Characterization of CdGAP/ARHGAP31 as a novel biological target in prostate cancer

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Abstract

Cdc42, Rac1 and RhoA are the most extensively studied Rho GTPases, a subfamily of proteins within the Ras-related superfamily of GTPases, that regulate many cytoskeleton dependent cellular functions such as cytokinesis, cell polarity, morphogenesis, and cell motility. Rho GTPases are regulated either positively or negatively by three families of proteins: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. In addition, the third family Guanine Dissociation Inhibitors (GDIs) sequesters Rho GTPases in an inactive form in the cytoplasm. As Rho GTPases are key organizers of the actin cytoskeleton, their regulators and effectors are prime targets for subversion in several pathologies including cancer.

Cdc42 GTPase-activating protein (CdGAP) is a RhoGAP that inactivates functions of the GTPases Rac1 and Cdc42. CdGAP is a well conserved protein that has been associated with a rare developmental disorder called Adams Oliver Syndrome characterized by limb anomalies and heart defects. In addition, there is compelling evidence implicating that CdGAP may regulate cancer related processes. First, CdGAP is required for normal angiogenesis. Second, CdGAP has been shown to play an important role in normal cell spreading, lamellipodia formation and cell migration. Third, CdGAP was shown to disrupt adherens junctions by altering the expression of E-cadherin; loss of which is a hallmark of cancer cell metastasis. Fourth, CdGAP was recently established as an important component required downstream of transforming growth factor-β signalling pathway to promote breast cancer cell migration and invasion. Notably, CdGAP was shown to act with Zeb2 as a co-transcriptional repressor of E-cadherin transcription resulting in tumorigenesis and metastasis. Collectively, CdGAP has been established as an oncoprotein in the context of breast cancer; however, whether CdGAP exerts a similar role at a global scale in other cancers remains elusive.

In this thesis, we have demonstrated a tumor promoting role of CdGAP in prostate cancer. We began by examining CdGAP levels and detected high levels of CdGAP protein and mRNA in the metastatic and aggressive PC-3 human prostate cancer cell line. Downregulation of CdGAP expression using short hairpin RNAs targeting CdGAP in PC-3 cells led to a significant reduction in cell motility, cell invasion, cell proliferation and colony-formation ability while an increase in cell apoptosis was observed. In addition, subcutaneous injection of PC-3 cells in mice revealed

that loss of CdGAP delays tumor onset, attenuates tumor growth, and results in reduced tumor size in mice. Consistently, using global gene expression approaches we have shown the pathways most significantly regulated by CdGAP in prostate cancer are associated with cell migration, cell cycle progression and cell apoptosis—further corroborating our experimental results. In addition, we also report a clinical significance of CdGAP in prostate cancer. Briefly, tissue microarray analysis of 285 prostate cancer patients demonstrated a correlation between the nuclear localization of CdGAP with an early relapse of prostate cancer. Lastly, a gain/amplification of the CDGAP/ARHGAP31 gene was associated with poor disease-free survival in prostate cancer patients. Collectively, the work presented in this thesis highlights a role of CdGAP in prostate cancer and yields it as a novel biomarker and potential molecular target to develop therapeutic strategies in the treatment of prostate cancer patients.

Résumé

Les protéines Cdc42, Rac1 et RhoA sont les petites GTPases les plus étudiées parmi les Rho GTPases, une sous-famille de protéines de la superfamille des GTPases apparentée aux Ras GTPases, qui régulent de nombreuses fonctions cellulaires dépendantes du cytosquelette, telles que la cytokinèse, la polarité cellulaire, la morphogénèse et la migration cellulaire. Les Rho GTPases sont réglementées positivement ou négativement par deux classes de protéines : respectivement les facteurs d'échange de nucléotide guanine (GEFs) et les protéines d'activation GTPasique (GAP). En outre, la troisième famille d'inhibiteurs de la dissociation de la guanine (GDI) séquestre les Rho GTPases sous une forme inactive dans le cytoplasme. Comme les GTPases Rho sont les principaux organisateurs du cytosquelette d'actine, leurs régulateurs et effecteurs sont des cibles de choix pour la subversion dans plusieurs pathologies, y compris le cancer.

La protéine d'activation de la GTPase Cdc42 (CdGAP) est une RhoGAP qui inhibe les fonctions des GTPases Rac1 et Cdc42. CdGAP est une protéine bien conservée qui a été associée à un trouble rare de développement, appelé syndrome d'Adams-Oliver ou AOS et caractérisé par des anomalies de développement des extrémités et des malformations cardiaques. Il existe aussi des preuves convaincantes impliquant que CdGAP peut réguler des processus liés au cancer. Tout d'abord, CdGAP est nécessaire pour l'angiogenèse normale. Aussi, CdGAP joue un rôle important dans la propagation normale des cellules, la formation de lamellipodes et la migration cellulaire. Également, CdGAP perturbe les jonctions cellulaires adhérentes en modifiant l'expression de la E-cadhérine, dont la perte est une caractéristique type des processus métastasiques des cellules cancéreuses. Finalement, CdGAP est une composante importante, requise en aval de la voie de signalisation du facteur de croissance TGFβ, pour favoriser la migration et l'invasion cellulaire dans le cancer du sein. En particulier, CdGAP peut agir avec le facteur de transcription Zeb2 comme un répresseur co-transcriptionnel de la transcription de la E-cadhérine, ayant pour résultat de promouvoir la tumorigénèse et les métastases au poumon. Collectivement, CdGAP a été établie en tant qu'oncoprotéine dans le contexte du cancer du sein. Cependant, de telles fonctions pour CdGAP dans le contexte d'autres cancers n'ont à ce jour pas été identifiées.

Dans cette thèse, nous avons démontré que CdGAP promeut la tumorigénèse dans le cancer de la prostate. Nous avons commencé par examiner l'expression de la protéine CdGAP et de son ARNm et avons détecté des taux élevés dans la lignée de cellules humaines métastasiques et agressives du cancer de la prostate PC-3. L'introduction d'un ARNsh destiné à réduire l'expression de CdGAP dans les cellules PC-3 a mené à une réduction de la motilité et de l'invasion cellulaire, de la prolifération et de la capacité de formation des cellules en colonie, parallèlement à une augmentation de l'apoptose cellulaire. En outre, l'injection sous-cutanée des cellules PC-3 déplétées de l'expression de CdGAP chez les souris a indiqué que la perte de CdGAP retarde l'apparition et atténue le développement des tumeurs, et a comme conséquence une réduction de la taille de la tumeur. De plus, en utilisant des approches globales d'expression génique, nous avons montré que les voies les plus significativement réglementées par CdGAP dans le cancer de la prostate sont associées à la migration cellulaire, à la progression du cycle cellulaire et à l'apoptose cellulaire. Par ailleurs, nous rapportons également un rôle clinique de CdGAP dans le cancer de la prostate. En effet, l'analyse « microarray » de tissus de 285 patients de cancer de la prostate a démontré une corrélation entre la localisation nucléaire de CDGAP avec une rechute précoce du cancer de la prostate. Enfin, un gain et/ou amplification du gène *CDGAP/ARHGAP31* a été associé à une faible survie chez les patients atteints de cancer de la prostate. En somme, les travaux présentés dans cette thèse mettent en lumière un rôle important de la protéine CdGAP dans le cancer de la prostate et révèlent CdGAP comme une nouvelle cible moléculaire potentielle dans le développement de stratégies thérapeutiques afin de traiter les patients atteints de cancer de la prostate.

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Contribution of Co-Authors

This thesis is a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by others must be accompanied with an explicit statement as to who contributed to such work and to what extent.

Chapter 2

My contribution to the manuscript entitled "The Rac1/Cdc42 regulator CdGAP is a positive modulator of prostate tumorigenesis" involves designing and performing all the experiments with the support of Dr Yi He and Dr. Nathalie Lamarche-Vane. The initial discussion of the project was conducted in collaboration with Karl-Phillippe Guérard and Dr. Jacques Lapointe. The TMA analysis was performed in collaboration with Véronique Ouellet and Dr. Fred Saad. The RNA-sequencing data was analyzed in collaboration with Dr. Carine Delliaux and Dr. Jean-François Côté. The mouse experiment was planned with the support of Dr. Nadia Boufaied and Dr. David Labbé. Lastly, I have written the manuscript with the support of Dr. Lamarche-Vane and it is currently in preparation for a publication.

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List of abbreviations

ACC	Aplasia cutis congenita
ADT	Androgen deprivation therapy
AOS	Adams-Oliver syndrome
BR	Basic residues
Cdc	Cell division cycle
CdGAP	Cdc42GTPase activating protein
cDNA	Complementary DNA
CRIB	Cdc42/Rac1-interactive binding
CRPC	Castrate-resistant prostate cancer
DAPI	4', 6-diamidino-2-phenylindole
DH	Dbl homology domain
DLC	Deleted in liver cancer
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
DTT	Dithiothreitol
E-cadherin	Epithelial cadherin
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
ERK-1	Extracellular Signal-regulated Kinase-1
FAK	Focal adhesion kinase
F-actin	Filamentous actin
FBS	Fetal bovine serum
GAPs	GTPase-activating proteins
GDIs	Guanine nucleotide dissociation Inhibitors
GDP	Guanine diphosphate
GEFs	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GSK-3	Glycogen Synthase Kinase 3

GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC	Human umbilical vein endothelial cells
mRNA	Messenger ribonucleic acid
NaF	Sodium fluoride
PAK	p21 protein (Cdc42/Rac)-activated kinase
PBR	Poly-basic region
PBS	Phosphate-buffered saline
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PI(3,4,5)P3	Phosphatidylinositol 3,4,5-triphosphate
PMSF	Phenylmethylsulfonyl flouride
PRD	Proline-rich domain
P-Rex1	Phosphatidylinositol-3,4,5- trisphosphate-dependent Rac exchange factor 1
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
QPCR	Quantitative real-time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
Rho	Ras homologous
Ras	Rat Sarcoma
Rac	Ras-related C3 botulinum toxin substrate
RBD	Rho-binding domain
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH3	Src-homology 3
shRNA	Short hairpin RNA
TIAM-1	T-lymphoma invasion and metastasis-inducing protein
TGFβ	Transforming growth factor β
TMPRSS2	Transmembrane serine protease 2
TRITC	Tetramethylrhodamine

VEGF	Vascular endothelial growth factor
WB	Western blot
ZEB2	Zinc Finger E-Box Binding Homeobox 2

Chapter 1

Introduction and Literature Review

1.0 Prostate Cancer: General Introduction

Prostate carcinoma is the most commonly diagnosed non-skin malignancy in men, surpassing lung cancer. The Canadian Cancer Society statistics showed that in 2017 an estimated 21,300 men would be diagnosed with prostate cancer, representing 21% of all new cancer cases in men in Canada [1]. Similar statistics prevail in other industrialized countries [2]. Prostate cancer is usually a slow growing tumor and men affected with a localized tumor have a long-term survival potential. However, metastasis of the cancer is a leading cause of prostate-cancer specific mortality with around 4,100 deaths recorded from metastasis of prostate cancer in 2017 [1]. Thus, despite the possibility of early detection and a host of available treatments for prostate cancer, the metastatic form of this disease remains a significant cause of mortality in men.

1.1 Prostate Cancer: Progression— prostatic intraepithelial neoplasia, localized tumor and castration resistant prostate cancer

A carcinoma is a cancer arising specifically from epithelial cells. An adenocarcinoma is a tumor resulting from malignant changes in epithelial cells lining a glandular tissue, for example the prostate ducts in men. Subsequently, one of the possible precursors of prostate cancer is development of prostatic intraepithelial neoplasia (PIN) which is characterized by abnormal activity of epithelial cells lining the secretory glands of the prostate [3]. Although the presence of PIN portends development of prostate cancer, it can take almost 10 years before a malignant tumor develops [3, 4]. Once the cancer forms it is localized in the areas of the prostate organ and can advance by invading the surrounding tissues such as seminal vesicles, bladder, ureters and the rectum [5]. Over time, tumors can spread to the rest of the body through access to the lymphatic or vascular system—known as metastases; with lymph nodes adjacent to the prostate often serving as the first site of metastasis [6] and the bone being the most common sites of prostate cancer metastasis [7]. Huggins and Hodges, were the first ones to recognize the hormone-responsive nature of prostate cancer and demonstrated that decreasing the activity of androgens through castration resulted in tumor regression [8]. Since then, one of the first lines of therapy for prostate cancer patients is androgen deprivation therapy (ADT). ADT is usually able to provide remission of the disease; however, in several cases the disease progresses further despite continuous doses of hormone depravation. At this state the disease is characterized as either castrate-resistant prostate cancer (CRPC) or metastatic castration-resistant prostate cancer (mCRPC), with

mCRPC in particular exhibiting a poor patient prognosis and a deplorable survival rate of about 2 years [9]. Collectively, prostate cancer is a multistep process beginning with the malignant transformation of cells forming a PIN, followed by a formation of a local tumor, then invasion of this tumor to surrounding organs, in some cases culminating into metastasis and eventually reaching a hormone-refractory state [10]. Since mCRPC is invariably fatal, the most active areas of research in this field is to elucidate the molecular mechanisms—by investigation of the various risk factors—underlying the evolution of prostate cancer. With the ultimate goal of using this knowledge to establish molecular subtypes of prostate cancer and guide towards the selection of more precisely targeted therapeutic strategies [11].

1.2 Prostate Cancer: Risk factors

A risk factor is an attribute that increases the susceptibility or likelihood of developing a disease and studying them allows understanding the pathways of progression in a given malignancy. The etiology of prostate cancer consists of various risk factors such as family history, genetic drivers, epigenetic modifications, age and lifestyle factors such as diet [11-14].

Some of the first studies associating family history and genetics to prostate cancer came from epidemiological and twin studies [10]. Epidemiological studies established that a family history of prostate cancer is a major predictor of development of this disease [15]. Furthermore, a twin study conducted between monozygotic and dizygotic twins stated that almost 42% of prostate cancer can be attributed to a heritable risk [16]. Moreover, some familial mutations such as in the *BRCA1* and *BRCA2* genes have also been associated with predisposing men to prostate cancer and are associated with poor prognosis [17]. Now, with the advent of next-generation sequencing techniques it has been easier to sequence the primary as well as metastatic tumors to accurately catalogue the different driver mutations that arise as the tumor naturally evolves [11]. To this end, several large-scale genomic studies have been conducted to identify the genetic drivers of this disease and the most common genomic alterations that have emerged include four pathways/genes: the rearrangements that place members of the E26 transformation-specific (ETS) transcription factor family under control of androgen-responsive promoter transmembrane serine protease 2 (TMPRSS2), loss of function of the prostate tumor suppressor NKX3.1, androgen receptor (AR) pathway and phosphatidylinositol 3-kinase (PI3K) pathway [11, 17].

Changes in the DNA sequence are characterized by gene mutations, amplifications, deletions and translocations. One of the most well characterized genomic alterations that is present in approximately 50% of localized prostate cancers is the chromosomal translocation involving a fusion between the androgen receptor-regulated TMPRSS2 with ERG, a member of the Ets family of transcription factors [11, 18]. This TMPRSS2-ERG fusion gene results in an over-expression of the oncogenic ERG transcription factor which promotes cancer progression [18]. In addition to this, another frequent mutation observed in almost 40% of prostate cancer cases lies in the androgen regulated homeodomain-containing transcription factor Nkx3.1. Briefly, it is considered as a tumor suppressor and loss of function of one allele has been classified as an initiating event of prostate tumorigenesis; acting as a strong biomarker of prostate cancer [11]. Moreover, the AR signalling is principal for development of the prostate; thus, it is unsurprising that the activities of this pathway are reported to be significantly subverted during cancer growth [17]. Another pathway which is commonly altered in prostate cancer is PI3K signalling. PI3K is a class of enzymes involved in basic cellular functions including cell growth, differentiation and survival. Prostate tumors often achieve aberrant activation of PI3K signalling through inactivating mutations in its negative regulator phosphatase and tensin deleted on chromosome ten (PTEN). In about 70% of prostate cancers a loss of heterozygosity of PTEN is observed and it always corresponds to poor prognosis [11, 17].

Besides genetic mutations, some epigenetic alterations such as changes in DNA methylation and histone modifications have been recently reported in prostate cancer [11, 19]. Briefly, DNA methylation is defined by the addition of a methyl group on the fifth carbon of cytosine residues in CpG dinucleotides (5-methylcytosine) resulting in silenced gene expression. Notably, the aberrant methylation of the CpG island at the glutathione S transferase pi (*GSTP1*) locus is observed in almost 95% of prostate cancer cases. GSTP1 represents a group of enzymes that mediate detoxification of exogenous substances thus this renders cells more prone to DNA damage [20]. Several other lifestyle factors that are also considered risk factors include ageing—99% of men that develop prostate cancer are over 50 years old, diet and ethnicity [14]. Most notably, several reports suggest that prostate cancer mortality is highest in men of African descent living in industrialized countries [13]. Collectively, the framework of triggers associated with prostate cancer progression comprises a large variety of genetic, epigenetic, transcriptomic,

lifestyle and environment stimulus and how these multiple factors intertwine remains a subject of intense research.

1.3 Prostate Cancer: Diagnosis, Current Standard of Care and Existing Limitations

Prostate cancer screening is initially performed using serum testing for prostate-specific antigen (PSA) or by performing a digital rectal exam. PSA is a protein that is secreted by the epithelial cells of the prostate and appears in the circulation only upon deregulation of cells lining the prostate gland; often a characteristic of PIN [3, 21]. These screening tests direct patients with potential prostate cancer symptoms towards a biopsy of the prostate organ which provides an accurate diagnosis of the extent and severity of the disease. Once diagnosed, the tumor is classified based on the grade and stage through histological analysis [22]. Based on the risk group stratification of the tumors biopsied, the treatments for prostate cancer can be classified as: active surveillance of the tumor, surgical removal of the prostate and radiation, chemotherapy and androgen deprivation therapy (ADT), and the recently emergent immunotherapies [11].

As prostate cancer is slow-growing, in the beginning active surveillance is used to monitor the indolent tumor—until screening tests and biopsy prove it to be a malignant growth [23]. The treatments used for the localized form of prostate tumor are surgery and radiation. The former refers to surgical radical prostatectomy—removal of the prostate gland, while the latter is the use of high energy rays to kill the areas affected with cancerous growth [23]. The AR signaling greatly influences the initial growth of the tumor; hence, androgen deprivation therapy is the gold standard of treatment once the tumor is locally advanced [9]. Briefly, ADT is administration of drugs that manipulate the production of testosterone thus resulting in a “chemical” castration. Particularly, they are gonadotropin-releasing hormone agonists, anti-androgens or CYP17A1 (an intermediate during the development of androgens) inhibitors. However, as previously stated most prostate cancers that respond to ADT eventually become castration resistant and reach the CRPC or mCRPC stage [9]. Here, chemotherapy is widely used whereby chemotherapeutic agents enter the bloodstream to reach metastatic sites and subsequently kill cancer cells. Currently, docetaxel and cabazitaxel are the principle forms of treatment and have shown to modestly prolong survival in patients with mCRPC [24, 25]. More recently, immunotherapy has emerged as a new age treatment where the immune system is manipulated to fight cancer cells [11]. For treatment of mCRPC robust immunotherapeutic strategies have not yet been identified;

however, a recent advancement was with the use of Sipuleucel-T which, is known to stimulate a T-cell immune response to fight the tumor cells in patients [26]. Collectively, despite the magnitude of research delved into prostate cancer each year there is no effective treatment for hormone-refractory disease, and it remains a compelling challenge in this field [11]. Although over the years several androgen-activity blocking agents, chemotherapies and now immunotherapies have shown to prolong the survival of mCRPC patients, it is clear that there is a need to look at some non-conventional players now that might lead to the development and progression of the disease in order to design effective therapeutic treatments.

