these two types of glioblastomas differ in multiple aspects like localization, the extent of necrosis, patient age group, and prognosis. (Ohgaki & Kleihues, 2013)

Due to its unique tissue composition and the presence of the blood-brain barrier, the brain offers a unique favorable microenvironment for glioblastoma which as a result rarely metastasizes out of the brain. Not only that, but this microenvironment encourages

other tumors in the body to metastasize into the brain, mainly breast and lung cancers, and melanoma. (Lah et al., 2020)

The standard of care for glioblastoma multiforme is surgical excision of the tumor followed by chemoradiotherapy; this approach, however, is proving futile in significantly improving the progression-free and overall survival of patients. Thus, scientists are shifting their focus to the field of targeted therapeutics whereby therapy is stratified according to each tumor's unique molecular biomarkers and genetic/epigenetic profile, as well as targeting the tumor microenvironment. (Le Rhun et al., 2019)

1.3 Cell invasion

Cancer cell invasion is a key process in metastasis as it allows cancer cells to penetrate neighboring tissue and even migrate to the blood (in a process called intravasation) where they circulate and can act as seeds to form secondary tumors at distant sites in the body. (Gerashchenko et al., 2019) This process is of the utmost importance to scientists and is a significant therapeutic target that, if inhibited, can decrease the severity of cancer and lead to a better prognosis. In the case of Glioblastoma, the tumor rarely ever metastasizes outside of the brain but greatly invades and damages the local tissue. This happens when glioblastoma cells start disseminating from the tumor into the perivascular spaces that surround the vasculature of the brain to sustain tumor growth. This process is called "Vessel Co-option" whereby the tumor cells utilize pre-existing blood vessels instead of forming new ones. In some rare cases, the tumor cells can breach the blood-brain barrier and reach circulation to metastasize. (Guyon et al., 2021)

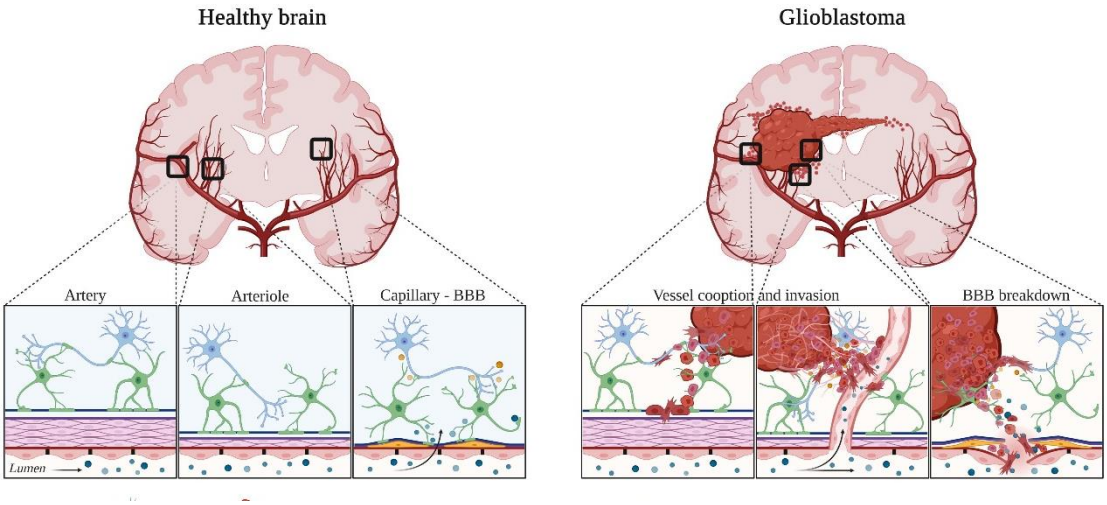

Figure 3. Glioblastoma Invasion. (Source: Guyon, 2021)

1.3.1 Invadopodia

Cancer cells are dynamic by nature which allows them to form a wide array of cellular protrusions that facilitate their migration and invasion; these structures are namely filopodia, lamellipodia, podosomes, and finally invadopodia, which are the focus of this paper. (Alblazi & Siar, 2015) While lamellipodia and filopodia are structural protrusions that aid in commanding the directionality of migrating cells, podosomes and invadopodia play a more catalytic role in cell invasion. Still, there are many differences between podosomes and invadopodia, which are mainly that podosomes can be present in normal cells and are short-lived and non-protrusive while invadopodia are long-lived protrusive structures that are exclusive to migrating cancer cells. (Eddy et al., 2017)

As mentioned before, invadopodia are long-lived stable protrusions that can last hours with a length of up to 2µm and a diameter ranging from 0.05 to 0.8µm. Composition wise, the most important feature of invadopodia is an F-actin rich core and a matrix-degrading ability that proteolyzes the ECM. (Alblazi & Siar, 2015) There is so much more

that goes into the structure and function of invadopodia, these include molecules like: actin regulatory proteins such as WASP and Arp2/3 along with upstream regulators like Cdc42 and cofilin, protein adhesion molecules like β1 integrins, membrane signaling proteins like focal adhesion kinase, and matrix degradation enzymes such as MMPs. (Augoff et al., 2020) Relevant to this paper are also TKS4 and TKS5 which are scaffolding proteins that are substrates of the SRC tyrosine kinase and are necessary for invadopodia formation and function. Both TKS proteins are key elements in mediating metastasis and can serve as markers for invadopodia in cancer cells. (Kudlik et al., 2020)

1.3.2 Invadopodia assembly

Invadopodia assemble in a series of three discrete yet complex stages which are: precursor core initiation, core stabilization, and invadopodia maturation. Invadopodia formation can be initiated by many events, such as stimulation from growth factors and signals from the ECM. (Eddy et al., 2017)

The initiation process involves the recruitment of WASP and cofilin to an actin-cortactin complex which drives actin polymerization and invadopodia protrusion. The activation of an Arp2/3 complex is also necessary for actin nucleation during the initiation phase. (Augoff et al., 2020) This leads to the formation of a precursor core that is however still unstable and thus needs stabilization. During the stabilization phase, TKS5 anchors the precursor core to the plasma membrane by binding to PI(3,4)P2 while Lamellipodin recruits a Mena-Arg-SHIP2 complex that produces more PI(3,4)P2 for TKS5 to bind to. This allows TKS5 to anchor the precursor core more steadily to the plasma membrane, thereby stabilizing it. (Eddy et al., 2017) At last, invadopodia maturation happens when

the stable core drives actin polymerization allowing the protrusion to elongate more. Mature invadopodia can now recruit proteolytic proteins like MMPs which lead to ECM degradation and subsequent cancer cell invasion and metastasis. (Augoff et al., 2020)

1.4 The Rho Family of small GTPases

1.4.1 Overview

The family of Rho GTPases encompasses 20 low molecular weight proteins that can be classified into 8 subfamilies, and which are part of the Ras superfamily of proteins. Rho GTPases are highly conserved among all eukaryotic cells and, by far, Rho, Rac, and Cdc42 are the best studied proteins out of the Rho GTPases family. (Hall, 2012)