1.4 Ras superfamily: historical overview

Investigation of the Rat sarcoma virus genes led to the discovery of Ras oncogenes around 1980, making Ras the founding member of the family of small GTPases [27]. Preliminary studies that identified these Ras oncogenes elucidated these proteins as small monomeric guanine triphosphatases (GTPases) encoding proteins of around 21 kDa. Today, this well characterized superfamily has grown to around 150 members and these have been implicated in the regulation of various cellular processes such as cell cycle progression, cell proliferation, differentiation, migration, polarity, regulation of gene expression and the cytoskeleton organization [28]. This highly conserved superfamily can be further divided into five subfamilies based on their structural and functional similarity: Arf, Rab, Ran, Ras and Rho (Figure 1.1). Thus far, the main functions associated with each family includes: The Ras proteins regulate gene expression, the Rho family predominantly modulates actin cytoskeletal organization, the Ran family is associated with nucleocytoplasmic transport and the Rab and Arf are involved with intercellular vesicle trafficking [27, 28]. Also, proteins from all the families mostly share a common molecular switch characteristic: cycling between an active guanosine triphosphate (GTP)-bound state and an inactive guanine diphosphate (GDP)-bound state. Their roles ranging from early development to day-to-day cellular activities emphasizes their importance to ensure proper development and maintain integrity within a tissue [29].

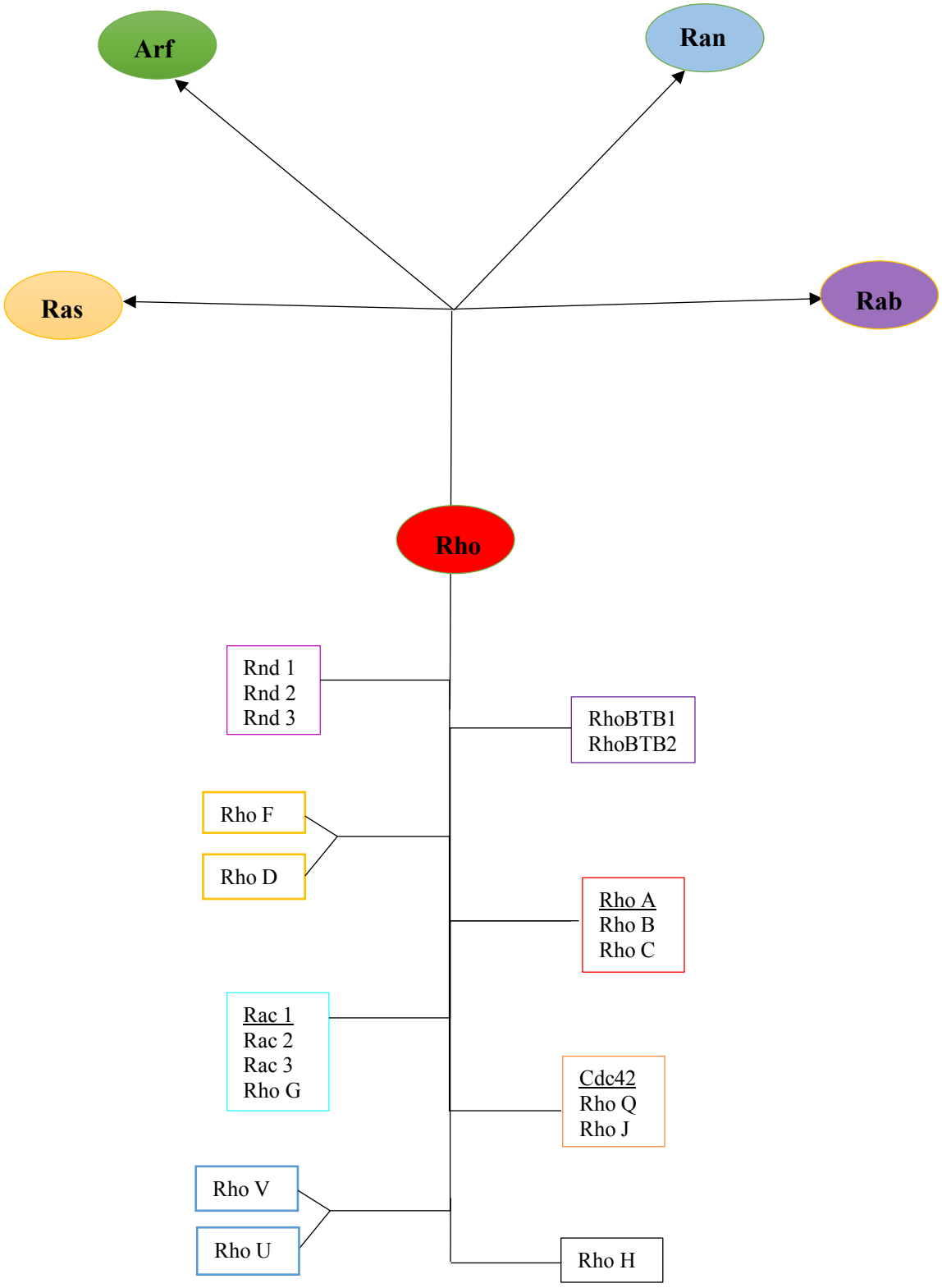
1.5 The Rho GTPase subfamily: Introduction

Ras homologA (rhoA) gene was initially isolated during the search for *ras* -related genes from sea mollusc *Aplysia*, thus it is the first member of the Rho family ever identified [30]. It is

Figure 1.1: Schematic of the Ras superfamily of GTPases.

Schematic of the mammalian Ras GTPase superfamily. Further classification of a subfamily within the Ras superfamily of GTPases: Rho guanosine triphosphatases (GTPases). The Rho subfamily of GTPases are comprised of 20 family members subdivided into eight families of which the best-characterized ones are Rho, Cdc42 and Rac1 (underlined).

Figure 1.1: Schematic of the Ras superfamily of GTPases



now known that the Rho subfamily is found in all eukaryotes and in mammals plays pivotal roles in cellular responses pertaining to the regulation of cytoskeletal dynamics, cell-cycle progression, axon guidance, motility and adhesion [29]. Thus far, 20 members (Figure 1.1) have been characterized that can be classified based on function, structural motifs and conserved primary amino acid sequences: the typical Rho proteins—the Rac1-related subfamily (Rac1, Rac2, Rac3 and Rho G), the RhoA-related subfamily (RhoA, RhoB and RhoC), the Cdc42-related subfamily [Cdc42, RhoJ (TCN), RhoQ (TCL)], the RhoBTB subfamily (RhoBTB1, RhoBTB2 and RhoBTB3), RhoD and RhoF; and the atypical Rho proteins—three Rnd isoforms: Rnd1, Rnd2 and Rnd3, RhoV and RhoU, and RhoH [31]. Further research characterized the typical family of Rho GTPases as conformational switches cycling between an active GTP-bound and an inactive GDP-bound state, much like their Ras counterparts [31]. The atypical proteins RhoV and RhoU are closely related in sequence to Cdc42 but display a high intrinsic guanine nucleotide exchange activity and are thus thought to be constitutively active [29, 31, 32]. Furthermore, RhoH and Rnd subfamilies lacks intrinsic -GTPase activity and cannot hydrolyze GTP therefore they are rendered as constitutively active [31]. The subsequent sections provide a brief overview of the structure, regulation and effectors of the typical members of the Rho family of GTPases.

1.6 The Rho GTPase subfamily: Structure

Rho proteins have a consensus sequence consisting of the core G-domain, a short insert region and a hypervariable C-terminal [33]. Briefly, the G-domain is a broadly conserved structure for the binding and hydrolysis of GTP and comprises of a six-stranded β -sheet surrounded by five α -helices. The difference between the GDP- and GTP- bound structural forms are attributed to switch I and switch II domains within this G-domain [34]. Furthermore, between the fifth β strand and fourth α helix is the landmark 13-residues long insert region that is a characteristic feature of Rho GTPases and distinguishes it from the Ras proteins [35]. Both the switch and the insert regions are able to interact with effectors proteins such as kinases, actin regulators and adaptor proteins in order to mediate downstream effects. Interestingly, knowledge of the structural property of Rho GTPases has made it possible to generate point mutations that allow selective interaction with effectors thus exerting only a limited number of downstream effects [36]. This has been useful to understand their function and has emerged as an attractive target for therapies to treat various malignancies. Lastly, the hypervariable C-terminus is essential for the spatiotemporal regulation

of the GTPases. In this domain is a -CAAX (C-cysteine, A-aliphatic residue, X-any amino acid) motif that enables Rho GTPases to undergo post-translation modifications by addition of lipophilic groups to the cysteine residue via prenylation (farnesylation or geranylgeranylation) and this anchors them to distinct membrane compartments which, is where they exert their functions [34].

1.7 The Rho GTPase subfamily: Regulation by nucleotide binding

In addition to being targeted to the subcellular membrane compartment, the Rho GTPases have to be in the GTP-bound state in order to allow interactions with downstream effectors (Figure 1.2). This is governed by three families of proteins: guanine-nucleotide exchange factors (GEFs) that facilitate exchange of GDP with GTP and render an active state; GDIs (Guanine Dissociation Inhibitors) which sequester Rho GTPases in an inactive form in the cytoplasm; and lastly, GTPase-activating proteins (GAPs) that reinstate the inactive conformation by accelerating hydrolysis of GTP-bound Rho proteins [29, 34]. These families of Rho regulators have been subsequently discussed in more detail.

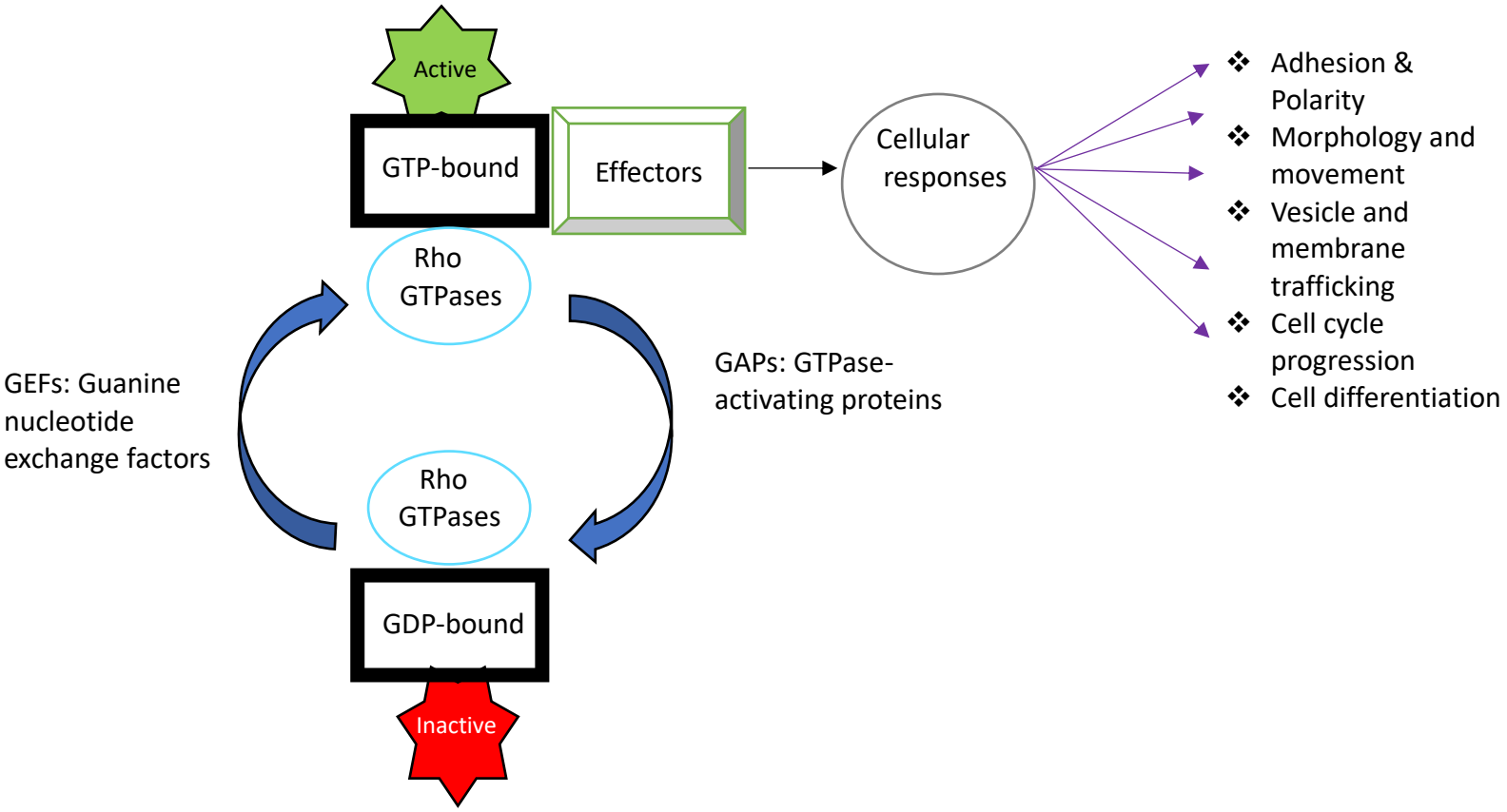
1.7.1 RhoGEFs

The first Rho guanine nucleotide exchange factor (GEF) to be identified was *Dbl* isolated as an oncogene from diffuse B-cell lymphoma cells [37]. Since then over 80 GEF's have been discovered and can be classified into the two following subfamilies based on structural similarity: Dbl and dedicator of cytokinesis (DOCK). The Dbl-family is by far the most represented and comprises of 69 members whereas for the Dock subfamily of GEF proteins around 11 members have been characterized to date. The Dbl GEFs subfamily contains two conserved regions: Dbl homology (DH) domain, followed by a Pleckstrin homology (PH) domain. The DH domain is responsible for catalyzing the exchange of GDP for GTP whereas the PH domain targets these proteins to plasma membranes—where they interact with Rho GTPases. The DOCK subfamily is structurally and functionally different than the DBL family of GEF proteins and it comprises of DHR1 and DHR2 domains. The former domain targets the GEFs to the plasma membrane while the latter domain is responsible for mediating the exchange of GDP to GTP [38]. Collectively, the main function of the RhoGEFs is to activate the Rho GTPases by mediating GDP-GTP exchange and this allows interaction of Rho GTPases with their effectors activating downstream signalling [39].

Figure 1.2: Rho GTPases act as molecular switches

This cycle is coordinated by two main classes of regulatory proteins: guanine-nucleotide exchange factors (GEFs) that facilitate exchange of GDP with GTP and render an active state; and GTPase-activating proteins (GAPs) that reinstate the inactive conformation by accelerating hydrolysis of GTP-bound Rho proteins. When the Rho GTPases are bound to -GTP it allows interaction with their target effectors, thus activating downstream signalling and mediating cellular responses.

Figure 1.2: Rho GTPases act as molecular switches



1.7.2 RhoGAPs

RhoGTPases when bound to GDP are inactive [34]. The rate of hydrolysis of GTP to GDP is slow and thus RhoGTPases require the help of another family of regulatory proteins known as the GTPase-activating proteins (GAPs) to increase the intrinsic ability of the GTPases itself to hydrolyze GTP and thereby inactivate downstream Rho GTP-driven signalling [40]. The first RhoGAP protein was identified in 1989 from the human spleen extract and since then 80 RhoGAP proteins have been characterized in humans [40, 41]. Unlike RhoGEFs which are sorted into two families, the RhoGAPs are mostly categorized by structure, function and sequence similarity. Most RhoGAPs have multidomain structures but the landmark conserved region is a 150 residue RhoGAP domain, which is comprised of an arginine residue (also known as the arginine finger) and is critical for binding to the GTP-bound Rho GTPases to stimulate GTP hydrolysis [34].

1.7.3 RhoGDIs

This subfamily of regulators of the Rho GTPases acts as inhibitors of the dissociation of GDP, as the name suggests. So far three proteins have been identified for the Rho family: RhoGDI1, RhoGDI2 and RhoGDI3, which are fairly conserved across eukaryotes. As mentioned, Rho proteins undergo lipid modifications at their COOH-terminus which allows these proteins to be anchored to membrane compartments. RhoGDI's are able to recognize the prenylated C-terminus of Rho proteins and this prevents localization of the GTPase at the membrane; thus impeding with its activation [42].

1.8 Rho Effectors

The regulation by GEFs and proper spatiotemporal targeting allows interaction of RhoGTPases with downstream effectors—implicating these proteins in various signalling pathways [34, 43]. Over 70 Rho effectors have been discovered so far and the most studied effectors are associated with the best characterized members of the Rho family of GTPases: RhoA, Rac1 and Cdc42. Briefly, a number of RhoA effectors have been identified such as Rho-kinase proteins, rhophilin, rhotekin, protein kinase N, mDia and more [34, 43]. These effectors generally comprise of a Rho-binding domain (RBD) which allows interaction with Rho when it is GTP-bound [44]. This interaction is typically characterized by the formation of stress fibres, generation

of contractile forces, regulation of microtubule dynamics, and cell adhesion [29]. Similarly, the effectors of Rac1 and Cdc42 contain a common sequence consisting of about 15 residues which is referred to as a Cdc42/Rac1-interactive binding motif (CRIB). The presence of this common domain allows them to share a number of effectors, although these initiate different cellular responses based on the GTPase [45]. Some examples of key effectors with the CRIB domain includes Wiskott-Aldrich-syndrome family scaffold proteins: Wiskott-Aldrich-syndrome Protein (WASP), WASP family Verprolin homologous proteins and p21-activated kinases, to name a few [43]. Briefly, the interaction of Cdc42 and Rac1 with their effectors typically results in the formation of actin-rich filopodia and lamellipodia, respectively; linking these two GTPases to actin polymerization [29]. Other cellular responses downstream of signalling from these two RhoGTPases include regulation of cell polarity, cell movement, cell cycle progression and the expression of genes. Owing to their key cellular roles, aberrations in Rho GTPase signalling has been implicated in neurological disorders, immunological diseases and oncogenic malignancies [29, 46, 47].

1.9 Rho GTPase influence in cancer

Recent progress in cancer research has ascribed classical hallmarks to cancer cells: 1) self-sufficiency in growth signals (oncogene activation), 2) insensitivity to growth inhibitory singles (loss of tumor suppressors), 3) unlimited proliferative potential, 4) evasion from apoptotic signals, 5) sustained angiogenesis and, 6) tissue invasion and metastasis [48]. Briefly, in order to become malignant, normal cells have to activate oncogenes and/or inactivate tumor suppressor genes—resulting in deregulated growth of cells. This growth originally starts in a localized region, then, expands to the surrounding tissues in a process coined epithelial to mesenchymal transition and is characterized by loss of cell-cell contacts and increased migration and invasiveness. Then, these cells eventually spread to other parts of the body through the vasculature or the lymphatic system in the process infamously classified as metastasis [49]. Rho GTPases play pivotal roles in day-to-day cellular signalling and it is unsurprising that they have been implicated in cancers [46]. Dysregulation of Rho proteins has been associated with all classical hallmarks of cancer [29, 46, 50]. Unlike their Ras counterparts such as K-Ras, N-Ras, and H-Ras,—frequently mutated in tumours—mutations in Rho GTPases themselves were not reported frequently until recently [46]. Instead, several lines of research alluded to altered expression levels or aberrant activity of either

Rho GTPases themselves or their regulators in human cancers [29, 46]. Now, with the advent of large-scale whole genome sequencing techniques this existing paradigm has shifted. By sequencing primary and metastatic tumors, it is evident that mutations in Rho GTPases also exist and are capable of predisposing to cancer development [50-52]. Subsequently, multiple mutations have also been reported in the classical Rho GTPases Rac1, Cdc42 and RhoA [50]. Thus, the following section summarizes two aspects in Rac1, Cdc42 and RhoA: 1) the historical evidence of aberrant activity or altered expression in the context of cancer and 2) the recent emerging evidence of the presence of driver mutations.

1.9.1 Rac1

Rac1 appears to be deregulated in terms of its level of expression and its activity in a multitude of human cancers [31, 52]. The initial evidence elucidating Rac1 as having a positive role in cancer was with the identification of a splice variant of Rac1 known as Rac1b. This variant is a result of an alternative splicing event that provides an extra 19 amino-acid residues. Functionally it has an increased ability to switch from the GDP to the GTP-bound form, often characterized as a "fast-cycling" GTPase [53]. Elevated expression of this isoform of Rac1 positively correlates with aggressiveness of the tumor and has a poor prognosis in lung, colon and breast cancer patients [54-56]. Moreover, genetic ablation of the *Rac1* gene itself was reported to impair the development of KRas-driven tumor formation in the skin [57]. In general, some cellular functions synonymous with Rac1 are: cell migration and invasion, vessel sprouting to induce angiogenesis and cell adhesion; all functions implicated in cancer [52, 56, 58-62]. Notably, blocking Rac1 in endothelial cells impairs vascular angiogenesis [63]. Attesting to this role, in lung cancer, vascular endothelial growth factor (VEGF) was shown to activate Rac1 and this had the potential for hematogenous metastasis [59]. Pertaining to its role in migration, decreasing the expression of Rac1 was shown to inhibit cell migration and invasion in colorectal cancer cells and gliomas, respectively [64, 65]. Also, in prostate cancer, a recent report demonstrated that Rac1 is overactivated and this positively correlates with disease progression of tumors [58]. By the same regard, in lung cancer, Rac1 inhibition has been suggested as a promising therapeutic target [61]. Lastly, in breast cancer, Rac1 provides resistance to drugs, thus its expression here again inversely correlated with patient survival [62].

Recently, as a result of large-scale genomic studies the two groups Krauthammer et al. and Hodis et al., have also identified a substitution of the amino acid proline to serine in Rac1 as a driver mutation enriched in about 5-10% of melanoma patients; making it the third most recurrent mutation in melanoma [66, 67]. This mutation renders the GTPase as fast-cycling and in agreement with this, the Rac1 (P29S) asserted a higher degree of binding with its effector p21-activated kinase (PAK) binding domain (PBD) [68].

1.9.2 RhoA

RhoA is critical for fundamental cellular functions such as gene expression, cell migration, adhesion, division and survival [34]. Subsequently, RhoA is implicated in almost all the hallmarks of cancer progression [46, 50]. However, there is contradictory evidence about whether RhoA plays a tumor suppressive or a tumor-promoting role in cancer [69]. Notably, in gastric cancer cell lines, inhibiting RhoA reduced proliferation by inducing a G1-S cell-cycle arrest [70]. Similarly, in cervical cancer, silencing RhoA also inhibits cell proliferation [71]. Additionally, an inhibitor of a downstream effector of Rho, Y-27632 has been shown to inhibit metastatic growth of PC-3 cells in immunocompromised mice [72]. Contrarily, sequencing analysis have identified tumor-suppressive functions of RhoA [51]. Briefly, in pediatric burkitt's lymphoma recurrent RhoA mutations were observed which caused a decrease in RhoA activity and this was associated with a poor prognosis in patients [73]. In agreement with this, in angioimmunoblastic T cell lymphomas and in peripheral T-cell lymphomas whole-genome sequencing in patients has identified a recurrent RhoA (G17V) mutation [74]. This mutation abolishes GTP-binding and thus impairs the ability of RhoA to interact with downstream effectors such as Rhotekin subsequently disrupting stress fiber formation and impairing migratory capacitates of the cell [74]. In addition, loss of RhoA was shown to exacerbate tumor growth in a lung cancer mouse model [75]. Another interesting *in vitro* study that corroborates with this notion was conducted in colon cancer cell lines where silencing of RhoA led to an increase in Wnt-signaling pathway and resulted in an increase in proliferation and tumor progression [76]. Lastly, in about 25% of diffuse-type gastric carcinoma patient's mutations were found in RhoA and these patients had poor prognosis [77]. There is a lack of mechanistic evidence available yet to reconcile the dual roles observed in the function of RhoA [69]. One possible explanation for this disparity may be the differential binding of downstream effectors leading to varied responses. Together, these findings address the high level

of regulation of the Rho family of proteins. Conventionally, RhoA and its downstream effectors were categorized as oncogenes, but emerging evidence proves that it may also function as a tumor suppressor in defined physiological conditions.

1.9.3 Cdc42

Cdc42 orchestrates actin remodelling, contributing significantly to cell migration and cell morphology. Thus, it is unsurprising that altered activity and expression of Cdc42 correlates with cancer mechanisms [78]. Several studies have indicated that Cdc42 is upregulated and induces tumorigenesis in melanomas, breast, colorectal and non-small cell lung cancers [79-83]. Notably, it was reported that overexpression of Cdc42 in colorectal cancer patients increases migration, invasion and proliferation which indicated that Cdc42 could be an anticancer drug target in this cancer model [81]. Furthermore, in melanoma patients Cdc42 expression inversely correlated with patient survival [82]. Moreover, in a breast cancer study it was deduced that ErbB2-driven tumors requires activation of the Cdc42 for their migratory abilities [83]. Contrary to this, the depletion of Cdc42 in liver cancers has been shown to promote tumour formation [84]. Interestingly, another study that reported a similar tumor-suppressor role of Cdc42 was observed in neuroblastomas [85]. Thus, much like RhoA, there is conflicting evidence about the role of Cdc42 in cells. There are two plausible explanations for this: 1) a positive or negative role of Cdc42 is confined to the tissue it is expressed in; 2) Perhaps it is the upstream regulators: the RhoGAPs and RhoGEFs, which might differently regulate Cdc42. Subsequently, this again reflects the complexity about expression, regulation and roles of these proteins in the context of cancer where a GTPase may promote one aspect of cancer progression but inhibit another.

1.10 Implication of Rho GTPase regulators in cancer

The Rho GTP-signaling pathways are quite complex. As mentioned that long-standing research highlights the role of Rho GTPases in cancer in three regards: 1) an alteration in protein levels; 2) changes in GTPase activity; and 3) direct mutations observed in human cancers [29, 46, 50, 52, 69, 78]. Evidently, perturbations in Rho-signalling can also be a direct consequence of aberrant activation of its effectors and/or regulators RhoGEFs and RhoGAPs. Thus, these regulators have also been implicated in several cancers [29, 46, 50]. Due to their ability to activate GTPases, RhoGEFs have generally been considered oncogenic whereas GAPs—inactivate

GTPases—have been assigned the role of a tumor suppressor [29, 46]. Now, various reports either attest to or contradict this existing dogma pertaining to the conventional roles of these proteins in cancer. In the next section are identified the GAPs and GEFs that have been implicated in cancer. As the focal point of this study is the role of a RhoGAP in cancer they have been discussed in more detail. Whereas, only four RhoGEFs—Ect2, Vavs, P-Rex1 and Tiam1—which have the strongest evidence in cancer metastasis and tumorigenesis [52], have been discussed briefly in this chapter.

1.10.1 RhoGEFs in Cancer

Ect2 is a RhoGEF specific for Rac1, Cdc42 and RhoA and was initially characterized for its role in cytokinesis. An increased expression of Ect.2 is observed in several tumor types including but not limited to lung, brain, bladder, pancreatic and ovarian cancers [86]. Some initial reports identifying its role in cancer were based on reports that suggested downregulation of Ect2 impaired the growth and invasion of lung and esophageal cancer cells [86]. Recently, it was shown that nuclear localization of Ect2 was correlated with transformation of ovarian cancer cells [87]. Subsequently, nuclear localization of Ect2 was shown to be required for Kras-driven lung tumorigenesis and cells devoid of Ect2 demonstrated reduced formation of tumors in these mouse models [88].

Phosphatidylinositol-3,4,5- trisphosphate-dependent Rac exchange factor 1 **(**P-Rex1) is a phosphatidylinositol 3-kinase–dependent Rac1-GEF that acts as a point of convergence for the Rho pathway and the PI3K signalling pathways. P-rex1 has been reported to be highly expressed in gliomas, melanomas, breast, thyroid, kidney, prostate, colon, pancreatic and ovarian cancers; making it a novel anticancer drug target [89, 90].

The VAV family of RhoGEFS is comprised of three members: VAV1, VAV2 and VAV3, and have activity towards the classical RhoGTPases Rac1, Cdc42 and RhoA. All three RhoGEFs have been reported to be significantly overexpressed in a cohort of cancers. Briefly, in patients with pancreatic ductal adenocarcinoma, VAV1 correlated with poor patient prognosis [91]. Subsequently, Vav1 was shown to be a driver of epithelial-mesenchymal (EMT) transition in ovarian cancer [92]. EMT is a key process allowing the dissemination of cancer cells and often results in metastasis which is usually considered the more aggressive form of a disease [93]. Also, some gain-of function mutations of VAV1 were found in peripheral T-cell lymphomas

[94]. Furthermore, Vav2 and Vav3 were shown to play critical roles in primary breast tumors and also during metastasis to the lungs [95]. Vav3 was shown to induce resistance to drugs and thus emerged as a novel therapeutic target to treat patients of ovarian cancer [96].

T-lymphoma invasion and metastasis-inducing protein (Tiam-1) is a GEF that displays exchange activity for Rac1, Cdc42 and RhoA. Crook et al., have summarized that Tiam1 is elevated in several malignancies, such as lymphoma, renal cell carcinoma, breast, colon, bladder, lung, cervical and prostate cancer [97]. However, contradictory reports suggest both tumor-promoting and tumor-suppressive functions of Tiam1; therefore, its function remains elusive. To demonstrate that point we can discuss the different reports arising from its role in colorectal cancer. Briefly, downregulation of Tiam1 expression was shown to decrease the migration of these cells, suggesting that it promotes cancer [98]. Concurrently, a recent report demonstrated that Tiam1 provides treatment resistance and thus enhances disease progression of colorectal cancer [99]. Alternatively, an interesting report elucidated that Tiam1 expression was shown to suppress the expression of YAP/TAZ and this corresponded to lower invasion of colorectal cancer cells [100]. Together, it is evident that TIAM1 exerts different roles—much like has been discussed about the GTPases, highlighting the complexity of Rho GTP-signalling in cancer.

1.10.2 RhoGAPs in cancer

ARHGAP1

ARHGAP1 (also known as p50RhoGAP or Cdc42GAP) was the founding member of the RhoGAP family, and has activity towards Cdc42, thus orchestrating actin remodelling and mediating cell motility [101]. ArhGAP1 has been implicated in cervical cancer in two cases: 1) Tumor samples had lower levels of this protein; 2) An *in vitro* study showed that overexpression of ArhGAP1 decreased cell proliferation, migration and invasion of cervical cancer cells—suggesting a tumor-suppressor role [102]. In contrast to this, a pro-invasive role of ArhGAP1 was identified in lung adenocarcinoma. Briefly, downstream of miR-34a, ARHGAP1 expression was suppressed and this attenuated tumor formation via a Rho GTPase–independent mechanism [103].

ARHGAP 3 (β2-chimaerin)

ARHGAP3/ β2-chimaerin exhibits activity towards Rac1 and has been implicated in cancer mostly as a tumor-suppressor [104]. Briefly, in breast cancer and gliomas a reduced expression of β2-chimaerin was observed [104]. Notably, downregulation of β2-chimaerin has been shown to promote proliferation and motility of breast cancer cells [105]. In agreement with this, overexpression of β2-chimaerin in breast cancer cells suppressed tumor initiation and reduced metastasis [106]. Alternatively, an interesting study by Medrano et. al. delineated an oncogenic role of β2-chimaerin in mammary tumorigenesis *in vivo*. Briefly, genetic ablation of β2-chimaerin in mice initially favored tumor initiation and increased the likelihood of development of breast cancer—similar to the *in vitro* results—but surprisingly delayed tumor progression [107]. Insight into why loss of β2- chimaerin delayed tumor progression needs further investigation and will enhance the understanding of the disparity observed in its role in breast cancer.

ARHGAP5 and ARHGAP35 (p190RhoGAPs)

p190A (ArhGAP35) and its closely related p190B (ARHGAP5) are structurally similar RhoGAPs with activity against RhoA. They are amongst the most studied RhoGAPs with significant functions relating to the actin cytoskeleton in the nervous system [108]. The first implication of p190A in cancer was because of its ability to associate with p120RasGAP, which was shown to regulate Ras-induced transformation of fibroblasts. Now, p190 proteins have been found to be mutated or having altered activity in various cancers [108].

p190A has been ascribed with tumor suppressor functions in various reports. One of the first implications to this end was described in a study conducted by Kusama et al., where p190RhoGAP reduced the invasion and metastasis of human pancreatic cancer cells [109]. Furthermore, in prostate cancer cells it was reported that inhibition of miR-20a activated p190 which then inhibited migration and invasion of prostate cancer cells [110]. Also, in melanoma, phosphorylation of p190RhoGAP led to its activation and subsequently reduced melanoma cell motility [111]. Moreover, in breast cancer, p190RhoGAP induced apoptosis in cells subsequently increasing sensitivity to drugs [112]. All the aforementioned tumor-suppressive activities of p190A have been relegated to its GAP-dependent inactivation of RhoA signalling. Additionally, a potential non-canonical tumor-suppressor function mediated by p190A which, could also serve as an explanation of its tumor-suppressor functions, was characterized in epithelial

cells. Briefly, p190A was shown to suppress canonical Hippo signaling which promoted contact inhibition of cell proliferation and reduced tumorigenesis [113]. Contrarily, there are a few studies reporting p190A as a potential oncogene [108]. Notably, in osteosarcoma and colorectal cancer, elevated expression of p190A was correlated with poor prognosis in patients [114, 115]. In accordance with this, in lung cancer a study reported that overactivation of p190A promoted proliferation, migration and invasion of the cells [116]. Additionally, in breast cancer cells, p190A was shown to increase cell motility and invasion [117]. Lastly, *ArhGAP35* gene emerged as altered in greater than 2% of cancers such as endometrial cancer, lung squamous cell carcinoma, lung adenocarcinoma, head and neck cancer and kidney cell carcinoma [108]. These mutations have been identified recently thus there is a lack of follow-up studies that implicate their roles in cancer progression.

As for p190B, in a cohort of cell lines comprising of: lung cancer, breast cancer, and hepatocellular carcinoma, and nasopharyngeal carcinoma, p190B was shown to have a pro-cancer role [108].

ARHGAP6

ARHGAP6 was identified in a human disease since part of this gene is deleted in microphthalmia with linear skin defects syndrome (MLS). Since then, it has been defined as a GAP with activities towards RhoA and Rac3 [118]. This RhoGAP acts as a tumor suppressor in cervical cancer where it is able to induce apoptosis and cell cycle arrest thereby suppressing cell proliferation, migration and invasion [119].

ARHGAP7, ARHGAP37 and ARHGAP38 (Deleted in Liver Cancer subfamily)

The DLC subfamily has been implicated with various cellular functions that include cell-matrix interaction, cell motility, actomyosin network remodeling, and regulation of cell polarity [120]. Each member of this family has been shown to mediate its activity against the GTPases RhoA, RhoB and RhoC [121]. *ARHGAP7/DLC-1* gene was first identified in 1998, on chromosome 8p with a homozygous deletion in a subset of highly metastatic liver cancers, designating it the name deleted in liver cancer [122, 123]. Subsequently, a liver cancer tumor model indicated that DLC1-negative tumors were more invasive and also a subset of metastatic liver cancer cell lines express low DLC levels [124]. These results conferred it a tumor suppressor

and over the years, it has been found as down-regulated or inactivated in a variety of other human cancers [125]. Since this is the most well-characterized GAP in cancer, its functions have been clearly established and only a few notable roles have been summarized in the following cancers: breast, prostate, and lung [125]. A simultaneous loss of the DLC1 and another tumor suppressor PTEN—which inactivates PI3K pathway—was shown to enhance breast cancer cell migration which emphasized that DLC1 is able to mediate cross-talk with other pathways to exert its tumor suppressive functions [126]. In prostate cancer, DLC1 was shown to modulate pathways downstream of RhoA resulting in an induction of the expression of E-cadherin and restoration of adherens junctions in cells [127]. In lung cancer, several *in vitro* and *in vivo* functional analysis have revealed DLC1 as a prognostic biomarker for lung cancer patients.

DLC2 (ARHGAP37) is another member of this subfamily which has contrasting evidence about its role [128]. In breast cancer, a study reported that lower levels of DLC2 expression corresponded with more aggressive tumors [129]. Furthermore, DLC2 was shown to inhibit cell proliferation and migration via inactivation of RhoA in hepatocellular carcinoma—suggesting that it has anti-cancer roles [130]. In contrast, in a study using mouse models in liver cancer revealed that genetic ablation of *DLC2* did not predispose patients to development of tumors [131]. A recent report provided slight clarity to the exact role of DLC2 in cancer. Notably, in a mammary cancer model the deletion of *DLC2* gene suppressed metastasis of tumors but it had no effect on the growth of primary tumors initially, suggesting a primarily metastasis-inhibiting rather than a tumor suppressive role of DLC2 in breast cancer and perhaps is also a plausible explanation for the results reported in hepatocellular carcinoma. [132]

DLC3 the third member of this subfamily, been assigned with anti-cancer roles and was reported to have lower mRNA levels in kidney, lung, ovarian, uterine and breast cancers [121]. Briefly, overexpression of DLC3 in breast and prostate cancer cells inhibited proliferation and colony-formation of cells [133]. Furthermore, in gastric cancer, DLC3 downregulation induced metabolic remodelling that allowed gastric cancer cells to migrate and invade [134]. Interestingly, this report suggested that DLC3 was able to transcriptionally repress the expression of certain genes such as MACC1 and this led to decreased tumorigenesis—a novel regulatory function for a GAP in the context of metabolism.

ARHGAP8

ARHGAP8 has been associated with several cytoskeletal remodeling functions and is a Cdc42-specific GAP [135]. Mutations in *ARHGAP8* have been observed in both breast and colorectal cancer. Interestingly, in some cases mutations were found in its RhoGAP domain potentially alluding to altered Rho activity; however, the clinical implications of these mutations have not been identified [136].

ARHGAP9

ARHGAP9 is a RhoGAP acting preferentially towards Cdc42 and Rac1. ArhGAP9 binds to the downstream players of the MAP kinase signaling pathway and prevents their activation; mediating crosstalk between the two pathways [137]. Interestingly, in hepatocellular carcinoma low expression of ARHGAP9 correlated with poor patient survival. Briefly, ARHGAP9 was shown to enhance the transcription of E-cadherin in these cells thereby inhibiting tumorigenesis [138]. In contrast to this, in breast cancer ARHGAP9 levels are inversely correlated with survival in patients [139]. However, further investigation is required to ascertain the role of this GAP in cancer.

ARHGAP10/ ARHGAP21/ (GRAF2)

GRAF2 inactivates RhoA and Cdc42. It has been identified with tumor-suppressor activity in glioblastoma, prostate, breast, ovary and lung cancer. [140-146]. Briefly, two mechanisms have been identified: 1) Overexpression of ARHGAP10 inhibited proliferation and tumorigenicity in ovarian cancer cells by inhibition of the activity of Cdc42 [145]. In agreement with this, in lung cancer overexpression of ARHGAP10 inhibits the motility and invasion of the cells through a mechanism dependent on Cdc42. [144]. Furthermore, downregulation of ARHGAP21 in glioblastoma cells resulted in migration of cells via increased Cdc42 activity [141] Concurrently, in prostate cancer cell lines its depletion increased migratory capacity of these cells [140]; 2) Beyond its conventional role of the suppression of Cdc42, a recent study identified miR-3174 as upstream target of ARHGAP10 in gastric cancer cells. They reported that ARHGAP10 promoted cell apoptosis in these cells [143]. Moreover, a genome-wide study in breast cancer linked a single-nucleotide polymorphism in ARHGAP10 to survival in patients [146]. Lastly, it was reported that

ARHGAP21 is overexpressed in head and neck squamous cell carcinoma expression and was suggested as a potential therapeutic target for this cancer type [142].