The Rho family of GTPases have been shown to be key components of a wide variety of cellular processes like cytoskeletal rearrangement, cell adhesion and spreading, cell migration, morphogenesis, cell cycle, gene expression… among many others. Accordingly, abnormal changes in Rho GTPase function are implicated in diseases like cancer and inflammation. (Hodge & Ridley, 2016)

Rho GTPases can also be classified into two categories, typical and atypical, depending on their mode of regulation and activity. Typical Rho GTPases like Rho, RhoF/RhoD, Cdc42, and Rac function as molecular switches as they alternate between a GDP-bound inactive form and a GTP-bound active form. Atypical Rho GTPases, like RhoBTB, are mostly bound to GTP and thus their activity is regulated by mechanisms other than molecular switching like post-translational modifications. (Haga & Ridley, 2016)

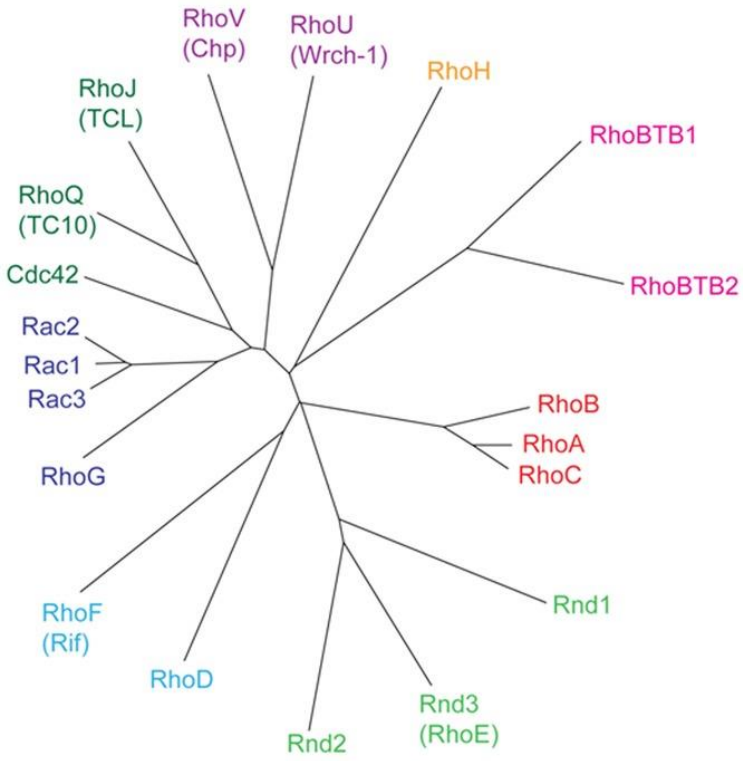

Figure 4. Human Rho Family of GTPases. (Source: Ridley, 2013)

1.4.2 Rho GTPases as molecular switches

As mentioned earlier, most Rho GTPases function as molecular switches alternating between active and inactive states by binding GTP and GDP, respectively. This switching is mediated by 3 groups of regulators which are: GTPase-activating proteins (GAPs), guanine exchange factors (GEFs), and guanine dissociation inhibitors (GDIs). (Hodge & Ridley, 2016)

The GEFs are considered the activators of typical Rho GTPases and they work by facilitating the release of the GDP molecule from the inactive GDP-bound GTPase and replacing it with a GTP molecule, thus shifting the GTPase into an active GTP-bound form. On the other hand, GAPs function as Rho GTPase inhibitors. GAPs work by catalyzing the hydrolysis of the GTP molecule in the active GTP-bound GTPases by

activating their intrinsic GTPase activity and shifting them into inactive GDP-bound states. GDIs also control the molecular switching by binding the inactive GDP-bound Rho GTPases, sequestering them in the cytosol, and preventing them from reaching the plasma membrane which is the site of their activation by GEFs and where they perform their function. (Haga & Ridley, 2016)

Note that the Rho GTPase GAPs, GEFs, and GDIs are themselves also regulated by multiple mechanisms which further highlights the complexity of the Rho GTPase signaling network. This also justifies how often this network is altered and mutated in diseases like cancer that rely on the multiple processes that Rho GTPases are involved in to facilitate invasion and metastasis. (Hodge & Ridley, 2016)

1.4.3 Upstream signaling of Rho GTPases

Rho GTPases have been shown to be activated by numerous extracellular stimuli that affect the receptors present on the cell's plasma membrane. Mainly, these extracellular stimuli regulate Rho GTPase activity inside the cells through 3 main types of receptors that are: G-protein coupled receptors (GPCRs), Receptor Tyrosine Kinases (RTKs), and Integrins. (Hanna & El-Sibai, 2013; Kjøller & Hall, 1999)

GPCRs were the first to be identified as receptors involved in Rho GTPase activation. Lysophosphatidic acid (LPA) is a ligand that when bound to GPCRs, activates them, and subsequently leads to the activation of Rho through the Gα subunit-mediated activation of Rho GEF. Rac and Cdc42 have also been shown to be activated by GPCRs through receptor agonists like Bombesin and Bradykinin, respectively. (Kjøller & Hall, 1999)

Receptor Tyrosine Kinases also play a very important role in the activation of Rho GTPases, mainly through the PI3K pathway. Activation of RTKs by growth factors like EGF and PDGF leads to an activation cascade whereby PI3K activates Rho and Rac through the activation of their GEFs. Note that the mentioned PI3K-mediated activation of the GEFs is less of a direct catalytic activation and more of a regulation of the intracellular localization of the GEFs through their PH domain. (Hanna & El-Sibai, 2013)

Rho GTPase activity can also be regulated by integrin-mediated cell-ECM interaction mainly through the activation of a downstream effector called the focal adhesion kinase (FAK). FAK activates Rac by activating proteins that form complexes between Rac and its GEFs, and inhibits RhoA by activating a RhoA GAP. (Hanna & El-Sibai, 2013; Kjøller & Hall, 1999)

1.4.4 Downstream effectors of Rho GTPases

There are more than 100 downstream targets for Rho GTPases most of which are kinases and adaptor and scaffolding proteins. Rho GTPases can bind up to 30 proteins which highlights how each Rho GTPase is involved in many different signaling pathways and not just a single one. (Kjøller & Hall, 1999)

Rho GTPases are involved in a wide array of functions among which is, most importantly, the regulation of actin polymerization. After a series of independent experiments, it became clear how each Rho GTPase performs a specific function that involves actin polymerization through their downstream effectors. (Bishop & Hall, 2000) Mainly, Rho is responsible for actomyosin formation and contractility, formation of stress fibers, and focal adhesions through the action of its downstream effector ROCK. It's also

involved in cytoskeletal remodeling through the ezrin–radixin–moesin proteins. Moreover, Rac and Cdc42 are responsible for lamellipodia and filopodia formation, respectively, through a family of scaffolding proteins called WASP/SCAR/WAVE and the downstream effector PAK. (Hanna & El-Sibai, 2013)