ARHGAP11A

ARHGAP11A is a GAP specific for RhoA. Recently, it was identified as an oncogenic GAP since it was essential for basal like breast cancer cell growth. Briefly, a depletion of ARHGAP11A lead to a cell cycle arrest mediated by p27 [147, 148].

ARHGAP13 and ARHGAP14

ARHGAP13 is also known as Slit-Robo GTPase-activating protein1 (SRGAP1) and has activity towards to Cdc42 and RhoA. SRGAP1 is implicated downstream of Slit-Robo pathway acting as a negative regulator of neuronal migration [149]. Subsequently, it has been shown to inhibit glioma cell invasion by inhibiting the activity of its target GTPase Cdc42 [150]. In support of this notion, in colorectal cancer it inhibited migration of these cells by reducing the activity of Cdc42 [151]. There have also been some mutations of SRGAP1 reported in ovarian and papillary thyroid carcinomas. Interestingly, the missense mutations of SRGAP1 observed in papillary thyroid carcinoma were proposed to impair its Cdc42 inhibition ability; thus, confirming a key regulatory role of SRGAP1 towards Cdc42 signalling in cancer [152]. In contrast to its tumor-suppressor function, SRGAP1 was identified as a promoter of metastasis of gastric cancer [153]. Briefly, it was reported that in regular conditions SRGAP1 expression was suppressed by miRNAs which resulted in inhibition of the growth of cancer cells [153].

The srGAP family consists of four members in total: srGAP1, 2, and 3, and a more distantly related member, srGAP4 [149]. Another member of this family that has been implicated in breast cancer as a tumor-suppressor is the Rac1-specific GAP srGAP3 (ARHGAP14) [154]. Briefly, it was reported that it negatively regulated Rac1 activity and subsequently activating Rho and its effector ROCK, thus orchestrating actomyosin contractility and inhibiting breast cancer cell invasion [154].

ARHGAP15

ArhGAP15 specifically promotes hydrolysis of Rac1-GTP and has been identified as a tumor suppressor in glioma, pancreatic ductal adenocarcinoma and colorectal cancers [155-

157]. Briefly, in gliomas, the forkhead transcription factor 3 upregulates the expression of ArhGAP15 resulting in an inhibition of Rac1 signalling and impeding the ability of these cells to migrate [156]. Interestingly, in colorectal cancer cell lines ARHGAP15 overexpression induced an increase in the expression of another tumor-suppressor PTEN which, decreased the phosphorylation of AKT leading to decreased tumorigenesis [155]. Furthermore, in pancreatic cancer, decreased expression of ARHGAP15 correlated with poor survival in patients[157]. Lastly, in a large-scale sequencing study found ARHGAP15 to be downregulated in urinary bladder cancer [158].

ARHGAP17/ Nadrin (RhoGAP interacting with CIP4 homologues, RICH1)

Nadrin (also called, RICH1 and ArhGAP17) targets the three most widely studied GTPases RhoA, Rac1 and Cdc42 and is well characterized for its role of regulating the actin cytoskeleton in the nervous system [159]. ARHGAP17 was identified as a tumor suppressor in colon cancer because it negatively regulated the Wnt/β-catenin signaling pathway resulting in decreased tumorigenesis [160]. Additionally, in cervical cancer, manipulation of ARHGAP17 expression increased expression of cell cycle arrest genes p21 and p27 resulting in suppressed tumorigenesis [161].

ARHGAP18

ARHGAP18 has GAP activities towards RhoA and RhoC. A recent report implicated ARHGAP18 as a promoter of triple negative breast cancer (TNBC) cell proliferation and migration. [162]. In concert with this, reports from hepatocellular carcinoma suggested that overexpression of ARHGAP18 increased cell migration. [163].

ARHGAP24

ARHGAP24 (FilGAP) preferentially inactivates Rac1 and is also implicated downstream of Rho, thus inhibiting cell protrusion and promoting cell contraction, respectively [164]. Several lines of evidence suggest that ARHGAP24 acts as a tumor suppressor. Subsequently, genetic depletion of ARHGAP24 impaired extravasation of breast cancer cells *in vivo* [165]. In concordance with this a study by Feng et al., in TNBC cells, reported that ARHGAP24 activity is negatively regulated leading to increased motility of these cells [166]. Also, ARHGAP24 was

reported to inhibit the migratory and invasive capacity of lung cancer cells via negative regulation of the Wnt/ B-catenin pathway[167]. Lastly, two independent studies have reported that overexpression of ARHGAP24 in renal cell carcinoma cells significantly reduces proliferation and promotes cell apoptosis [168, 169].

ARHGAP26 (GRAF1)

GTPase regulator associated with focal adhesion kinase (GRAF1), also known as ARHGAP26 is a RhoA and Cdc42 GAP. GRAF1 has been associated with clathrin-mediated endocytosis, membrane fusion, cell migration and cell-cell contacts [170]. Several lines of evidence allude to a tumor-suppressive function of ArhGAP26 in cancer. Firstly, ablation of ARHGAP26 was shown to result in the development of promyelocytic leukemia [171]; Second, a mutation in ARHGAP26 inversely correlated with survival in acute myeloid leukemia patients [172]; Third, downregulation of ARHGAP26 in glioblastoma was associated with increased cell proliferation and migration [173]; Fourth, in ovarian cancer cells ARHGAP26 was shown to suppress the motility and invasion of ovarian cancer cells [174]; Fifth, in gastric cancers, mutations in *ARHGAP26*, resulted in disruption of cellular epithelial structures and correlated with increased invasiveness [175].

ARHGAP29

ARHGAP29 or PTPL1-associated RhoGAP (PARG1) displays activity towards RhoA. In renal cell carcinoma one report alluded a tumor-promoting role of PARG1 where it was able to increase proliferation and invasion of these cells [176].

ARHGAP30

ARHGAP30 is a RhoA and Rac1-specific RhoGAP. ARHGAP30 has recently been implicated with tumor suppressive functions in colorectal and lung cancer [177, 178]. Briefly, a novel regulatory role for a RhoGAP was identified in colorectal cancer where ArhGAP30 enhanced the acetylation of p53 by binding to p53 and P300 (acetyltransferase), thereby facilitating P300-mediated acetylation of p53 resulting in induction in cell cycle arrest and apoptosis pathways—inhibiting tumorigenesis [177]. Further attesting to its anti-cancer role, in

lung cancer cells, ARHGAP30 was shown to repress the Wnt-signalling pathway and thereby suppressing migration, invasion and proliferation of these cells[178].

ARHGAP31

ARHGAP31 or Cdc42 GTPase-activating protein (CdGAP) is specific towards Rac1 and Cdc42. CdGAP has been implicated in the context of metastatic breast cancer cells. Notably, the level of CdGAP protein and mRNA levels were increased in mammary tumour explants expressing an activated form of the ErbB2 receptor [179]. In response to TGF-β, CdGAP can promote the motility and invasion of these cells. Interestingly, knockdown of CdGAP in these cells induced expression of E-cadherin [179]. More recently He et al., demonstrated that in breast cancer cells, CdGAP mediates the repression of E-cadherin transcription by forming a complex with Zeb2 which is a well identified co-repressor of E-cadherin. Surprisingly, this is a GAP-independent function of CdGAP and highlights another novel regulatory and unconventional role of a RhoGAP [180].

ARHGAP43/ SH3BP1 (SH3 Domain Binding Protein 1)

SH3BP1 (also known as 3BP1) displays a GAP activity toward Rac1 and Cdc42 [181]. It has been implicated in cervical and hepatocellular carcinomas. In both cases downregulation of SH3BP1 increased levels of active Rac1, and its effector WAVE2, leading to increased aggressiveness of the tumors [182, 183].

ARHGAP45/HMHA-1 (Human Minor Histocompatibility antigen-1)

HMHA-1 also known as ARHGAP45 is a recently characterized RhoGAP with activities towards Rac1 and RhoA [184]. It encodes the minor histocompatibility antigen-1 (HA-1), which is directly correlated with acute myeloid leukemia, thus implying a connection of HMHA-1 with hematological malignancies [185].

ArfGAP with RhoGAP domain, Ankyrin repeat and PH domain (ARAP3)

ARAP3 regulates both Arf- and Rho-GTPases, mediating cross-talk between two classical GTPase pathways [186]. Overexpression of ARAP3 in a metastatic gastric carcinoma cell line suppressed tumor cell dissemination in mice [187]. Interestingly, a mutation in the RhoGAP

domain of ARAP3 inhibited dissemination thus hinting towards a pathway involving the regulation of the GTPases [187]. Contrarily, a whole exome sequence analysis identified ARAP3 as a promoter in papillary thyroid carcinoma; however, the functional significance of this mutation has to be causally defined [188].

RACGAP1 (MgcRACGAP1)

RacGAP1 can inactivate Rac1 and Cdc42 and has been identified as an oncogenic GAP in several malignancies such as meningiomas, uterine carcinosarcoma, colorectal, gastric, breast and ovarian cancer [147, 148, 189-193]. Interesting studies on basil-like breast cancer revealed another mechanism of RacGAP1 where depletion of this protein led to an increase in the cyclin dependent kinase inhibitor CDKN1A/p21 and this was associated with an increase in senescence in these cells [147]. Furthermore, Ke et al. reported that RacGAP1 correlated with tumor recurrence and poor prognosis in meningioma [190]. Moreover, a study of gastric cancer patients revealed that in a subset of these tumors, RacGAP1 expression was associated with particularly invasive tumors [189]. Additionally, in colorectal cancer, opposing prognostic outcome was identified in patients based on RacGAP1 nuclear or cytoplasmic expression where the former corresponded to poor survival and the latter was associated with favorable prognosis; however, there is a lack of mechanistic evidence to explain the differential outcome of patient survival based on expression [191]. Also, in uterine cancer, RacGAP1 was reported to promote the motility invasion of the cells by regulating the signal transducer and activator of transcription 3 (STAT3) pathway [193]. Lastly, in ovarian cancer the expression of RacGAP1 positively correlated with lymph node metastasis [192].

RALBP1 (RIP1, RLIP76)

RalBP1 was cloned as a Ral effector protein mediating the crosstalk between Ras and Rho GTPases pathways [194]. One of the first roles ascribed to RalBP1 was associated with glutathione-conjugated electrophile transport. Further characterization of its activity as a transporter revealed that it influences not only transport of glutathione-conjugates but also of chemotherapy drugs such as doxorubicin [195]. Subsequently, it was demonstrated that RalBP1 inferred drug resistance in certain cell lines and was able to defend cancer cells from apoptosis [196]. Concurrently, inhibition of RalBP1 via antibodies or anti-sense oligonucleotides in various

cell lines increased cytotoxicity and cell death [195, 197]. Over the years, apart from its role as a transporter it has been allotted other pro-oncogenic roles. Particularly, its depletion diminished the ability of prostate cancer PC-3 cells to form a tumor and also abrogated the ability of these cells to metastasize [198]. In good agreement with this, RalBP1 protein expression was deemed a predictor of poor survival of colorectal cancer patients [199].

Summary

Conventionally been considered as tumor suppressors RhoGAPs inactivate the functions of Rho GTPases—terminating downstream signalling [34]. However, as is evident from the discussion above RhoGAPs, can be ascribed with both tumor-suppressor and oncogenic functions (Table 1.1). Some plausible explanations for this disparity include: Firstly, RhoGAPs can have a tissue-specific pattern of expression thus depending on the cancer type, they exert different functions; Second, the number of RhoGAPs identified thus far outnumbers the 20 Rho GTPases that exist in humans by an almost 4:1 ratio. This allows some RhoGAPs to impart their functions towards either one or several members of the Rho GTPase subfamily; Third, GAPs might selectively activate pathways downstream of the Rho GTPase; fourth, GAPs can themselves act as scaffold proteins, effectors or transcription factors, all of which allow cross-talk between Rho GTPases and other signalling pathways in cancer [40]. Conclusively, given the involvement of the RhoGAPs in cancer described above, makes them attractive and novel targets for therapies.

The goal of my project was to investigate the role of a member of this large family of RhoGAP's: Cdc42 GTPase-activating protein (CdGAP), and its role and function identified thus far has been described in the following sections.

Table 1.1 RhoGAPs implicated in cancer

Blue- tumor suppressor role; Red- Oncogenic role; Purple- Mutations identified

Symbol	Name	Rho Target	Cancer type
ARHGAP1	Rho GTPase activating protein 1	Cdc42	Cervical cancer, Lung cancer
ARHGAP3	Rho GTPase activating protein 3	Rac1	Breast cancer, Gliomas, Breast cancer
ARHGAP5	Rho GTPase activating protein 5	RhoA	Lung cancer, Breast cancer, Hepatocellular carcinoma, Nasopharyngeal carcinoma
ARHGAP6	Rho GTPase activating protein 6	RhoA	Cervical cancer
ARHGAP7	Rho GTPase activating protein 7	RhoA, RhoB, RhoC	Breast cancer, Prostate cancer, Lung cancer
ARHGAP8	Rho GTPase activating protein 8	Cdc42	Breast cancer, Colorectal cancer
ARHGAP9	Rho GTPase activating protein 9	Rac1, Cdc42	Hepatocellular carcinoma, Breast cancer
ARHGAP10	Rho GTPase activating protein 10	Cdc42, RhoA, RhoC	Glioblastoma, Prostate cancer, Breast cancer, Ovary cancer, Lung cancer, Head and neck cancers

ARHGAP11A	Rho GTPase activating protein 11A	RhoA	Basel like-breast cancer
ARHGAP13	Rho GTPase activating protein 13	Cdc42, RhoA	Glioma, Colorectal cancer, Ovarian cancer, Papillary thyroid carcinomas, Gastric cancer
ARHGAP14	Rho GTPase activating protein 14	Rac1, Cdc42	Breast cancer
ARHGAP15	Rho GTPase activating protein 15	Rac1	Glioma, Colorectal cancer, Pancreatic cancer, Urinary bladder cancer
ARHGAP17	Rho GTPase activating protein 17	Rac1, Cdc42, RhoA	Colorectal cancer, Cervical cancer
ARHGAP18	Rho GTPase activating protein 18	RhoA, RhoC	Breast cancer, Hepatocellular carcinoma
ARHGAP24	Rho GTPase activating protein 24	Rac1	Breast Cancer, Lung cancer, Renal cell carcinoma
ARHGAP26	Rho GTPase activating protein 26	RhoA, Cdc42	Promyelocytic leukemia, Glioblastoma, Ovarian cancer, Gastric cancer, Acute myeloid leukemia
ARHGAP29	Rho GTPase activating protein 29	RhoA, Rac1, Cdc42	Renal cell carcinoma

ARHGAP30	Rho GTPase activating protein 30	RhoA, Rac1	Colorectal cancer, Lung cancer
ARHGAP31	Rho GTPase activating protein 31	Rac1, Cdc42	Breast cancer
ARHGAP35	Rho GTPase activating protein 35	RhoA	Pancreatic cancer, Prostate cancer, Melanoma, Breast cancer, Osteosarcoma, Lung cancer, Breast cancer, Colorectal cancer, Endometrial cancer, Lung cancer, Head and neck cancer and Kidney cancer
ARHGAP37	Rho GTPase activating protein 37	RhoA, RhoB, RhoC	Breast cancer, Hepatocellular cancer, Breast cancer, Hepatocellular carcinoma
ARHGAP43	Rho GTPase activating protein 43	Rac1, Cdc42	Cervical cancer, Hepatocellular carcinoma
ARHGAP45	Rho GTPase activating protein 45	Rac1, RhoA	Leukemia
ARAP3	ArfGAP with RhoGAP domain, Ankyrin repeat and PH domain 3	RhoA	Gastric cancer, Papillary thyroid carcinoma

RacGAP1	MgcRACGAP	RhoA	Meningiomas, Uterine cancer, Colorectal cancer, Gastric cancer, breast cancer and ovarian cancer
RALBP1	RalA binding protein 1	Rac1, Cdc42	Prostate cancer, colorectal cancer
DLC3	Deleted in liver cancer 3	RhoA	Breast cancer, prostate cancer, gastric cancer, ovarian cancer, uterine cancer

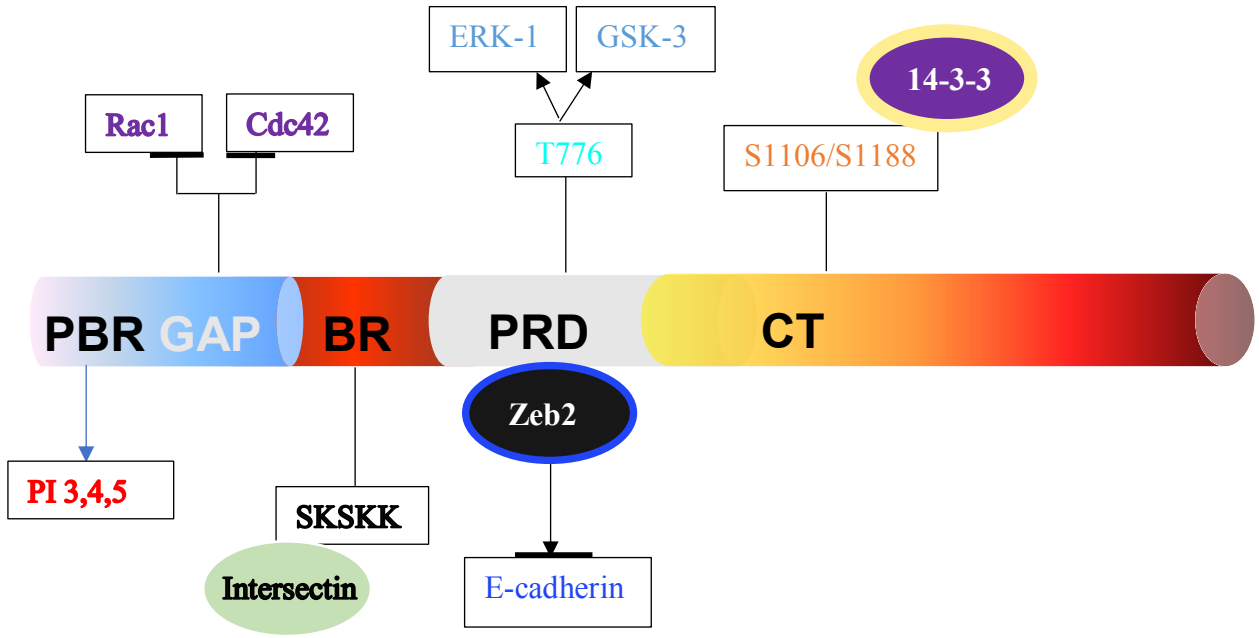
1.11 Cdc42 GTPase-activating protein/ARHGAP31: Overview

CdGAP was first cloned from a yeast-two- hybrid screen using Cdc42L61-Y40C mutant as bait and identified as a murine proline-rich 820 amino acid GAP [36]. CdGAP has activities towards Rac1 and Cdc42 but not RhoA, both *in vivo* and *in vitro* [36, 200]. Later, this protein was found to be of a larger 1425 amino acid full-length protein. CdGAP is now characterized in both humans (1444aa) and the mouse [201]. It is expressed ubiquitously and enriched in heart and muscle tissues in humans [201]. Initial studies revealed that overexpression of this protein in diverse cell lines is known to induce cell rounding, reduce cell spreading and decrease lamellipodia formation through disruption of actin and focal adhesions; identifying it in regulation of actin cytoskeleton [36]. Over the years the multidomain structure of CdGAP has been characterized (Figure 1.3). Briefly, it is comprised of a N-terminal Polybasic region (PBR) followed by a GAP domain, a central region rich in basic residues (BR), a proline-rich domain (PRD) and an extended C-terminal region. The various functional domains within its structure allow cross-talk with players of a multitude of signaling pathways; many of which modulate its conventional GAP activity (Figure 1.3). At the N-terminal region of CdGAP is the PBR that can bind phospholipids such as phosphatidylinositol 3, 4, 5-triphosphate (PIP3), which allows CdGAP to be targeted to the plasma membrane and is essential for GAP activity of CdGAP [202]. The highly conserved RhoGAP domain has activity towards the GTPases Rac1 and Cdc42 [36, 201]. Followed by the GAP domain is BR and this region of CdGAP can interact with actopaxin—a focal adhesion protein—and this induces the localisation of CdGAP to focal adhesions where it inhibits spreading of osteosarcoma cells [203]. The PRD of CdGAP is a key domain for the regulation of CdGAP due to its interaction with various kinases thus allowing cross-talk with other signalling pathways [179, 180, 204, 205]. Glycogen Synthase Kinase-3 (GSK-3) and Extracellular Signal-regulated Kinase-1 can phosphorylate CdGAP on residue T776, located in its PRD; this inhibits its activity [204, 205]. Additionally, the PRD of CdGAP is an essential component in the synergistic interaction between TGFβ and ErbB-2 signaling pathways during breast cancer cell migration and invasion which will be described in detail in the next section [179, 180]. The C-terminus of CdGAP is involved in its regulation and implicated in a human disease [206]. Briefly, two major phosphorylation sites—Ser-1093 and Ser-1163—located in the extended C-terminus region of CdGAP were shown to be phosphorylated by the AGC kinase RSK1, leading to the recruitment of 14-3-3 adaptor proteins and subsequently sequestrating CdGAP in the cytoplasm; negatively

Figure 1.3: **Schematic representation of the structure of CdGAP and summary of CdGAP functions.**

PBR: Polybasic Region-The PBR of CdGAP associates with phosphatidylinositol 3,4,5 triphosphate with high affinity that positively regulates the GAP activity of CdGAP; GAP: GTPase activating protein- specific for Rac1 and Cdc42, BR: Central region rich in Basic Residues-BR region of CdGAP binds a scaffold protein called intersectin; PRD: Proline-Rich-Domain- The Glycogen Synthase Kinase-3 and Extracellular Signal-regulated Kinase-1 can phosphorylate CdGAP on residue T776; this inhibits its activity. Also, the PRD of CdGAP can associate with Zeb2 in breast cells and together they co-repress E-cadherin transcription, thus promoting breast cancer metastasis; CT: C-terminal- is able to associate with 14-3-3 adaptor proteins and this restricts CdGAP in the cytoplasm inhibiting its membrane targeting and this association inhibits the GAP activity of CdGAP.