Rho GTPases also play a big role in cell motility. As mentioned earlier, Rac and Cdc42 are responsible for lamellipodia and filopodia formation through N-WASP and Arp2/3 respectively. These structures aid in cell polarity, cell directionality, chemotaxis, and subsequent cell motility. (Hanna & El-Sibai, 2013)

Just like Rho GTPases can be activated through adhesions to the ECM, this activation in turn plays a downstream role in adhesion formation. Active Rac activates PAK which in turn forms focal complexes at the cell's leading lamellipodium; these focal complexes mature into focal adhesions which strengthen the cell-ECM adhesion and gives the cells contractility. (Hanna & El-Sibai, 2013)

It is important to highlight again here that Rho GTPases do not function in discreet separate downstream pathways and there is a lot of crosstalk that happens between the different Rho GTPases. Rho GTPases can share the same downstream effector molecules and can either function synergistically or antagonize each other just like we see with Rho and Rac whereby whenever one of them is active, it inactivates the other. (Guilluy et al., 2011)

1.4.5 Rho GTPases in cancer cell invasion and metastasis

For the longest time, Rho GTPases were thought not to be mutated in human cancers but now, increasing evidence is proving otherwise. Constitutively active Rho

GTPases can transform normal fibroblasts; Not only that, but the Rho GTPases Rho A, Rac1, and Cdc42 are either mutated and/or overexpressed in numerous different types of human tumors like melanomas, breast cancer, gastric cancer, liver cancer… etc. and this disruption plays an important role in cancer invasion and metastasis. (Haga & Ridley, 2016; Sahai & Marshall, 2002)

RhoA controls cell migration and the actin cytoskeleton through the action of ROCK1 and ROCK2 proteins. The depletion of RhoA in breast and prostate cancer cells made the cells more elongated, increased lamellipodia formation, and enhanced the invasiveness of the cells. (Ridley, 2013)

RhoC promotes the formation of invadopodia in cancer cells, which are proteolytic actin rich protrusions that degrade the ECM. Accordingly, RhoC was proven to increase the invasiveness of prostate and breast cancer cells through a specific downstream effector called Formin-like Protein 3 (FMNL3). (Haga & Ridley, 2016; Ridley, 2013)

RhoB increases cell adhesion to the ECM through β1 focal adhesions, subsequently preventing cell migration. This explains why RhoB tends to be downregulated in cancer cells and how its overexpression decreases cancer cell migration and invasion. (Haga & Ridley, 2016; Ridley, 2013)

Rac1 tends to be overexpressed in multiple tumor types like colorectal cancer, breast cancer, and lung cancer. This is of particular importance since active Rac1 can transform normal epithelial cells and activate the epithelial-to-mesenchymal transition (EMT) which is a key process in cancer invasion and metastasis. (Haga & Ridley, 2016)

Cdc42 has an important role in increasing cancer cell invasion in two ways: increasing the formation of invadopodia and increasing the activation and production of MMPs. Cdc42 activation also plays a role in EMT, amoeboid migration, and collective cancer cell migration behind fibroblasts. (Stengel & Zheng, 2011)

1.5 Rho GTPases in glioblastoma

The Rho protein has multiple isofroms including RhoA, RhoB, RhoC, and RhoG, each of which is regulated differently in glioblastoma and works through different effectors. In accord with the previously mentioned roles of each isoform, RhoA and RhoB expression decreases while RhoC tends to be over overexpressed in the high-grade glioblastoma. (Al-Koussa et al., 2020)

ROCK and mDia are key effector proteins downstream of RhoA. The knockdown of mDia and ROCK decreases glioblastoma cell migration and motility. The inhibition of FAK, another RhoA effector, inhibits glioma cell migration. (Al-Koussa et al., 2020; Kwiatkowska & Symons, 2020) Since active RhoB increases β1 integrins that interact with the ECM, the depletion of RhoB in glioblastoma cells increases invasion and motility. (Baldwin et al., 2008) Another study on glioblastoma cells showed that the knockdown of RhoC significantly increased cell migration, leading to a migration rate that is faster than what is seen with RhoA and RhoB knockdowns. (Tseliou et al., 2016)

Rac1 is overexpressed at the mRNA and protein levels and shows increased activation in high-grade glioblastoma tumors. (Kwiatkowska & Symons, 2020) The depletion of Rac1 and Rac3 leads to a significant decrease in glioblastoma cell invasion, but only Rac1 knockdown inhibits cell motility too. (Chan et al., 2005, p. 3) RhoG is a

Rho GTPase that belongs to the Rac subfamily and is responsible for cancer cell invasion and migration. In glioblastoma cells, RhoG increases invadopodia formation and its knockdown inhibits glioma cell invasion. (Kwiatkowska et al., 2012)

Cdc42 plays similar roles to Rac1 in GBM in terms of invasion and migration due to shared domains, GEFs, and subcellular localization. (Al-Koussa et al., 2020) The overexpression of Cdc42 increases the invasiveness of glioblastoma cells in vivo. Cdc42 also plays a role in a clinical radiotherapy complication in GBM termed "X-ray promoted invasion" whereby the radiation conversely increases the invasiveness of the tumor. (Okura et al., 2016, p. 42; Xiong et al., 2017) Interestingly, Cdc42 was found to increase the formation of newly discovered invasive structures called flectopodia that turn pericytes in the tumor microenvironment into tumor-promoting cells. (Caspani et al., 2014)

1.6 Palladin

1.6.1 Structure and role

Palladin was first described by two independent research groups: the Otey lab in the year 2000 and the Carpen lab in 2001. (Mykkänen et al., 2001; Parast & Otey, 2000) Palladin belongs to the family of myotilin-myopalladin-palladin proteins and is highly conserved among vertebrates as well as present in many developing and mature tissues. This protein family is characterized by the presence of multiple copies of an Ig-like domain. Palladin has multiple isoforms originating from the PALLD gene; these isoforms differ by tissue distribution and the copy number of the distinctive Ig-like domain in their structure. (Beck et al., 2013; S. M. Goicoechea et al., 2008) Palladin also has proline-rich

regions called PR1 and PR2 which bind to a wide array of proteins that regulate actin dynamics and cytoskeletal rearrangement. (Najm & El-Sibai, 2014) Recently, there has been increased interest in palladin in the context of cancer due to its involvement in many pathways that affect the actin cytoskeleton, in addition to findings that showed the expression of different palladin isoforms in HeLa cells and tumor-associated fibroblasts. (S. M. Goicoechea et al., 2008)

The main role of palladin is to bind F-actin and crosslink the actin filaments to form actin bundles through its actin-binding Ig-like domain Ig3. (Beck et al., 2013) Palladin also acts as a cytoskeletal molecular scaffold as it binds to a wide array of actin-binding proteins that regulate actin dynamics. Palladin binds an actin cross-linking protein called α-actinin which is a key regulator of cell adhesions, whether they are cell-cell or cell-ECM interactions. α-actinin was also shown to be upregulated in metastatic cancer cells. (S. M. Goicoechea et al., 2008) Palladin also binds an actin-associated scaffold called ezrin which was shown to play a role in cancer cell metastasis. (Curto & McClatchey, 2004) Moreover, palladin controls the cytoskeletal rearrangements downstream of Src that are involved in podosome formation. (M. Rönty et al., 2007)

Increasing evidence is highlighting the role of palladin in cell motility due to the effect it has on the actin cytoskeleton and subsequently the cell motility and contractility. Studies on cultured fibroblasts show that the knockdown of palladin produces a wide array of defects in actin dynamics, cellular adhesion to the ECM, integrin stability, and cell motility. (Liu et al., 2007) The knockdown of palladin in fibroblasts also leads to cell rounding due to the loss of focal adhesions and actin stress fibers. (Parast & Otey, 2000)

Palladin was also shown to localize to focal adhesions in osteoblasts to mediate their adhesion and migration. (Lai et al., 2006)

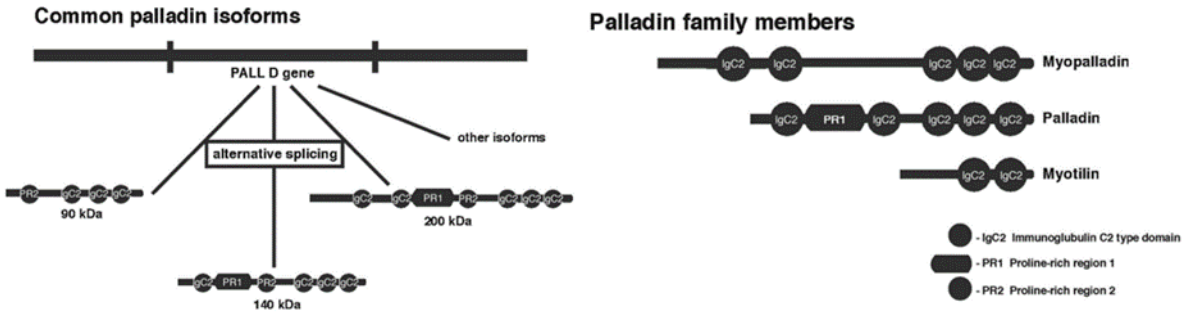

Figure 5. Palladin Isoforms and Family Members. (Source: Najm & El-Sibai, 2014)

1.6.2 Palladin in cancer

Since the role of palladin in regulating the actin cytoskeleton as well as cell adhesion and motility has been established by now, one can suspect palladin to be involved in cancer cell invasion and metastasis as well. Gene expression analysis conducted by Ryu *et al* showed that the gene coding for palladin is present in a cluster of genes that is responsible for pancreatic and colorectal cancer invasion. (Ryu et al., 2001) Moreover, Wang *et al* conducted microarray analysis on human breast cancer tumors and showed that palladin is overexpressed up to 3-fold in the migrating invasive cells. (Wang et al., 2004)

Palladin was of extreme interest during the studies concerning "Family X", which is a family that suffered from strangely high incidence of pancreatic cancer. A palladin mutation was present exclusively in the family members with pancreatic cancer and palladin was highly expressed in the sporadic pancreatic tumors. (Pogue-Geile et al., 2006)

As mentioned before, palladin promotes the formation of podosomes which are invasive structures in cancer cells. The depletion of palladin in invasive breast cancer cells attenuated the formation of podosomes and decreased the invasion of these cells. (S. M. Goicoechea et al., 2009) Palladin is also highly expressed in cancer-associated fibroblasts (CAFs) that surround many aggressive tumors like pancreatic and breast cancer, thereby activating them to promote tumor invasiveness. (M. J. Rönty et al., 2006) In addition, some palladin binding proteins like Eps8 and ezrin are highly expressed in invasive tumors like pancreatic and breast cancer as well as metastatic tumor cells. (Akisawa et al., 1999; Welsch et al., 2007)

1.7 Palladin crosstalk with Rho GTPases

Since Palladin and Rho GTPases appear to be involved in the same processes within the cell like actin cytoskeleton rearrangement, cell adhesion, and cell motility, it is speculated that there must be crosstalk between the two to perform these functions.

In a study performed by Brentnall *et al,* palladin overexpression activated human dermal fibroblasts, transforming them into myofibroblasts which are more invasive and contractile. This process is probably mediated by Rho signaling since the level of RhoA almost doubled in the palladin-overexpressing cells. (Brentnall et al., 2012) The knockdown of palladin in mouse embryonic fibroblasts prevented stress fiber formation which is also a phenotype that we see when Rho signaling is inhibited. Palladin knockdown also increases the phosphorylation of cofilin, which is a downstream effector of Rho. (Liu et al., 2007) Moreover, Rho signaling appears to affect the subcellular localization of palladin since palladin has been shown to relocate to the nucleus upon the inhibition of Rho by cAMP. (Niedenberger et al., 2014)

Palladin also seems to affect Rac activation and signaling. As previously mentioned, palladin increases podosome formation through binding the protein Eps8 that is known to regulate the Rac activity within the cell. Goicoechea *et al* performed a study which showed that the activation of Rac significantly decreased in HeLa cells where palladin was knocked down. (S. Goicoechea et al., 2006, p. 8)

Now that palladin was shown to affect Rac activation, and since Rac1 and Cdc42 tend to have similar roles and upstream regulators as well as regulate similar pathways in cells, palladin might affect Cdc42 regulation after all. Cdc42 is known to increase invadopodia formation in CAFs upon PMA treatment; Accordingly, the knockdown of palladin in CAFs reduced the activity of Cdc42 by more than 60% as well as reduced invadopodia formation. (S. Goicoechea et al., 2014)

1.8 Purpose of this study

The aim of this study is to investigate the role of palladin in the invasion of Glioblastoma multiforme (GBM) through invadopodia formation. Also, based on sufficient data that suggest crosstalk between palladin and Rho GTPases in cellular processes like cell invasion, motility, and adhesion in different types of cells, we sought to investigate how palladin and Rho GTPases can work together to promote GBM invasion through invadopodia. This was done mainly through analyzing the localization of TKS4 and TKS5 which are invadopodia markers, as well as studying the expression and activation of Rho GTPases like Cdc42 and Rac1 in GBM cell lines upon changing palladin expression.

Chapter 2

Materials and Methods

2.1 Cell culture

Cell Culture. Human glioblastoma cell line SNB-19 was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS and 100 U penicillin/streptomycin and were maintained under standard cell culture conditions at 37°C and 5% CO 2 in a humid environment.

2.2 Antibodies and reagents Cell culture

The following primary antibodies were used in this study: Rabbit polyclonal antibody against β-Actin, goat polyclonal antibody against palladin, mouse monoclonal antibody against Rac1, and rabbit polyclonal antibodies against TKS4 were purchased from Abcam (Abcam Inc., Cambridge, UK). Rabbit polyclonal antibodies against TKS55 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, Dallas,Texas). Anti-rabbit, anti-goat, and anti-mouse HRP-conjugated secondary antibodies were obtained from Promega (Promega Co., Wisconsin). To stain for the actin cytoskeleton in cells, Rhodamine-phalloidin was purchased from (Invitrogen).