Figure 1.3: Schematic representation of the structure of CdGAP and summary of CdGAP functions



regulating its GAP activity [206]. Furthermore, Ajuba—a scaffold protein—is able to bind the C-terminus of CdGAP and this preserves the junctions in a GAP-dependent manner in keratinocytes [207]. Lastly, gain-of-function mutations in the C terminal of CdGAP in patients has been associated with the Adams-Oliver syndrome (AOS) characterized by limb and heart anomalies. This finding was instrumental as it deciphered a novel role of a RhoGAP in human disease and implied that aberrant activity of CdGAP can result in malignancies [208].

AOS is characterised by the congenital absence of skin in the scalp referred to as aplasia cutis congenita (ACC) and terminal transverse limb defects, typically affecting proper formation of distal phalanges or digits [208]. The mutations in CdGAP associated with AOS are autosomal dominant truncation mutants lacking portions of the C-terminal domain which results in enhanced GAP activity of CdGAP leading to a reduction in active Cdc42 [208]. This is characterized by a disruption in Cdc42-induced actin cytoskeletal structures which, serves as a rationale for the defective early organogenesis observed in patients with these mutants. In addition to the skin, AOS patients also show defects in angiogenesis [208, 209]. Angiogenesis is a process that can be described as the formation of new blood vessels and one of the most important factors for this process is vascular endothelial growth factor (VEGF) [210]. Also, it is well known that Rac1 is activated resulting in protrusions in the leading edge of endothelial cells that guide the newly formed sprouts during angiogenesis [211]. As mentioned previously, RhoGAPs have a tissue specific pattern of expression and CdGAP expression is enriched in the heart; however, no cardiac defects were seen in AOS patients harboring CdGAP-specific mutations thus indicating that mutations in CdGAP do not account for the cardiac abnormalities observed in AOS. Despite this, an increased CdGAP expression was recently reported in endothelial cells notably, the human umbilical vein endothelial cells (HUVECs) [212]. Accordingly, in HUVEC cells, CdGAP was shown to interact with the VEGF receptor-2 (VEGFR2) and this interaction is key to allow Rac1 activation which is necessary for sprouting of vessels. Agreeably, a depletion in the expression of CdGAP either *in vitro* or *in vivo* was associated with severe defects in VEGF-dependent angiogenesis. These data have strongly suggested a role of CdGAP in angiogenesis as well [212]. Remodelling of the cytoskeleton and cell migration are two key cellular processes that endothelial cells undergo in order for new capillaries to form. Thus, it is not surprising that CdGAP which has been independently implicated in both the processes in other contexts, also has a role in angiogenesis. Collectively, extensive studies by means of genetic and biochemical approaches has

emerged CdGAP has a versatile protein which has been shown to be involved in basic cellular functions, developmental processes such as angiogenesis, in human disease and recent reports suggest a strong link of CdGAP in breast cancer.

1.11.1 Implication of Cdc42 GTPase-activating protein in cancer

Cdc42 GTPase-activating protein (CdGAP) dons many hats by interacting with different pathways and has also been implicated in cancer [179, 180]. First reports of CdGAP in cancer were characterized in a breast cancer model with mouse mammary cancer cell explants that express the activated Neu/ErbB-2 receptor. Interestingly, in these cells, elevated CdGAP levels were reported. Downstream of the TGF-β pathway the ErbB2-transformed breast cancer cells have increased invasion. Subsequently, downregulation of CdGAP expression by short interfering RNA (siRNA) specifically inhibited the ability of TGFβ to induce cell motility and invasion in these cells. Interestingly, this effect of CdGAP was found to be a GAP-independent function and mediated through the PRD region. Moreover, the expression of CdGAP in these cells was found to be inversely correlated with E-cadherin protein levels [179]. Cancer cells progress and metastasize via loss of intercellular contacts which is characterized by downregulation of proteins such as E-cadherin; a hallmark of cancer. Previously, CdGAP was shown to act as a negative regulator of cell-cell contacts in keratinocytes and in these cells the levels of CdGAP inversely correlated with E-cadherin [207]. Accordingly, downregulating CdGAP expression in breast cancer cells resulted in an increase in E-cadherin levels and a reinstatement of intercellular junctions. The mechanistic basis for this inverse correlation was identified in a recent report where CdGAP was shown to form a functional protein complex with the transcriptional repressor Zeb2 to suppress E-cadherin expression in these cells. Again, the function identified here is independent of the GAP domain and mediated via the PRD of CdGAP. Moreover, loss of CdGAP in ErbB2-transformed breast cancer cells impaired tumor growth and suppressed metastasis to the lungs in mice. Lastly, immunohistochemical analysis of human breast cancer tissue specimens indicated that CdGAP is frequently overexpressed in these tumors and elevated CdGAP expression is associated with a poor prognosis for patients [180]. In agreement with all these findings, CdGAP can be classified as an oncogenic GAP and surfaces as a promising therapeutic target for treatment of breast cancer.

1.12 Rationale and Objectives for this study

In cancer, the cytoskeletal organization can be perturbed by the increased activation of oncoproteins, or loss-of-function of tumor suppressor proteins. As Rho proteins are major determinants of cytoskeletal organization, the actions of their regulators and effectors are tightly constrained to ensure equilibrium during normal cellular responses and to avoid subversion during oncogenic transformation. A link between Rho GTPases along with their regulators and cancer has been clearly established. The existing paradigm in the field ascertains Rho GTPases and their positive regulators RhoGEFs as oncogenes whereas the RhoGAPs—negative regulator—are conventionally viewed as tumor suppressors. As discussed previously, it is now clear that RhoGAP proteins are implicated in a lot more than the GTPase-Activating Protein role. In particular, CdGAP serves as an oncogenic GAP, where increased CdGAP protein levels correlated to tumor progression and metastasis in breast cancer, making it a potential therapeutic target and a possible biomarker to screen breast cancer patients for this protein. Apart from the direct implication in cancer CdGAP has been associated with several hallmarks of cancer such as cell migration, disruption of cell-cell contacts and angiogenesis. Report from the cbioportal has also implicated the presence of highest *CdGAP* gain/amplification in prostate cancer tumors (Figure 1.4). Thus, the goal of my project was to extend these studies and investigate the role of CdGAP in prostate cancer, in two main objectives:

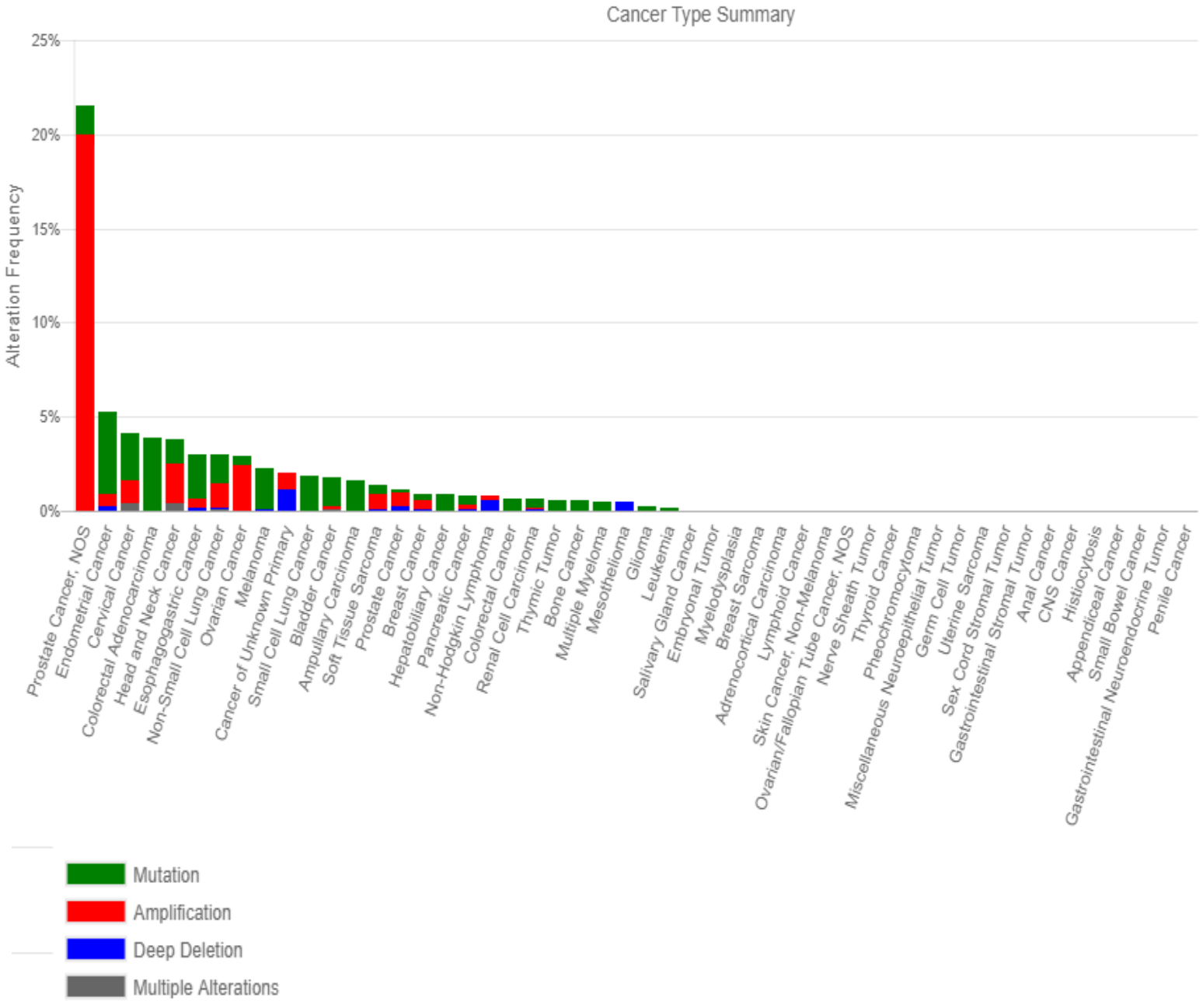
1: To demonstrate a potential role of CdGAP in prostate cancer we investigated its regulation of human prostate cancer cell proliferation, adhesion, migration and invasion through *in vitro* and in vivo experiments.

2: To elucidate and characterize the mechanism of action of CdGAP in prostate cancer cell via investigation of signalling pathways under the influence of CdGAP expression in prostate cancer cells we performed a global analysis of CdGAP gene targets in prostate cancer cell through quantitative-deep RNA sequencing.

Figure 1.4: Alterations in CdGAP/ARHGAP31 gene in human cancers (cbioportal).

Cohort of cancer patients with alterations of CdGAP/ARHGAP31 gene from www.cbioportal.org represents highest gene amplification/ gain in prostate cancer.

Figure 1.4: Alterations in CdGAP/ARHGAP31 gene in human cancers (cbioportal)



1.13 Preface to Chapter 2

As alluded to in chapter 1, CdGAP is a multidomain GAP specific to Rac1 and Cdc42, two well-characterized small GTPases. CdGAP was shown to be required for migration and invasion induced by TGF-β in breast cancer cells. Furthermore, a previously unknown nuclear function for CdGAP was characterized where it cooperates with the transcriptional repressor to represses E-cadherin transcription in breast cancer cells; a GAP-independent function of CdGAP. Currently the oncogenic functions of CdGAP within the vast regulatory network of Rho GTPase signalling are unclear at a global biological level and largely confined to breast cancer. Whether CdGAP exerts similar or different roles in other cancer remains elusive. In the second chapter, we are extending this analysis to prostate cancer with the aim that the proposed studies will highlight a novel and as yet uncharacterized molecular function of CdGAP in regulation of prostate cancer cell behavior and migratory processes. Using human prostate cancer cell lines, we have identified a novel role of CdGAP in the aforementioned processes and revealed it as a potential therapeutic target and probable biomarker of prostate cancer.

Chapter 2

The Rac1/Cdc42 regulator CdGAP is a positive modulator of prostate tumorigenesis

The Rac1/Cdc42 regulator CdGAP is a positive modulator of prostate tumorigenesis

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2.0 Abstract

Rho GTPases are major determinants of the actin cytoskeleton which makes these proteins a target for subversion during oncogenic transformation. The existing paradigm is that Rho GTPases and guanine nucleotide exchange factors (proteins that activate RhoGTPases) act as oncogenes whereas GTPase-activating protein (proteins that inactive RhoGTPases) are predominantly tumor suppressors. However, recent evidence implicates altered expression and activity of many RhoGAPs in a multitude of human cancers and/or cell lines. Cdc42 GTPase-activating protein (CdGAP), a GAP for Rac1 and Cdc42, has been established as an essential component required downstream of transforming growth factor-β signalling pathway to promote breast cancer cell migration and invasion. In addition, CdGAP was shown to act with Zeb2 as a co-transcriptional repressor of E-cadherin transcription in breast cancer. Here, we examined CdGAP levels in prostate cancer (PCa) and report high levels of CdGAP protein and mRNA in the metastatic and aggressive PC-3 human prostate cancer cells compared to low levels in the androgen receptor positive LNCaP cells. Interestingly, downregulation of CdGAP expression using short hairpin RNAs targeting CdGAP in PC-3 cells led to a significant reduction in cell motility, invasion, proliferation and colony-formation ability and an increase in cell apoptosis was observed. Conversely, overexpression of CdGAP in the castration resistant and metastatic DU-145 cell line increased cell migration and invasion. Moreover, GST-CRIB pull down assays revealed an increase in the levels of active GTP-bound Rac1 in CdGAP depleted PC-3 cells. Furthermore, subcutaneous injection of PC-3 cells into mice revealed that loss of CdGAP delayed tumor initiation and attenuated tumor growth. Lastly, using global gene approaches we uncovered that novel genes such as p21, and pathways such as cell cycle, cell apoptosis and cell migration were significantly regulated by upon CdGAP-depletion in PC-3 cells. Additionally, tissue microarray analysis of 285 PCa patients demonstrated a positive correlation between strong nuclear localization of CdGAP with early biochemical relapse in patients. Lastly, gain/amplification of the CDGAP gene was associated with poor disease-free survival in PCa patients. Collectively, these findings further support the pro-oncogenic role of CdGAP in prostate cancer and unveil CdGAP as a novel biomarker and therapeutic target in drug discovery for the development of individualized strategies to treat PCa patients.

2.1 Introduction

Prostate cancer is the most commonly diagnosed non-skin cancer in men all round the world with an estimated 1,600,000 new cases and 366,000 cases of mortality each year [2]. Prostate cancer has a high survival rate for a localized tumor; however, once the tumor advances and metastasizes there are fewer effective therapies. In early stages of the disease prostate cancer cells are dependent on the androgen receptor signalling pathway, hence androgen deprivation therapy (ADT) has proven useful for its early management until the point at which tumors become resistant to ADT leading to a fatal outcome characterized as castration-resistant prostate cancer (CRPC) [9]. Thus, understanding the molecular mechanisms underlying prostate cancer progression is a pressing unmet need and further investigation will lead to the identification of novel targets in order to improve therapeutics for the treatment of this disease.

Rho guanosine triphosphatases (GTPases) are a subfamily of small G proteins within the Ras superfamily of GTPases [29]. They have important roles in cytoskeletal remodelling, cytokinesis, cell polarity, cell motility, cell invasion, and apoptosis [213]. Rho GTPases act as molecular switches mediating downstream effects in an active, GTP-bound state that is positively regulated by guanine nucleotide exchange factors that catalyse the exchange of bound GDP for GTP and negatively controlled by Rho GTPase-activating proteins (GAPs) that increase the intrinsic ability of Rho GTPases to undergo GTP hydrolysis [213]. Given their key roles in normal cellular processes, it is unsurprising that aberrant Rho GTP-signalling is frequently implicated in human tumors [31, 46]. However, as activating mutations in Rho protein genes are rare, the regulators of Rho GTPases have emerged as targets of subversion in cancer [31]. Particularly, GAPs have been assigned tumor-suppressor roles in cancer due to their ability to inactivate Rho GTPases. However, recent evidence has emerged contradicting the existing dogma and implicated RhoGAPs as oncoproteins in several cancers, including prostate cancer [140, 147, 179, 180].

Cdc42 GTPase-activating protein (CdGAP) is a RhoGAP specific for Rac1 and Cdc42, but not RhoA [201]. Previous reports have identified gain-of-function mutations in *CdGAP* gene in a human disease called the Adams-Oliver syndrome (AOS) which is characterized by aplasia cutis congenital and terminal transverse limb defects [208]. CdGAP is also highly phosphorylated on serine and threonine residues in response to growth factors and is a substrate of extracellular signal-

regulated kinase (ERK) , GSK-3 and RSK, mediating cross talk with the Ras/MAP kinase pathways [204-206]. There is compelling evidence to support the involvement of CdGAP in several steps of cancer progression. Notably, CdGAP is a serum-inducible gene and modulates cell spreading, lamellipodia formation, focal adhesion turnover, matrix-rigidity sensing and durotaxis—implicating a role in cytoskeletal remodelling and cellular migration [203, 214, 215]. Furthermore, the loss of CdGAP severely compromises embryonic vascular development and is associated with superficial vessel defects and subcutaneous edema; angiogenesis is one of the hallmarks of cancer [48, 212]. Moreover, CdGAP has been implicated in the regulation of the expression of E-cadherin—loss of which is a key step of epithelial-to-mesenchymal transition—via two mechanisms: Firstly, the expression of CdGAP has been shown to significantly disrupt mature epithelial cell-cell contacts [207]; Second, in breast cancer cells CdGAP was shown to translocate to the nucleus and form a functional complex with Zeb2 to co-repress the expression of E-cadherin [180]. Importantly, CdGAP mediates transforming growth factor (TGFβ)-and ErbB2-induced cell motility and invasion in a GAP-independent manner [179]. In addition, loss of CdGAP in breast cancer cells attenuated ErbB2-mediated tumor growth and metastasis to the lungs [180].

In this report, we provide insight into another novel role of CdGAP in human prostate cancer migration, invasion, proliferation and tumorigenesis. Briefly, we report high endogenous CdGAP protein and mRNA expression in mCRPC human prostate cancer cell line PC-3, moderate expression in mCRPC DU-145 cells and significantly lower expression in the androgen-responsive LNCaP cells. Notably, downregulation of CdGAP expression using short hairpin RNAs abrogates the ability of PC-3 cells to migrate and invade. Interestingly, CdGAP downregulation in PC-3 cells reduces the colony-formation ability and inhibits cell proliferation. Global gene analysis of CdGAP-depleted cells enabled us to correlate this effect with an increase in p21 levels and a concomitant arrest in the G0/G1 phase of the cell cycle of PC-3 cells upon CdGAP depletion. Furthermore, loss of CdGAP delays PC-3 cells driven tumor-initiation, decreases tumor volume and tumor size in mice. In addition, high expression of CdGAP is negatively correlated with biochemical relapse free survival in patients. Conclusively, we provide strong preliminary evidence of a tumor-promoting role of CdGAP in human prostate cancer yielding it as an attractive molecular target to improve the treatments of prostate cancer.

2.2 Experimental Procedures

Cell culture and transfection

PC-3, LNCaP, and DU-145 prostate cancer cells were cultured in RPMI 1640 (Wisent: 350-000-CL) supplemented with 2 mM L-Glutamine, 10% FBS, 1% penicillin/streptomycin, and maintained in a humidified incubator at 37°C with 5% CO_2. To generate a stable CdGAP-knockdown PC-3 cell line, PC-3 cells were infected with short hairpin RNA (shRNA) targeting CdGAP lentiviruses (5'-CCTCATTTAGTTCACCTGGAACTCGAGTTCCAGGTGAACTAAATGAGG-3') (Sigma: TRCN0000047639) or control shRNA (Sigma: SHCON 001) purchased commercially. 48 hours after infection, puromycin [(0.1 ug/ml) Sigma: P8833] was added to the medium to efficiently select cells. These cells were then plated in a 96-well plate at 1 cell/well and selected until single cell clones were achieved. For CdGAP overexpression, DU-145 cells were transfected with full-length pEGFPC1-mCdGAP and pEGFP-EV constructs using jetPRIME transfection reagent (Polyplus:114-07) following manufacturer's instructions. All experiments were carried out 24 hours post-transfection.

Western Blotting and antibodies

Cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Hepes buffer (pH 7.5), 0.1% sodium dodecyl sulfate, 1% triton X-100, 1% sodium deoxycholate, 50 mM sodium fluoride, 150 mM sodium chloride, 10 mM EDTA (pH 8.0), 50 mM sodium orthovanadate, 20 mM leupeptin, 20 mM aprotinin and 1 mM phenylmethylsulfonyl fluoride. Protein lysates were subjected to centrifugation at 10,000 x g for 15 min at 4°C to remove insoluble materials and protein concentrations were determined using the Bicinchoninic Acid Assay (BCA) protein kit (Thermo-Scientific). Equal amounts of protein samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes for western blotting with the indicated antibodies, and visualized by enhanced chemiluminescence (ECL) using Clarity^{TM} western ECL substrate (BioRad: 1705061) and the ChemiDoc^{TM} MP imaging system. All quantitative densitometry analysis on the obtained images were carried out using Image Lab software. Antibodies used include: α -Tubulin (Sigma), CdGAP (Sigma: HPA036380), E-cadherin (Cell Signaling: 3195), Snail1 (Cell Signaling: 3879),

Rac1 (AbCAM: 23A8), Slug (Cell Signaling: C19G7), N-cadherin (BD Biosciences: 610920), Anti-rabbit IgG (ThermoFisher: 45-000-682) and Anti-mouse IgG (ThermoFisher: 45-000-679).

Reverse transcription and Quantitative real time polymerase chain reaction (Q-PCR)

Total RNA was extracted using Qiagen RNeasy kit (Qiagen: 74104). mRNA was reverse transcribed using the 5X All-In-One RT MasterMix kit (AbCAM: G485). Next, quantitative real time polymerase chain reaction (Q-PCR) was performed with SYBR Green PCR Master Mix (Applied Biosystems), using primers specific to the genes of interest: CdGAP (Qiagen: QT00076671), 18S (Qiagen: QT00095431) and the remainder are listed in Table 1. Q-PCR reactions were carried out at 95°C for 3 min, followed by 40 cycles at 95°C for 20 sec, then at 60°C for 30 sec and finally at 72°C for 30 min. Gene expression was normalized to 18S ribosomal RNA [179, 180]. RNA-sequencing was performed and analyzed as previously described [180]. Each sample was analyzed in triplicates.

Immunofluorescence

Cells grown on glass coverslips were fixed for 30 minutes in 3.7% formaldehyde in PBS before permeabilization for 5 minutes with 0.25% Triton-X-100. After blocking for 30 minutes in a solution of 1% bovine serum albumin (BSA), coverslips were incubated overnight at 4°C with anti-CdGAP antibodies, followed by a 45-minute incubation with Alexa Fluor 488-conjugated anti-rabbit and rhodamine-conjugated phalloidin to stain for F-actin filaments. 4′,6′-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Between each step, coverslips were washed three times with PBS. Coverslips were mounted on glass slides using Prolong Gold antifade reagent (Invitrogen: P3696). Cells were examined with a motorized inverted Olympus IX81 microscope using a 40x Plan-S-APO oil objective lens. Images were recorded with a CoolSnap 4K camera (Photometrics) and analyzed with Image J software. At least 100 cells per condition were analyzed in at least 3 independent experiments [179, 180].

Cell migration and Invasion assays

For migration assays, 100 000 cells were resuspended in serum-free medium and seeded in the top chamber of transwell inserts (Falcon: 353097). For invasion assays, 150 000 cells were plated onto a 5% matrigel (ThermoFisher: 356234) layered over the top chamber. Cells were

incubated at 37°C overnight which allowed migration towards the bottom chamber containing complete medium with 10% FBS. After 24 h, cells on the bottom surface of the insert were fixed in 10% formalin (BioShop: 8G56294) and stained with a crystal violet solution. Then, the excess dye was washed out, cells on the top of the insert were removed with a cotton swab and four images of cells stained at the bottom of each insert were obtained using a Nikon inverted microscope camera with a 10 X (Nikon Eclipse TE300 Inverted microscope). Quantitative densitometry was assessed using Image J software. The data represent the average derived from three independent experiments relative to that of shcontrol PC-3 cells [179].

Cell adhesion assay

An *in vitro* adhesion assay was performed by resuspending 40 000 cells in complete media and seeding them on 10ug/ml type 1 collagen (BD Bioscience: 354246) or 10ug/ml fibronectin (Sigma: F1141) coated 96-well plates for 30 min at 37 °C. Cells were fixed using 3.7% formaldehyde for 15 minutes, washed twice with washing buffer (0.1% BSA in RPMI media) and stained with a crystal violet solution. After washing the excess dye out, the plates were allowed to dry for 1 hour. Then the crystal violet stain absorbed by the cell nuclei was extracted with 10% acetic acid and the optical density was measured at 570 nm using a spectrophotometer for each well [179].

Cell proliferation assay

To assess cell proliferation, the cell growth determination MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit (AbCAM: 211091) was used. Briefly, 250 control PC-3 or shCdGAP PC-3 cells were seeded in triplicates in 96-well plates and grown over a period of five days. MTT solution was added to each well for the last 4 h of treatment on each day as per manufacturer's protocol. Absorbance was measured at 590 nm [179].

Colony formation assay

250 cells per well in 6-well plates were resuspended in complete media for 10 days at 37 °C in a humidified incubator. On day 10, the 6-well plates were washed with PBS, fixed in 10% formalin (BioShop: 8G56294) and stained with a crystal violet solution. Then, the excess dye was washed out with ddH_20 twice and the plates were then left to dry overnight. Images were obtained with

a Nikon Eclipse TE300 Inverted microscope and 50 cells were counted as one colony. The data represent the averages of all the images per condition obtained from three independent experiments [216].

Rac1 activation assay

The CRIB domain of mouse PAK3 (amino acids 73–146) fused to glutathione S-transferase (GST-CRIB) was used to isolate GTP-bound Rac1 and was purified as previously described [206, 217]. Briefly, bacterial pellets were resuspended in the lysis buffer (buffer A) containing 20 mM HEPES pH 7.5; 120 mM sodium chloride (NaCl); 2 mM EDTA pH 8; 10% glycerol; and 1% Triton-X 100, sonicated and centrifuged at 3000 RPM at 4°C. Then, 30ug of purified GST-CRIB was coupled to glutathione–agarose beads (50%) (Sigma) for 3 hours at 4°C. were centrifuged at 1000 RPM for 1 minute, and the washed in buffer A twice. Meanwhile, control or shCdGAP PC-3 cells were lysed in RIPA buffer. 1mg of cell lysates were added to the eppendorfs containing the GST-CRIB proteins coupled to the glutathione–agarose beads. The samples were left at 4°C for 45 minutes on a rotator. Finally, the samples were centrifuged at 1000 RPM at 4°C for 1 minute to collect the beads and washed three times in cold RIPA buffer before resuspension in SDS sample buffer, heated at 95°C and then examined by western blotting. The levels of Rac1-GTP were assessed by densitometry and normalized to the total amount of Rac1 detected in the total cell lysates.

Cell cycle and Apoptosis assay

Control or shCdGAP PC-3 cells were serum starved overnight to regulate their cell-cycle phases and then cultured in regular serum for 24 hours Then, 1x 10^6 cells were harvested, counted, and washed twice in ice-cold PBS and fixed in 70% ethanol for 1 hour at 4°C. Then, cells were washed with PBS and incubated with RNase A for 1 hour at 37°C in a humidified incubator. Finally, cells were stained with 10µg/ml propidium iodide (PI) (Sigma: P4170). Cells were subjected to flow cytometry analysis with BD FACSCanto II system. The cell cycle distribution was analyzed using FlowJo Analysis software [206]. To assess apoptosis, control or shCdGAP PC-3 cells were serum-starved overnight. Then, 1x 10^6 cells were harvested and washed in 1X binding buffer. Cells were stained simultaneously with Annexin V-fluorescein isothiocyanate (FITC) and PI using an FITC Annexin V Apoptosis Detection Kit (Invitrogen: V13241). Harvested cells were analyzed by flow

cytometry using the BD FACSCanto II system. The analysis was carried out using the FlowJo Analysis software according to the manufacturer's protocol.

Tumorigenesis assay

To assess primary tumor growth of control or shCdGAP PC-3 cells, 1×10^6 cells were diluted in 100 ul of serum-free RPMI containing 50% Matrigel (ThermoFisher: 356234) and injected subcutaneously using BD disposable syringe with Leur-Lok Tips (ThermoFisher: 14-823-30) into the right flanks of 7-weeks old male athymic mice. Tumors were measured every two days with a digital caliper and the tumor volume was calculated using the following formula: $V = \pi$ (length $\times$ width^2)/6. After 34 days, mice were sacrificed, and the tumors were harvested, fixed in 4% formalin and subjected to weight analysis. All animal protocols were approved by McGill University Animal Use and Care Committee, in accordance with guidelines established by the Canadian Council on Animal Care.

Statistical Analysis

Statistical analysis was performed using a two-sample unequal-variance Student's t-test. Data are presented as the mean +/- SEM and the p value of less than 0.05 was considered to be statistically significant. Data are representative of at least three independent experiments.

2.3 Results

CdGAP is a negative prognostic biomarker for human prostate cancer survival

To assess the clinical relevance of CdGAP in prostate cancer, we examined CdGAP protein levels in a panel of prostate tumors (n=256) using tissue microarray analysis (TMA). Tumor tissues were compared with adjacent unaffected prostate tissues from each patient. We found CdGAP is abundant in prostate cancer patients (Figure 2.1a) and that elevated levels of nuclear CdGAP correlated with decreased biochemical relapse-free survival (Figure 2.1b). To further investigate the prognostic effect of CdGAP, a Kaplan-Meier analysis was conducted to determine the correlation between CdGAP expression and the probability of disease- free survival in prostate cancer patients (n=485). We report a correlation between the gain and amplification of the *CDGAP* gene (Figure 2.1c) with a lower probability of disease-free survival in prostate cancer patients (P value = 0.00211). Consistently, this has been previously reported for breast cancer patients, where elevated CdGAP expression correlated with a poor prognosis in patients [180]—thus alluding to a role of CdGAP as an oncoprotein on a global scale. Taken together, disease-free survival inversely correlates with high CdGAP expression in prostate cancer patients.

CdGAP is endogenously expressed in a cohort of metastatic human prostate cancer cell lines

LNCaP, PC-3 and DU-145 cell lines are derived from the lymph node, brain and bone metastasis, respectively, and are the most common human cell lines used to study prostate cancer [218]. To elucidate a potential role of CdGAP in prostate cancer, we examined the expression of endogenous CdGAP across these three human prostate cancer cell lines using western blot analysis (Figure 2.2a). Compared with DU-145 cells, endogenous CdGAP protein expression was 9-fold higher in metastatic PC-3 cells, whereas, the level of CdGAP expression in the indolent LNCaP cells was below detectable threshold (Figure 2.2b). As well, the mRNA levels of CdGAP were determined using quantitative real-time PCR (Q-PCR). Compared with DU-145 cells, PC-3 cells displayed a 10-fold higher CdGAP mRNA level whereas LNCaP cells expressed low CdGAP mRNA levels (Figure 2.2c). Furthermore, we analyzed the localization of CdGAP in PC-3 cells by indirect immunofluorescence. We found that CdGAP was ubiquitously expressed throughout the cell and

localized to both the nucleus and the cytoplasm of these cells (Figure 2.2d, 2.2e). Taken together, these data confirmed variable levels of expression of CdGAP with higher expression in metastatic human prostate cancer cells.

CdGAP depletion increases the levels of active Rac1 in PC-3 cells

To investigate whether CdGAP was involved in protumorigenic behaviors such as cell motility and invasion, proliferation and tumorigenesis of PC-3 cells, we first downregulated the levels of CdGAP with short-hairpin RNA (shRNA) lentiviruses targeting CdGAP. Successful downregulation of CdGAP protein expression was confirmed by western blot when compared with control shRNA PC-3 cells (figure 2.3a, 2.3b). In addition, the intensity of CdGAP staining was lower in shCdGAP cells in contrast with control cells, further confirming successful downregulation of CdGAP expression mediated via shRNA approach (Figure 2.3c). We then examined the effect of CdGAP depletion on the levels of active Rac1 by performing a GST-CRIB pull down assay (Figure 2.3d). Indeed, downregulation of CdGAP—GAP against Rac1 and Cdc42—resulted in increased Rac1-GTP (Figure 2.3e) in PC-3 cells associated with increased membrane ruffles (Figure 2.3f). These results demonstrate that CdGAP acts as a major GAP for Rac1 in prostate cancer cells.

CdGAP is required for the motility and invasion of human prostate cancer cells

To assess the role of CdGAP in prostate cancer cell migration, we performed transwell migration and invasion assays. For cell migration assays, control PC-3 cells or CdGAP-depleted cells were counted, the same number of cells were suspended in serum-free medium and plated in transwell filters. These cells were allowed to migrate towards the bottom chamber which contained media with 10% fetal bovine serum during a period of time of 24 hours. (Figure 2.4a). Migration ability was inhibited in shCdGAP PC-3 cells by 60% (Figure 2.4b). In addition, a transwell invasion assay (Figure 2.4c) with Matrigel, demonstrated that the invasiveness of the CdGAP-depleted PC-3 cells was also decreased by 70% compared to control cells (Figure 2.4d). To confirm promigratory and invasive effects of CdGAP in human prostate cancer cells, we chose another cell line DU-145 to further test the effects. Taking advantage of lower CdGAP expression in these cells we transfected full length CdGAP in DU-145 cells and consistently observed increased cell migration by 50%

(Figure 2.4e, 2.4f) and cell invasion by 20% (Figure 2.4g, 2.4h). Taken together, these results suggest that CdGAP is a promoter of prostate cancer cell migration and invasion.

CdGAP regulates the expression of epithelial-to-mesenchymal genes in PC-3 cells

Cancer cells progress and metastasize in a process known as epithelial-to-mesenchymal transition, which is characterized by the loss of intercellular contacts and downregulation of proteins such as E-cadherin and increase in mesenchymal markers such as N-cadherin [219]. Interestingly, in keratinocytes, CdGAP was shown to localize to mature adherens junctions and its expression resulted in disruption of these structures through negative regulation of E-cadherin expression [207]. Furthermore, it has been previously reported that CdGAP works as a co-transcriptional repressor with Zeb2 in breast cancer cells and suppresses E-cadherin expression. Conversely, CdGAP protein downregulation in human breast cancer MDA MB 231 cells, resulted in increased E-cadherin levels and a reinstatement of intercellular junctions [180]. Therefore, this research was extended to prostate cancer. Interestingly, there existed an inverse correlation between CdGAP and E-cadherin protein and mRNA (Figure 2.5a-c) levels in the AR-positive LNCaP and AR-negative DU-145 and PC-3 human prostate cancer cells. We next assessed the levels of E-cadherin upon downregulation of CdGAP in our stable CdGAP-depleted PC-3 cells; which would also explain the effects observed during the motility and invasion assays. In contrast to the effects observed in breast cancer, in prostate cancer, CdGAP depletion led to a further decrease in E-cadherin mRNA and protein levels (Figure 2.5d-f). We were intrigued by this observation and further investigated the transcriptional repressors of E-cadherin to understand why this change in E-cadherin expression was induced. Interestingly, an increase in the transcriptional repressor of E-cadherin and a mesenchymal marker Snail1 was observed in PC-3 cells (Figure 2.5g-i). Upregulation of Snail1 explains the marked decreased in E-cadherin levels [220]; however, this is contrary to the observed decreased motility and invasion suggesting other molecular players have key roles here instead. Testing further, we investigated the levels of two other mesenchymal markers N-cadherin and Slug. Knockdown of CdGAP resulted in a significant decrease of both in shCdGAP PC-3 cells (Figure 2.5j-m)—reduced levels of both have been consistently reported with decreased cell motility [220]. Based on our findings, we observed different regulation of EMT genes compared to the role of CdGAP in breast cancer, thus revealing a novel role in prostate

cancer. Collectively, CdGAP has been shown to contribute to the migratory and invasive activities of metastatic prostate cancer cells through regulation of EMT genes.

Loss of CdGAP mildly affects the adhesion of PC-3 cells when plated on fibronectin.