2.3 Cell transfection with siRNA

Flexi Tubes siRNA for palladin oligos 1 and 7, Cdc42 oligo 4 and 7, and RhoC oligos 1 and 5 were obtained from Qiagen (Qiagen, USA). The cells were transfected with the

siRNA at 23 final concentration of 10 nM using Hiperfect (Qiagen, USA) as indicated by the manufacturing group. Control cells were transfected with siRNA sequences targeting GL2 Luciferase (Qiagen, USA). After 72 hours, protein expression levels in total cell lysates were examined by western blotting using the appropriate antibodies or the effect of the corresponding knockdown was assayed.

2.4 Western blot

Whole cell lysates were obtained by scraping the cells with Laemmli Sample Buffer (LSB) consisting of 4% SDS, 20% glycerol, 10% β 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 palladin (Abcam, 1:1000 dilution), or against Rac1 (Abcam 1:1000), or against actin (Abcam, 1:2500). After the incubation with the primary antibody, the membranes were washed with PBS – 0.5% Tween20 and incubated with secondary antibody at a concentration of 1:2500 for 1 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with Western blotting electrochemiluminescent reagent (ECL) (GE Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.5 Gelatin Invadopodia Assay

QCM Gelatin Invadopodia Assay (Green) kit from Sigma-Aldrich was used to measure the invasion ability of SNB-19 cells. Briefly, cells were treated with siRNA against palladin, plated on fluorescently labeled gelatin (matrix), and incubated at 37 °C

and 5% CO2 for 24 hours. Then cells were visualized on a 63x objective lens of the Zeiss Observer Z1 microscope. The area devoid of fluorescence and intensity of the signal was analyzed using Image J software.

2.6 Immunostaining

Cells were plated on cover slips and treated with palladin oligo 1 for 72 hours. Cells were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X 100 for 15 minutes on ice. For blocking, cells were incubated with 1% BSA in PBS for 1 hour. Samples were stained with primary antibodies for 24 hours 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 Förster Resonance energy transfer (FRET)

SNB-19 cells were transfected with 2.5 μg of the Cdc42 Fluorescence resonance energy transfer (FRET)-based Biosensor plasmid using FuGene HD Transfection Reagent (Promega Co., Wisconsin). FRET images were obtained with an automated using a 63X objective lens on Zeiss Observer Z1 microscope supplemented with a computer-driven Roper cooled CCD camera and operated by Zen software (Zeiss, Oberkochen, Germany). A CFP/YFP FRET filter cube was used: YFP was imaged with exciter S500/20 and emitter S535/30 (YFP/acceptor image), CFP was imaged with exciter S430/25 and emitter S470/30 (CFP/donor image) or S535/30 (FRET image).

2.8 Quantification of FRET images

The FRET image sequences that were obtained consisted of a CFP image, YFP image and FRET image. To analyze the obtained images, ImageJ software was used. (National Institutes of Health, MA, USA). Images were background corrected and the 25 YFP images were thresholded to generate a binary mask with values of 1 within the cell and 0 for the background. This was used to remove the background from ratio calculations, by multiplying CFP and FRET images by the mask. The increase in FRET signal due to activation was detected by dividing the FRET image (CFP excitation- YFP emission) by the donor image (CFP excitation- CFP emission). FRET signals were quantified by averaging the mean FRET ratio in all the cell area and values were then normalized to control cells (untreated) and expressed as fold change.

Chapter 3

Results

3.1 Palladin expression and knockdown in glioblastoma

Before diving into studying the role of palladin in invadopodia formation in GBM cells, we had to make sure that palladin is indeed expressed in these cells to begin with. Therefore, we performed western blots to verify palladin protein expression in two glioblastoma cell lines, U87 and SNB-19. We detected palladin expression in both cell lines through protein bands that resolved at ~90 KDa. Then, to establish a standard knockdown protocol to manipulate palladin expression for upcoming experiments, we used siRNA oligo 1 and oligo 7 targeted against palladin. Both oligos showed significant palladin knockdown.

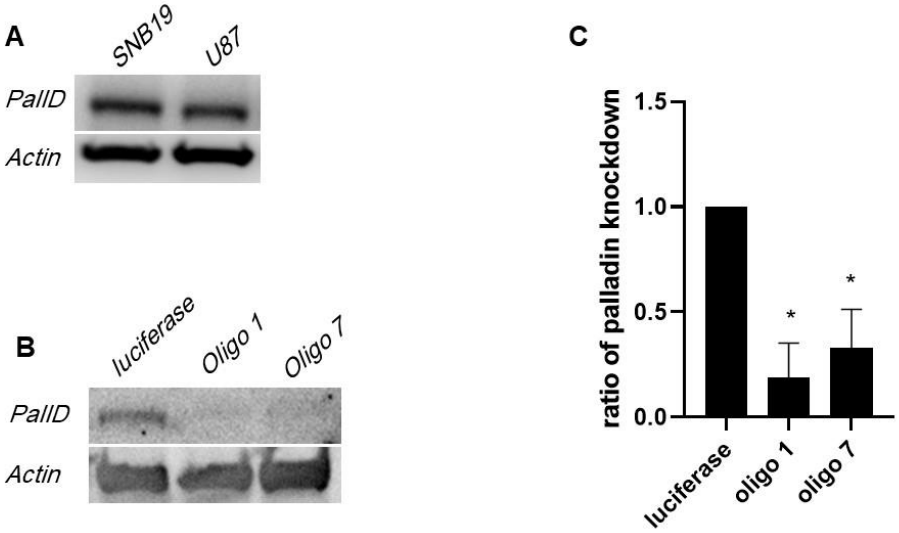

Figure 6. Palladin expression and knockdown in glioblastoma. A. Western blot for palladin expression in glioblastoma cell lines SNB-19 and U87 (normalized against actin). **B.** Western blot for palladin expression following knockdown with oligo 1 or oligo 7 in SNB-19 (normalized against actin). **C.** The average intensity rate was quantified using ImageJ. Data are the mean ± SEM. * indicates that the values are significant with $p<0.05$.

3.2 Palladin knockdown increases invasion on gelatin matrix

After establishing a successful palladin knockdown protocol, we sought to test the role of palladin on the invasion of SNB-19 cells. We depleted palladin in SNB-19 cells using oligo 1 and performed a gelatin invadopodia assay by plating the cells on a fluorescently labeled gelatin (matrix) and incubating them at 37 ℃ and 5% CO2 for 24 hours. The cells were then imaged with 63X objective to assess invasion based on area and intensity of fluorescence. The palladin knockdown significantly increased the invasion of the SNB-19 cells which is detected through a decrease in gelatin fluorescence intensity (an indication of gelatin degradation). Some cells were also more elongated and formed multiple protrusions where invasion was concentrated. Note that the average measured area between the luciferase and palladin knockdown groups was almost the same, verifying that the change in the gelatin fluorescence intensity observed between the two groups was indeed due to the palladin knockdown and not due to difference in the measured area.