It has been reported that increased migratory capacity of a cell depends on its ability to rapidly attach and detach with the extracellular matrix [221]. Thus, we next assessed whether the effects of reduced motility and invasion correlated with decreased cell adhesion. To this end, we performed a cell adhesion assay to examine the effects of CdGAP depletion on the ability of PC-3 cells to adhere to type I collagen and fibronectin. We found that loss of CdGAP caused a mild but significant decrease in cell adhesion compared to control PC-3 cells when plated on fibronectin (Figure 2.6a). However, the loss of CdGAP had no effect on the adhesion ability of the cells plated on collagen type I (Figure 2.6b). Thus, CdGAP differentially regulates the adhesion of PC-3 cells plated on different extracellular matrix proteins.

Downregulation of CdGAP suppresses cell proliferation, induces cell cycle arrest and increases apoptosis in PC-3 cells.

We next assessed the impact of CdGAP on prostate cancer cell proliferation. MTT assay revealed that loss of CdGAP significantly inhibited proliferation of PC-3 cells over a span of 5 days (Figure 2.7a). We extended this analysis and performed a colony formation assay that revealed a decrease in the number and size of colonies formed upon CdGAP downregulation (Figure 2.7b, 2.7c), indicating that anchorage-independent growth is promoted by CdGAP in PC-3 cells. To gain insight into how CdGAP depletion inhibits cell growth of PC-3 cells, a cell cycle flow cytometry analysis was conducted by staining cellular DNA with propidium iodide. Flow cytometry assay revealed a significant increase in cell population in the G0/G1 phase (from 53% to 61%) cell cycle in CdGAP-depleted PC-3 cells, therefore reducing the proportion of cells present in the S (from 21% to 16%) and G2 (from 26% to 22%) phases (Figure 2.7d, 2.7e). To examine whether CdGAP could also induce cell death, we performed Annexin-V staining to observe a potential effect on cell apoptosis. Flow cytometry analysis revealed a significant increase in cell apoptosis in shCdGAP PC-3 cells (Figure 2.7f, 2.7g). Collectively, the loss of CdGAP results in a cell cycle arrest with a concomitant increase in cell apoptosis in PC-3 cells.

Global gene expression analysis of CdGAP-depleted PC-3 cells reveals the biological functions associated with CdGAP

To define the molecular pathways regulated by CdGAP, we performed next generation RNA sequencing (RNA-seq) on control PC-3 cells compared to CdGAP-depleted PC-3 cells. Global analysis of the gene data sets from RNA-sequencing results revealed a significant enrichment of genes in pathways associated with epithelial-to-mesenchymal transition, apoptosis, cell-cycle regulation and p53 pathway genes (Figure 2.8a-d). The heat map (Figure 2.8e) depicts the changes in expression of the cell cycle checkpoint genes between control and shCdGAP PC-3 cells. Interestingly, accordingly to the heat map of GSEA analysis of cell-cycle pathways, we found increased levels of the cyclin-dependent kinase (cdk) inhibitor p21 (Figure 2.8e), which is crucial in the regulation of G1 cell cycle progression [222, 223]. Consistently, this was confirmed by Q-PCR which represented that mRNA levels of p21 was increased by 3-fold in shCdGAP PC-3 cells when compared to control cells (Figure 2.8f). This is in good agreement with our observed effects in cell proliferation and cell cycle arrest. Thus, these analyses corroborated our results and revealed CdGAP as a key regulator of prostate cancer cell migration, invasion and proliferation.

The loss of CdGAP delays tumor formation and attenuates tumorigenesis induced by prostate cancer PC-3 cells in vivo

To validate a potential role of CdGAP in vivo, we performed subcutaneous injection of shCdGAP and Con PC-3 cells into mice (Figure 2.9a). Briefly, one million cells of each condition were injected into the right flanks of 23 athymic mice (representing 12 of the shcontrol cohort and 11 in the shCdGAP group). After 7 days post-injection, tumors were measured using a caliper every 2 days until 34 days. Tumor growth in the shCdGAP group was significantly delayed than in control group whereby it took 10 days for half the control cohort to form tumors and around 26 days for 50% of the shCdGAP group to form tumors (Figure 2.9b). Additionally, three of the mice in the shCdGAP cohort did not form tumors at all (Figure 2.9b). This result was correlated with the reduction in tumor volume in the shCdGAP cohort by half to that of control—observed consistently throughout tumor progression (Figure 2.9c). Additionally, the endpoint tumors were subjected to weight analysis and the shCdGAP group weighed less than half the size of

control PC-3 tumors (Figure 2.9d). Collectively, these data show that CdGAP plays a significant role as an oncoprotein in the ability of PC-3 cells to form a tumor.

2.4 Discussion

Prostate cancer remains the most commonly diagnosed cancer and the second cause of cancer-related mortality in men in Canada. The gold standard for treatment of prostate cancer remains targeting the androgen receptor axis characterized as androgen deprivation therapy (ADT) [9]. However, response to these agents is limited and many patients ultimately progress and develop tumors become resistant to ADT leading to a lethal stage of this disease known as castration-resistant prostate cancer (CRPC). Thus, despite the scientific advances in pharmacological drugs, increasing the understanding of the molecular mechanisms that lead to prostate cancer development and treatment resistance remains essential in order to improve the success of targeted therapies [11]. Herein, we present the first study demonstrating a novel role of CdGAP in prostate cancer via its investigation in the castration-resistant and metastatic cell line PC-3.

Several lines of evidence implicate that CdGAP may have a pro-tumorigenic role in cancer. As a GAP for Rac1 and Cdc42, it is a key regulator of the actin-cytoskeletal remodeling conferring pro-migratory roles to CdGAP [36, 201]. Also, CdGAP was shown to have a key role in the regulation of directional membrane protrusions of migrating osteosarcoma cells [214, 215]. Furthermore, a recent study found that CdGAP is the major RhoGAP expressed in HER2/ErbB2-induced mouse breast tumors [179]. In line with this, downstream of transforming growth factor β (TGFβ) and ErbB2 signaling pathways, CdGAP was shown to be regulating cell migration and invasion in the ErbB2-induced breast cancer cell model, NMuMG-NT (mouse mammary cancer cells expressing constitutively activated Neu/ErbB-2 receptor) [179]. In a follow-up study, the mechanism of action of CdGAP in these cells was revealed. Briefly, a novel nuclear role of CdGAP was reported where via its PRD region—in a GAP-independent manner—CdGAP interacted and formed a complex with the transcriptional repressor Zeb2 and in turn inhibited E-cadherin expression. In addition, loss of CdGAP suppressed the ability of these cells to metastasize to the lungs [180].

Here, we found elevated levels of CdGAP expression in a cohort of human prostate cancer cells. In particular, the castration-resistant and metastatic cell line PC-3 expressed high levels of CdGAP mRNA and protein. Immunohistochemistry staining revealed that nuclear CdGAP expression correlated with poor biochemical relapse-free survival in prostate cancer patients. Interestingly, we also observed nuclear as well as cytoplasmic localization of CdGAP in PC-3 cells

through immunofluorescence staining. To validate its role in prostate cancer we applied shRNA lentiviral vectors to interfere with CdGAP expression in PC-3 cells. Consistent with its pro-tumor effects in breast cancer, downregulation of CdGAP inhibited the ability of PC-3 cells to migrate and invade *in vitro*. Conversely, overexpression of CdGAP protein in DU-145 cells enhanced the migratory and invasive capacities of these cells. Another characteristic of cancer cell motility is the reduction in cell-matrix adhesion which in turn allows tumor cells to invade surrounding tissues and metastasize [179]. Therefore, we next investigated whether there is a decreased ability of these cells to adhere to extracellular matrix collagen type 1 or fibronectin and observed a mild but significant reduction in the ability to attach to fibronectin whereas no effect was observed for collagen. Thus, CdGAP is essential for the motility and invasion of prostate cancer cells but the effect on cell adhesion seems to be minimal.

It is well known that in order for cells to migrate and invade they have to undergo a process well-characterized as epithelial-mesenchymal transition. Some hallmarks of this process include upregulation of the expression of mesenchymal markers Snail, Slug, N-cadherin and downregulation of epithelial markers such as E-cadherin, ZO-1 and claudins. Interestingly, downregulation of CdGAP resulted in a further decrease of E-cadherin levels and an increase in the level of Snail. When we investigated further, we observed a decrease in other mesenchymal markers such as Slug and N-cadherin. Expression of both Slug and N-cadherin has been correlated in several reports with increased motility and an aggressive cancer phenotype [224, 225]. Thus, although the marked decrease in E-cadherin levels upon CdGAP downregulation is in contrast to the findings in breast cancer, the regulation of other genes hints at a differential mechanism of action of CdGAP in prostate cancer. Whether N-cadherin and Slug are direct targets of CdGAP during the regulation of EMT in prostate cancer, needs to be further investigated. However, the differential gene regulation highlights a novel role of CdGAP in the migration and invasion of prostate cancer cells.

Further investigation of the migratory and invasive capacities via *in vivo* experiments demonstrated that CdGAP-depleted tumors exhibited delayed tumor onset, reduced tumor volume and tumor weight, in comparison to control tumors in PC-3 cells and this further substantiated the results obtained through the *in vitro* experiments. Moreover, the prognostic significance of CdGAP was supported by immunohistochemistry analysis as there was a clear association between high nuclear localization of CdGAP and decreased biochemical relapse-

free survival. Lastly, in a cohort of about 500 patients high CdGAP mRNA expression was also indicative of poor survival in patients. In future clinical studies CdGAP may be used as a prognostic marker when predicting the outcome of prostate cancer patients as well as breast cancer patients.

We also looked at cell proliferation and we observed a robust attenuation of cell proliferation. Consistently, we observed a decrease in colony-formation ability in CdGAP-depleted PC-3 cells. The colonies in CdGAP-depleted PC-3 cells were loose and scattered from one another and unable to form compact ones as observed in control PC-3 cells. Furthermore, when we investigated the decrease in cell proliferation, we observed that these cells displayed an arrest in the G0/G1 phase with an increase in the levels of p21 mRNA and concomitant increase in cell apoptosis. By the same token, RNA-sequencing analysis between CdGAP depleted and control PC-3 cells revealed increased p21 levels and the associated pathways regulated were that of cell migration, cell-cycle and apoptosis—further corroborating our data. Previous reports have implicated RhoGAPs in the regulation of cyclin-dependant kinase inhibitors [147, 148]. Notably, a depletion of ARHGAP11A was shown to lead to a cell-cycle arrest mediated by p27 in basal-like breast cancer cell growth. In the same report depletion of another RhoGAP RacGAP1 led to an increase in the cyclin dependent kinase inhibitor CDKN1A/p21 and this was associated with an increase in senescence in these cells [148]. This report identified both these RhoGAPs as an oncogenic GAP essential for the regulation of cell proliferation [147, 148]. Alternatively, ARHGAP24 (FilGAP) emerged as a tumor-suppressor in renal cell carcinoma by promoting G1/S phase cell cycle progression, increasing apoptosis and inhibited tumor growth in renal cell carcinoma cells [168]. Consistently, another RhoGAP, ARHGAP10 has been consolidated as a tumor-suppressor in ovarian cancer cells by inhibiting cell cycle progression and inducing apoptosis resulting in suppression of tumorigenesis [145]. Furthermore, the mechanism identified in these cells was associated with the GAP activity of ARHGAP10. Briefly, by interacting and inhibiting Cdc42, ARHGAP10 mediated its tumor suppressive functions. [145] Collectively, the emerging role of RhoGAPs in the regulation of cancer cell proliferation is interesting and may identify novel therapeutic targeting strategies.

We provide preliminary evidence of novel pro-cancer actions of CdGAP in prostate cancer. Together, our results suggest that loss of CdGAP significantly impairs migration, invasion and proliferation of PC-3 cells both *in vivo* and *in vitro*. It remains to be determined whether these

roles of CdGAP are GAP dependent or independent. In breast cancer, the novel nuclear role of CdGAP responsible for the positive regulation of breast tumorigenesis and metastasis was independent of its GAP activity. Briefly, CdGAP was shown to translocate to the nucleus and act as an E-cadherin transcriptional co-repressor with Zeb2. Furthermore, a recent study suggested that ARHGAP30 suppressed tumorigenesis in colorectal cancer independently of its GAP function and primarily through acetylation of p53 [177]. Moreover, another report correlated a GAP-independent function of a RhoGAP, DLC1, with reduced metastasis [226]. Interestingly, we have presented increased Rac1-GTP levels upon CdGAP downregulation in PC-3 cells. Therefore, it will be interesting to further investigate whether CdGAP exerts its functions in prostate cancer in a GAP-dependent or independent manner. This will also provide a better understanding of the oncogenic domains of CdGAP and will prove useful to design drugs when targeting these tumor-promoting roles for potential treatments of cancer.

As Rho proteins organize the cytoskeleton, their regulators and effectors are involved in maintaining normal homeostasis and are prone to alteration due to oncogenic transformations [46]. A negative regulator of Rac1 and Cdc42 activities suggests that CdGAP should act as a tumor suppressor; however, the opposite has been observed in breast cancer cells where CdGAP has emerged as a promoter of breast cancer metastasis [179, 180]. Furthermore, elevated CdGAP expression correlated with poor prognosis in patients with breast cancer. In this study, a Kaplan-Meier analysis of 495 patients revealed an inverse correlation between CdGAP-expression and survival in prostate cancer patients. This is in agreement with the results we have reported and this pro-oncogenic role of CdGAP challenges the existing paradigm that views GAPs primarily as tumor-suppressors. Recent evidence has also implicated other RhoGAPs as tumor-promoters. In a study conducted with basal-like breast cancer ARHGAP11A and RacGAP1 emerged as oncogenic GAPs [147]. Consistently, in ovarian cancer the expression of RacGAP1 positively correlated with lymph node metastasis [192]. Additionally, in colorectal cancer, nuclear RacGAP1 expression corresponded to poor survival [191]. This is in line with the inverse correlation observed between nuclear localization of CdGAP and biochemical relapse-free survival in prostate cancer patients. Interestingly, some GAPs traditionally viewed as tumor-suppressors have now also been identified with contrary tumor-promoting roles. Notably, p190A, a RhoGAP for RhoA has been implicated as an oncogenic GAP in osteosarcoma, colorectal, lung and breast cancer. Briefly, in both lung

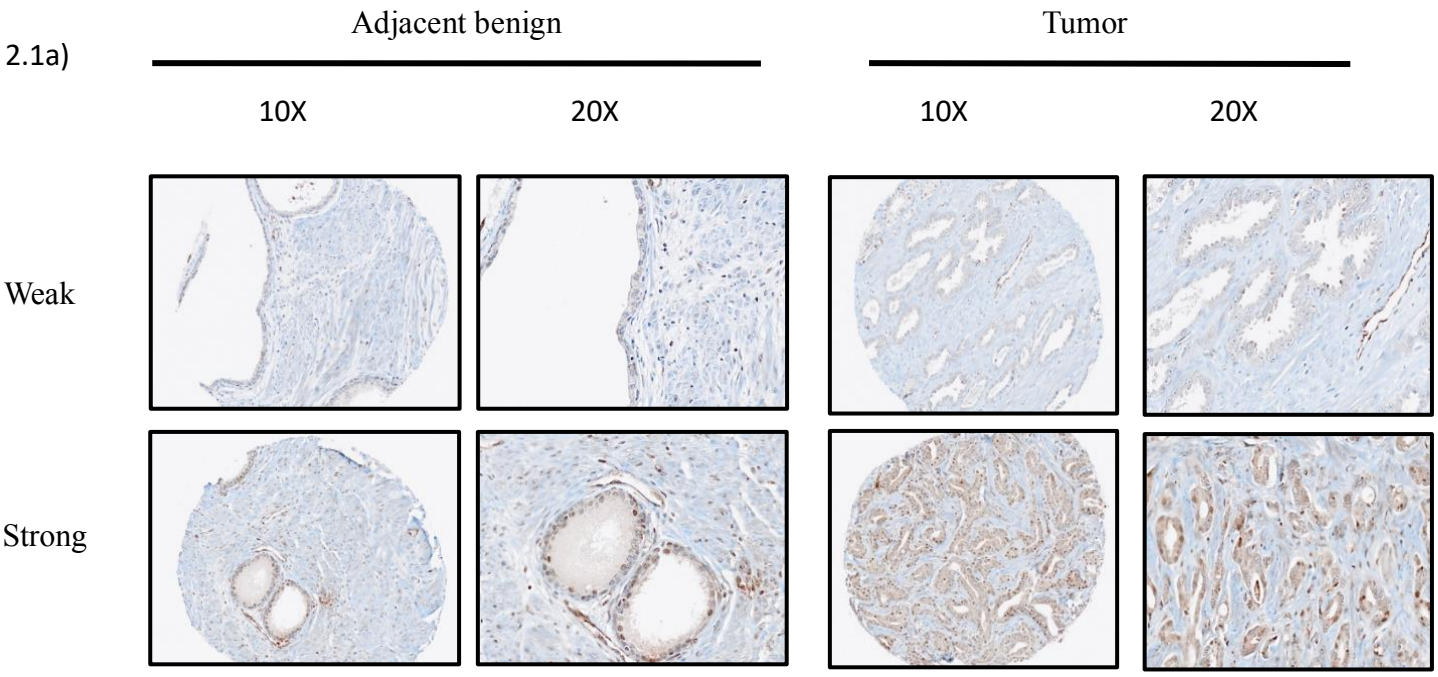
and breast cancers overexpression of p190A promoted the migration and invasion of these cells [108].

In conclusion, the current study demonstrated a drastic impact of CdGAP silencing on cell migration, invasion and proliferation of prostate cancer PC-3 cells. We suspect that CdGAP mediates these effects partially via its ability to translocate to the nucleus. It remains to be identified whether the effects are GAP-dependent or independent, and to find interacting partners of CdGAP that are involved in this regulation. The studies reported here have highlighted the molecular function of CdGAP in cancer cell behavior and migratory processes revealing it as a potential promoter of prostate cancer, and we propose CdGAP as a valuable prognostic biomarker and novel drug target in the treatment of prostate cancer.

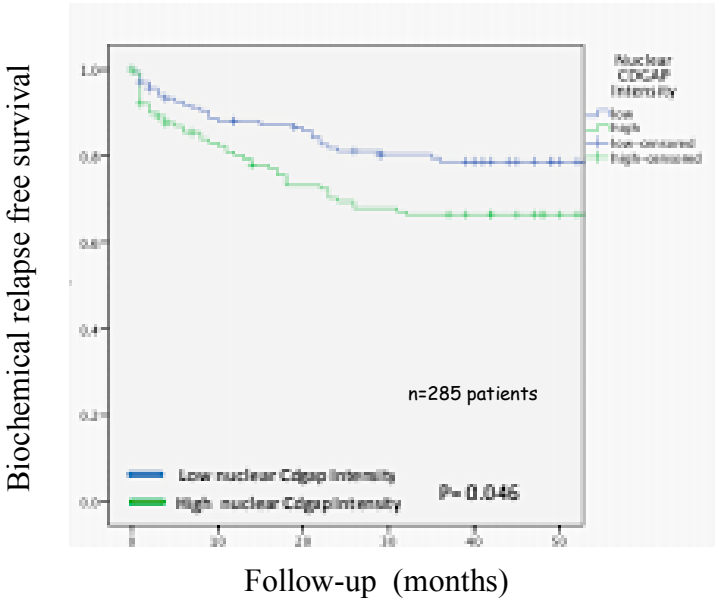
2.5 Figures for chapter 2

Figure 2.1: CdGAP is a negative prognostic biomarker for human prostate cancer survival. (a) Representative images of strong and weak staining of CdGAP nuclear expression on prostate tumor microarray (TMA) containing 285 human prostate cancer specimens. **(b)** Kaplan-Meier curves representing the probability of biochemical relapse free survival at 36 months of prostate cancer patients, based on the relative levels of CdGAP nuclear staining on a prostate TMA containing 285 human prostate cancer specimens. **(c)** Kaplan-Meier curves representing the probability of disease-free survival of prostate cancer patients with or without CdGAP/ARHGAP31 gains or amplifications (TCGA, provisional) from www.cbioportal.org.