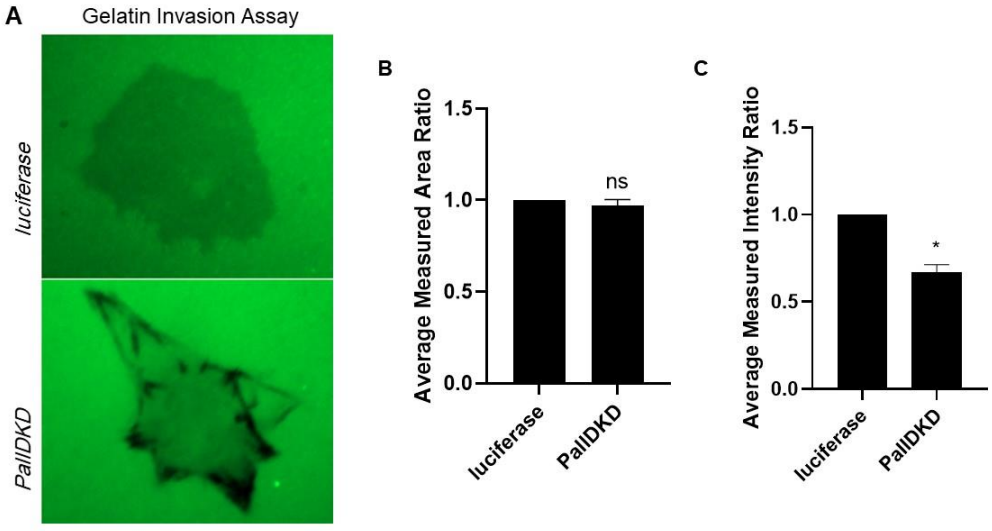

Figure 7. Palladin knockdown increases invasion on gelatin matrix. A. Representative images for gelatin degradation following treatment with luciferase siRNA (upper panel) and palladin knockdown (lower panel) **B.** Average measured area of invasion. ns indicates that difference in measured area was nonsignificant **C.** Average measured intensity from cells. Data are the mean ± SEM. * indicates that the values are significant with $p<0.05$.

3.3 Palladin knockdown increases invadopodia as seen by TKS4/5 staining

Now, we wanted to verify that the observed palladin knockdown-mediated increase in SNB-19 cell invasion was in fact due to increased invadopodia formation. This was done by immunostaining against TKS4 and TKS5 which are invadopodia markers. Upon palladin knockdown, the expression of TKS4 significantly increased and that of TKS5 almost doubled, indicating that palladin most likely controls SNB-19 cell invasion through the formation of invadopodia.

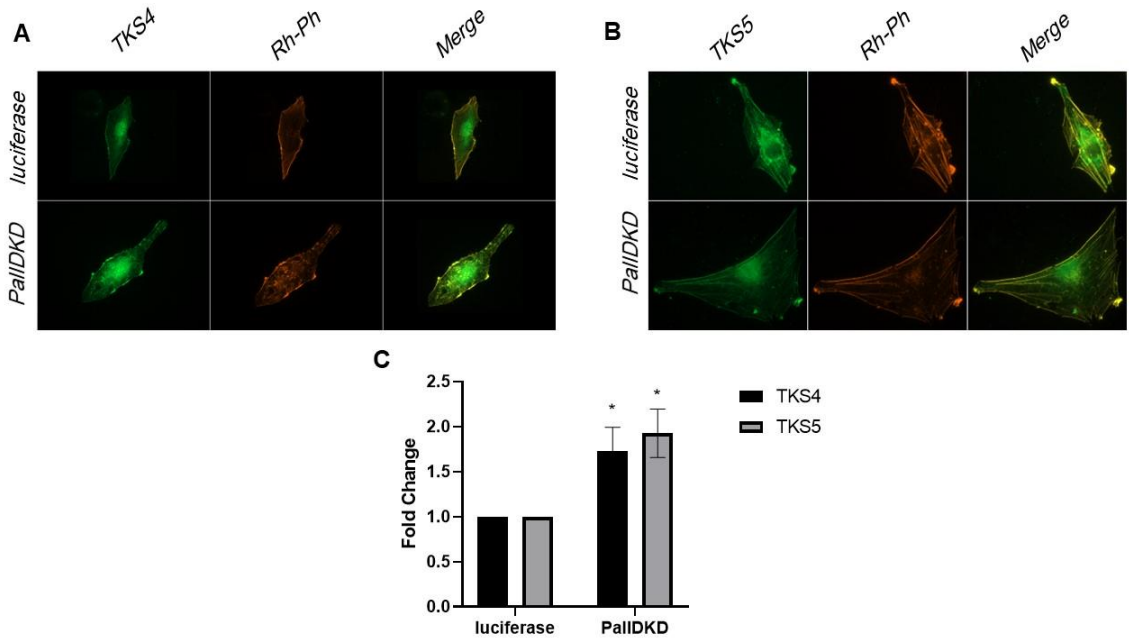

Figure 8. Palladin knockdown increases invadopodia as seen by TKS4/5 staining. A. Representative micrographs of SNB-19 cells transfected with luciferase siRNA (upper panel) palladin oligo 1 (lower panel) that were fixed and immunostained with antibodies against TKS4 as well as vinculin and Rhodamine phalloidin. Cells were imaged using a 63x objective **B.** Representative micrographs of SNB-19 cells transfected with luciferase siRNA (upper panel) palladin oligo 1 (lower panel) that were fixed and immunostained with antibodies against TKS5 as well as vinculin and Rhodamine phalloidin. Cells were imaged using a 63x objective **C.** The average number of invadopodia was quantitated using image J. Data are the mean ± SEM. * indicates that the values are significant with $p<0.05$.

3.4 The effect of Cdc42 and RhoC deprivation on invadopodia formation

Cdc42 and RhoC, among other Rho GTPases, have been shown to be implicated in invadopodia formation based on previous findings from our lab. Accordingly, we sought to investigate the crosstalk between palladin and the mentioned Rho GTPases by comparing knockdown phenotypes. Based on immunostaining analysis, the knockdown of both Cdc42 and RhoC significantly decreased the levels of TKS4 and TKS5 which is indicative of decreased invadopodia formation and opposite to what we saw with the

palladin knockdown. This shows that palladin, Cdc42, and RhoC affect invadopodia formation in SNB-19 cells.

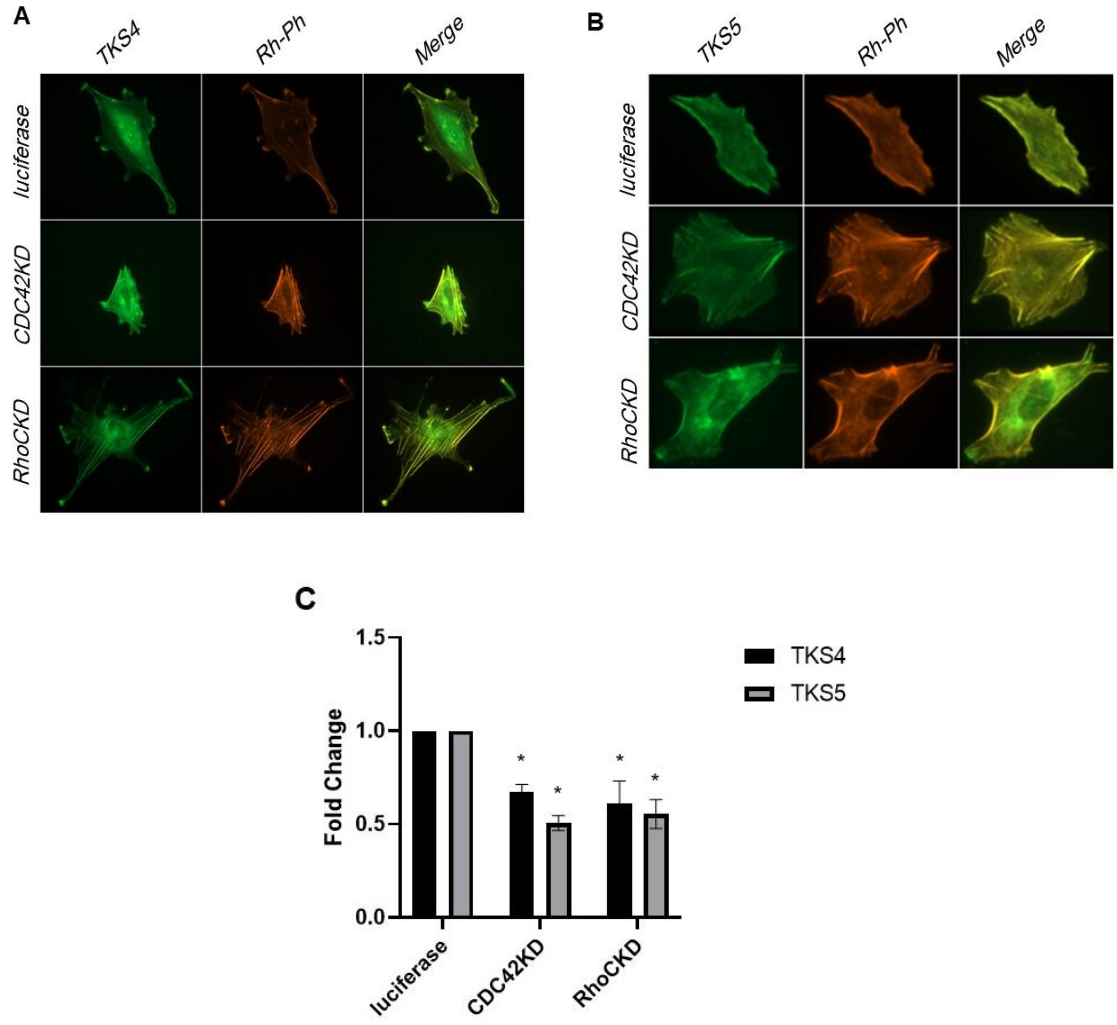

Figure 9. The effect of Cdc42 and RhoC deprivation on invadopodia formation. A. Representative micrographs of SNB-19 cells transfected with luciferase siRNA (upper panel), Cdc42 oligo 4 (middle panel), and RhoC oligo 5 (lower panel) that were fixed and immunostained with antibodies against TKS4 as well as vinculin and Rhodamine phalloidin. Cells were imaged using a 63x objective **B.** Representative micrographs of SNB-19 cells transfected with luciferase siRNA (upper panel), Cdc42 oligo 4 (middle panel), and RhoC oligo 5 (lower panel) that were fixed and immunostained with antibodies against TKS5 as well as vinculin and Rhodamine phalloidin. Cells were imaged using a 63x objective **C.** The average number of invadopodia was quantitated using image J. Data

are the mean ± SEM. * indicates that the values are significant with $p<0.05$. ** indicates that the values are significant with $p<0.005$.

3.5 Palladin deprivation leads to a decrease in Cdc42 activity

Since now we know that palladin and RhoC are both involved in invadopodia formation, we want to check whether palladin affects Cdc42 activation. We knocked down palladin in SNB-19 cells and transfected them with a Cdc42 single chain optimized FRET biosensor. Image analysis based on FRET/CFP rationing showed that the knockdown of palladin decreased the activation of Cdc42 by almost 25%.

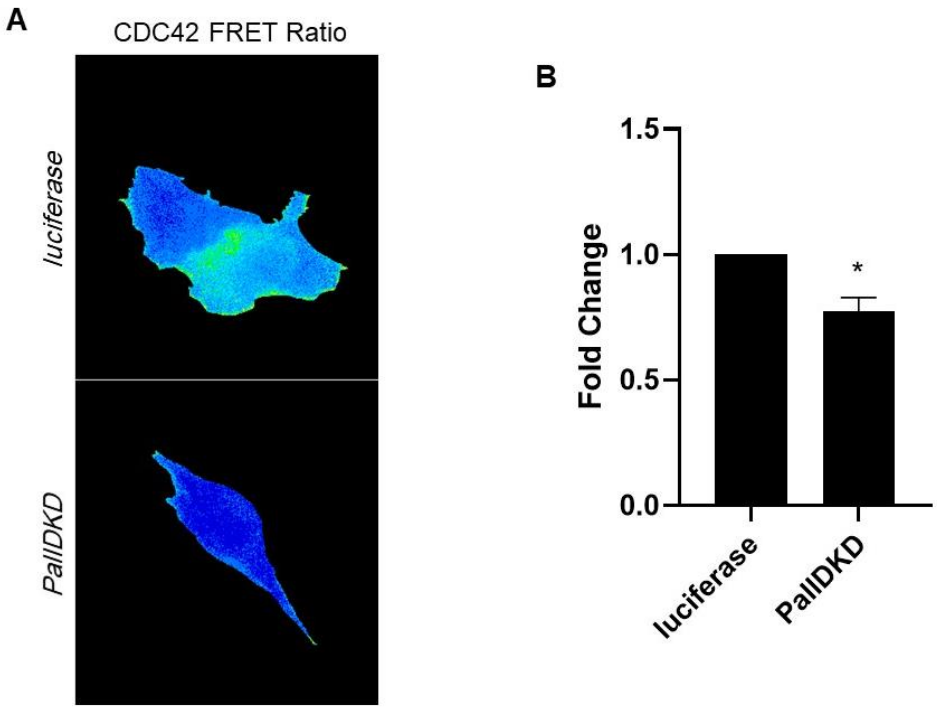

Figure 10. Palladin deprivation leads to a decrease in Cdc42 activity. A. Representative images of SNB-19 cells transfected with siRNA for palladin and Cdc42 FRET biosensor and fixed. Cells were imaged on the FRET channel and raw FRET images were ratioed to CFP image for normalization. **B.** The average intensity rate was quantified using ImageJ. Data are the mean ± SEM. ** indicates that the values are significant with $p<0.005$.

3.6 Novel role for palladin in the expression of Rac1

Moving further away from investigating the activation and more into the expression of Rho GTPases, we wanted to see if palladin affects the expression of Rac1 especially that no previous studies have been done concerning this. Accordingly, we knocked down palladin in SNB-19 cells and performed a western blot to determine the expression levels of Rac1. Indeed, the palladin-depleted SNB-19 cells showed a significant decrease in Rac1 expression.

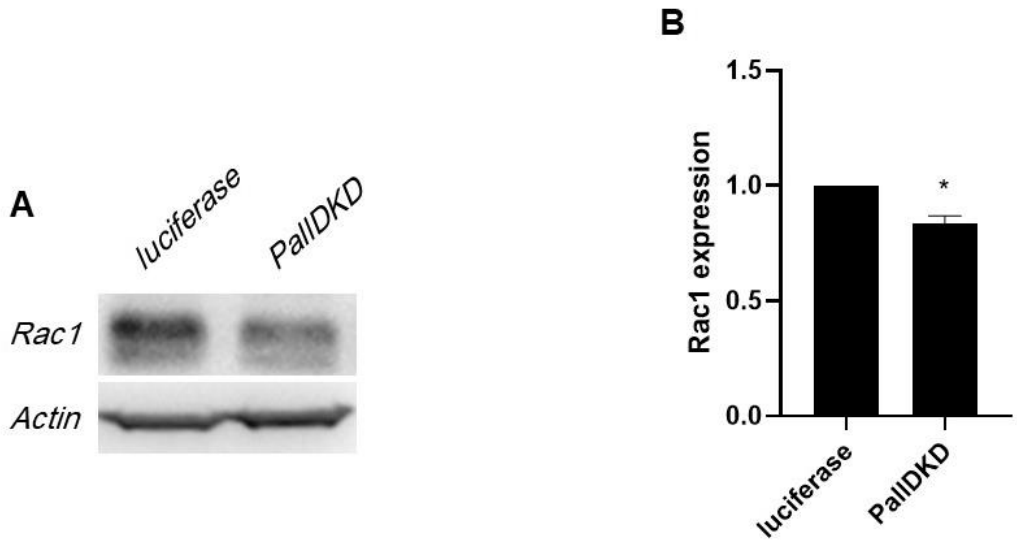

Figure 11. Novel role for palladin in the expression of Rac1. A. Western blot Rac1 expression normalized against actin following palladin knockdown. **B.** The average intensity rate was quantified using ImageJ. Data are the mean ± SEM. * indicates that the values are significant with $p<0.05$.

Chapter 4

Discussion

Palladin is a protein that has recently been gaining the interest of many scientists, especially in the context of cancer and metastasis. Ever since it was discovered and characterized, palladin has proved to be a key regulator of actin dynamics within the cell, in addition to its role in the formation of invasive structures like podosomes. (S. M. Goicoechea et al., 2009) Palladin expression has also been implicated in many invasive cancers like breast and pancreatic cancer. (Pogue-Geile et al., 2006) Nevertheless, little studies were done to investigate the role of palladin in Glioblastoma multiforme, and no studies were conducted to link palladin to invadopodia formation in this type of cancer. Our lab has produced valuable findings concerning the role of Rho GTPases in invadopodia formation and glioblastoma; Therefore, in this paper we were able to fit palladin, Rho GTPases, and invadopodia into a context that is the first to be established in glioblastoma, using SNB-19 cell lines.

In this study, we performed a western blot to verify the expression of palladin in SNB-19 cells. Then we performed a gelatin invasion assay to study the effect of palladin on SNB-19 cell invasion; the results showed that the knockdown of palladin increases gelatin degradation, verifying that palladin is involved in glioblastoma cell invasion and that its depletion increases this invasion. Now, in an attempt to uncover the mechanism by which palladin affects glioblastoma invasion, we assessed the molecular markers of invadopodia TKS4 and TKS5 using immunostaining experiments. The knockdown of

palladin increased TKS4 and TKS5 in SNB-19 cells, suggesting that palladin controls invasion through invadopodia formation. To verify this statement, further studies should be done to analyze other invadopodia-associated molecules like WASP, Arp2/3, and cofilin upon palladin depletion. (Eddy et al., 2017)

Based on previous data that highlighted the important role of Rho GTPases in cellular processes like cytoskeletal rearrangement and cell invasion (Haga & Ridley, 2016), we sought to determine the role of RhoC and Cdc42 in invadopodia formation in GBM cells. Immunostaining experiments showed a decrease in TKS4 and TKS5 upon the knockdown of RhoC and Cdc42 in SNB-19 cells which is a phenotype that is opposite to what we saw with the palladin knockdown.

Palladin was previously shown to be involved in Cdc42 activation in PMA-stimulated CAFs. (S. Goicoechea et al., 2014) To see if this is the case in glioblastoma cells, we performed FRET experiments using a Cdc42 biosensor to assess its activity following palladin knockdown. Indeed, the depletion of palladin in SNB-19 cells decreased the activation of Cdc42. However, since the palladin knockdown and Cdc42 knockdown showed opposing phenotypes on TKS4 and TKS5, we speculate that palladin regulates invadopodia formation in a pathway other than that of Cdc42.

Previous studies have also shown that palladin affects the activation of Rac1 (S. Goicoechea et al., 2006). Here, we wanted to take a different route and check if palladin affects Rac1 expression. We performed a western blot and whereby the knockdown of palladin decreased the expression of Rac1. This is interesting because it shows that not

only can palladin regulate Rho GTPases at the activation level, but it can also regulate their expression.

A limitation of this study is that it only looks at the relationship of palladin with Cdc42 and Rac1 out of all the Rho GTPases. Future studies are needed to investigate a relationship between palladin and other Rho GTPases like RhoC and RhoA which have been implicated in invadopodia formation and cancer invasion. Also, such studies could focus on the effect of palladin on both the activation and the expression of the Rho GTPases. StarD13 is a tumor suppressor that has recently been shown by our lab to regulate invadopodia (Al Haddad et al., 2020) so it would be interesting to check if there is a crosstalk between palladin and StarD13 that affects invadopodia formation in GBM cells.

α-actinin is one of the main binding partners of palladin that is overexpressed in metastatic cancer cells (S. M. Goicoechea et al., 2008) and recent studies showed that dynamin and α-actinin promote pancreatic cancer invasion by stabilizing invadopodia (Burton et al., 2020). Therefore, it would be interesting to see if palladin is involved in the dynamin-α-actinin signaling pathway that regulates invadopodia in glioblastoma cells. Eps8 is another palladin-binding protein that is overexpressed in metastatic cancer cells and that has recently been shown to promote the 3-dimensional invasion of glioblastoma cells (Cattaneo et al., 2012). Again, further studies can focus on revealing previously unknown interactions between palladin and Eps8 that play a role in GBM cell invasion.

Chapter 5

Conclusion

The results of this study show that palladin negatively regulates cell invasion, as the depletion of palladin in SNB-19 glioblastoma cells increased invasion and invadopodia formation. In the context of Rho GTPases, we demonstrated that palladin positively regulates Cdc42 activation and Rac1 expression. These findings provide valuable insights concerning the regulation of invadopodia at the molecular level. Future studies could focus on the relationship between palladin and other Rho GTPases in GBM in terms of activation and expression. Also, these studies can focus on other palladin-binding proteins that regulate actin dynamics and have been implicated in other cancers. This would further characterize the role of palladin in the signaling networks that modulate cancer invasion and metastasis.

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