2.1a)



2.1b)



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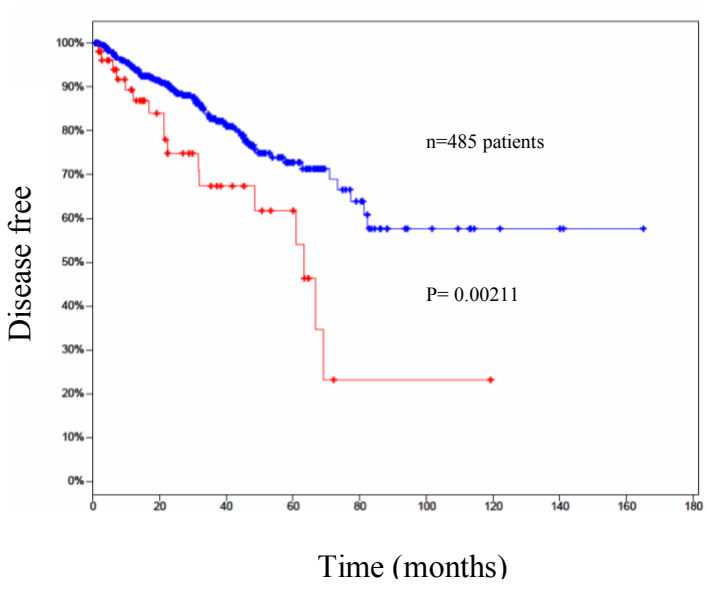
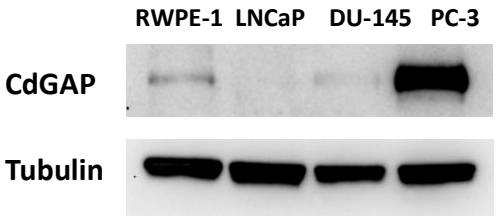


- Cases without ARHGAP31 Gain/Amplification
- Cases with ARHGAP31 Gain/Amplification

Figure 2.2: CdGAP is endogenously expressed in a cohort of metastatic human prostate cancer cell lines (a) Western blot analysis of CdGAP protein levels in a panel of human prostate normal and cancer cell lines. RWPE-1 is an immortalized and non-tumorigenic epithelial cell line. Tubulin was used as a loading control. **(b)** Quantitative analysis of blots presented in (a) and the values are normalized to tubulin and plotted relative to the expression in DU-145 cells. **(c)** mRNA was extracted, subjected to reverse transcription and this was followed by quantitative PCR analysis to measure CdGAP levels. Analysis was normalized to 18S ribosomal RNA. **(d)** The localization of CdGAP in PC-3 cells was characterized using immunofluorescence. Cells were fixed and stained for: CdGAP (green) with anti-CdGAP antibody; nucleus (blue) with (DAPI); and F-actin (red) with TRITC-phalloidin. Scale bar represents 10 μm. The images represent localization of CdGAP classified according to nuclear, cytoplasmic or nuclear + cytoplasmic staining. **(e)** Percentage of cells with CdGAP localizing to nuclear, cytoplasm or both. Error bars represent SEM. n=3 (**P<0.01, *P<0.05).

2.2a)



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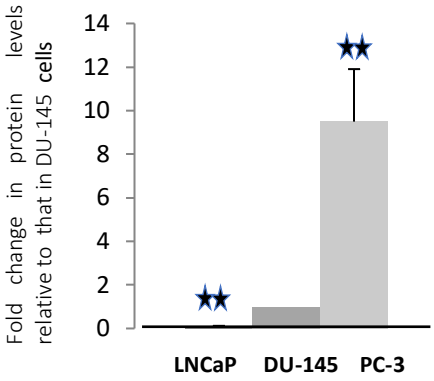

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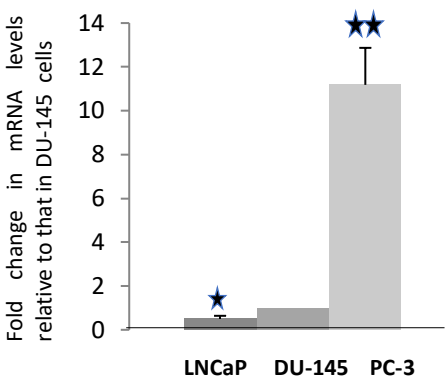
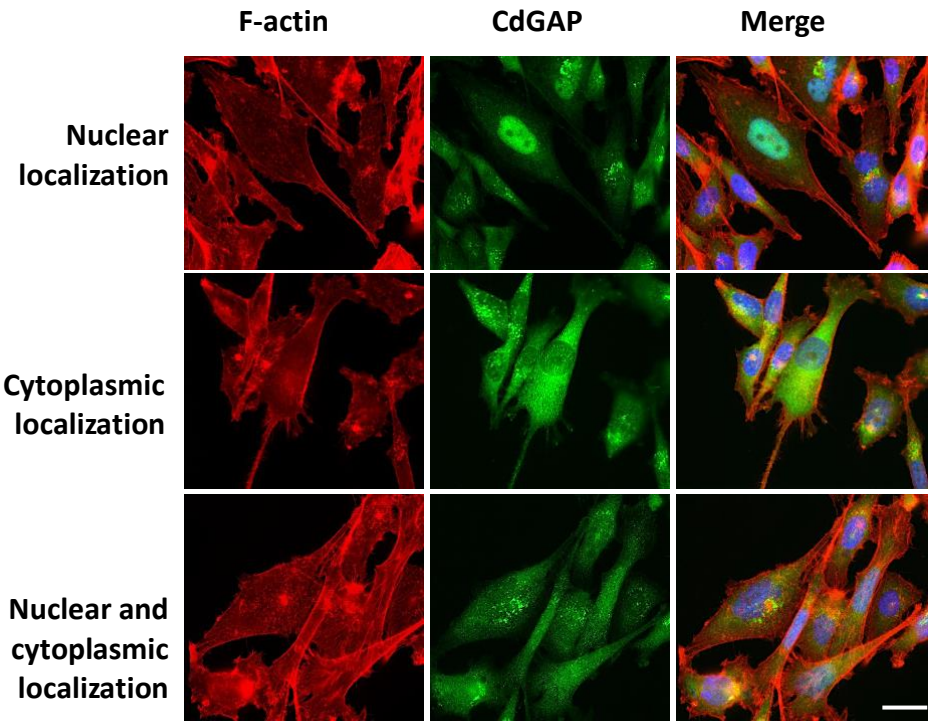


2.2d)



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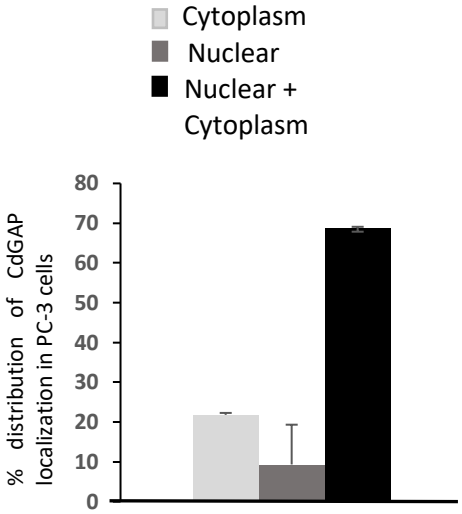
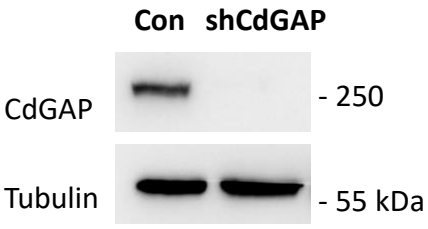


Figure 2.3: CdGAP depletion increases the levels of active Rac1 in PC-3 cells. PC-3 cells were transfected with shRNA targeting CdGAP (shCdGAP) or scrambled shcontrol (Con) to downregulate its expression in PC-3 cells. **a)** Western blot analysis of CdGAP expression using anti-CdGAP antibody. Tubulin was used as loading control. **(b)** Quantitative analysis of blots shown in (a) normalized to tubulin. **(c)** Cells were fixed and stained for: CdGAP (green) with anti-CdGAP antibody; nucleus (blue) with (DAPI); and F-actin (red) with phalloidin. (d) GTP-bound Rac1 was pulled down using GST-CRIB from control (Con) or CdGAP-depleted PC-3 (shCdGAP) cell lysates. Rac1 was detected with an anti-Rac1 antibody. TCL: total cell lysates. **(e)** Quantitative analysis of GTP-bound Rac1/total Rac1 relative to control cells is represented. (**f)** Control and shCdGAP PC-3 cells were plated on coverslips coated with collagen type I. Cells were fixed and stained for F-actin (red) with TRITC-phalloidin. Arrow points to membrane ruffles. Scale bar represents 10 μm. Error bars represent SEM. n=3 (*** $P < 0.001$, *$P < 0.05$).

2.3a)



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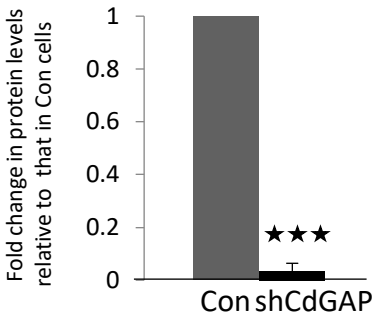

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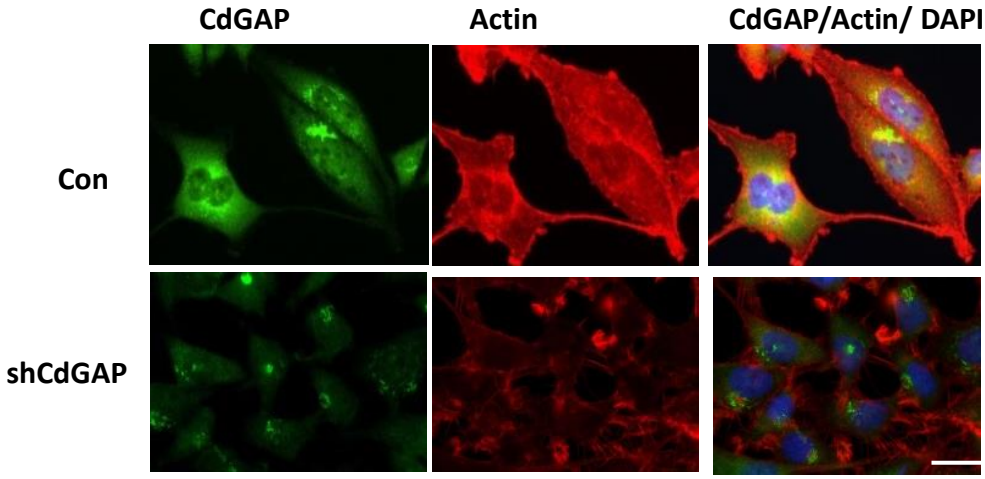

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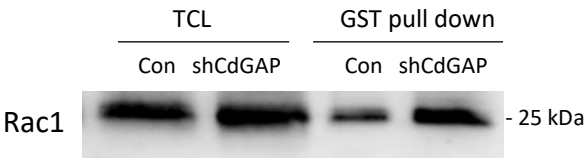

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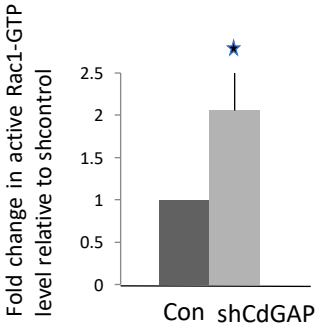

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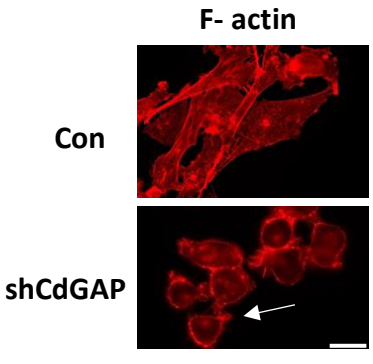
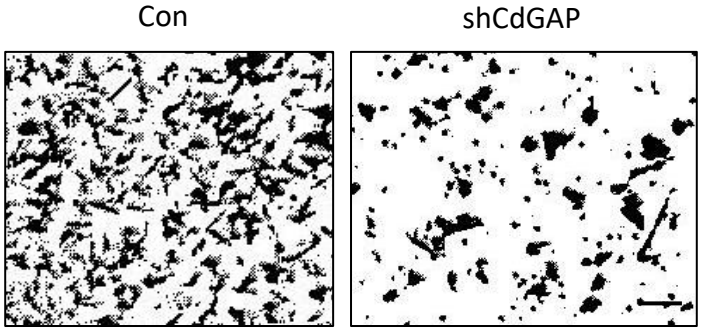


Cells plated on fibronectin-coated coverslips

Arrow-points to ruffles

Figure 2.4: CdGAP is required for the motility and invasion of human prostate cancer cells. Representative images of shCdGAP and shcontrol PC-3 cells that penetrated through transwell filters when subjected to motility assays **(a)** and invasion assays **(c).** Quantification of images from motility **(b)** and invasion **(d)** assays is relative to that of control PC-3 cells. Images are representative of DU-145 cells transfected with either control vector or GFP-mCdGAP subjected to motility assays **(e)** and invasion assays **(g).** Fold changes for motility and invasion are quantified in **(f)** and **(h),** respectively, relative to DU-145 transfected with empty vector. Error bars represent SEM. n=3 (*** $P < 0.001$, *$P < 0.05$).

2.4a)



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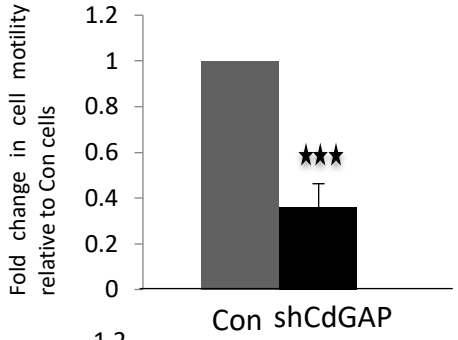

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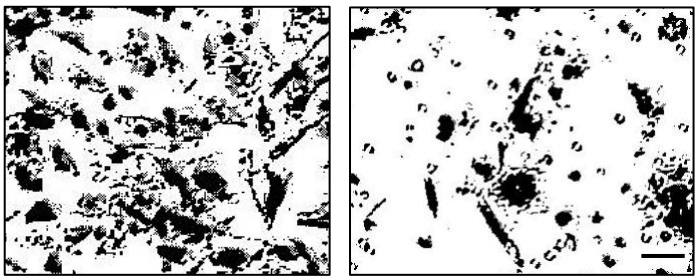

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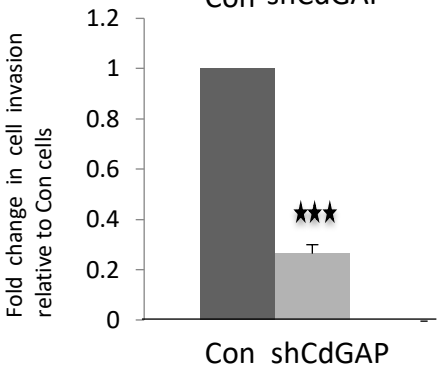

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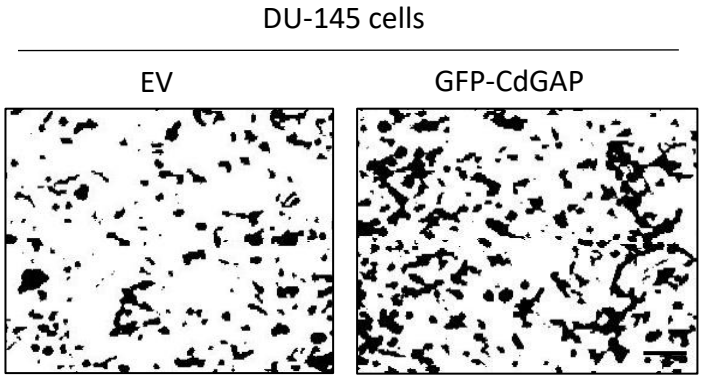

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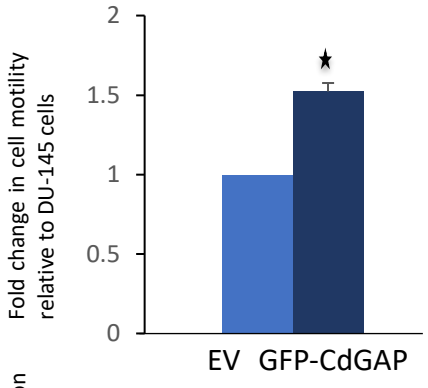

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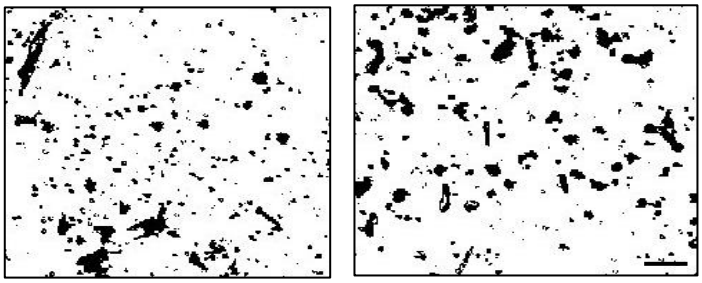


2.4h)

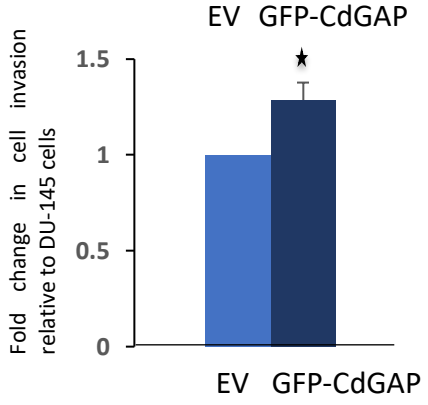
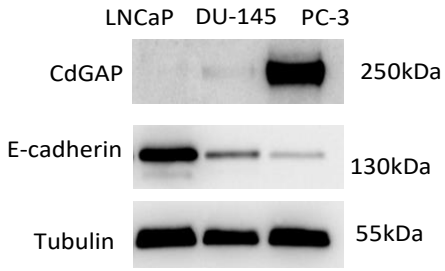


Figure 2.5: Regulation of EMT genes upon downregulation of CdGAP. (a) Using antibodies against E-cadherin and CdGAP, the level of protein expression was examined by western blot analysis in LNCaP, DU-145 and PC-3 cells. Tubulin was used as a loading control. **(b)** Data from western blot representative of E-cadherin shown in (a) was quantified normalized to tubulin and relative to DU-145 cells. **(c)** The mRNA levels of E-cadherin were measured by Q-PCR in LNCaP, DU-145 and PC-3 cells, relative to 18S ribosomal RNA. **(d)** Using antibodies against E-cadherin and CdGAP, the level of protein expression was examined by western blot analysis. Tubulin was used as a loading control. **(e)** Data from western blot representative of E-cadherin shown in (a) was quantified relative to shcontrol PC-3 cells and normalized to tubulin. **(f)** The mRNA levels of E-cadherin were measured by Q-PCR, relative to 18S ribosomal RNA. **(g)** Downregulation of CdGAP increases the level of Snail as represented by the level of protein expression examined by western blot. Data from western blot representative of Snail shown in (g) was quantified in **(h). (i)** The mRNA levels of Snail1 were measured by QPCR, relative to 18S ribosomal RNA. **(j)** The level of n-cadherin and slug protein **(l)** expression was examined by western blot analysis and quantified in **(k)** and **(m),** respectively**.** All data represented is compared to shcontrol PC-3 cells. Error bars represent SEM. n=3 (**P<0.01, *P<0.05).

2.5a)



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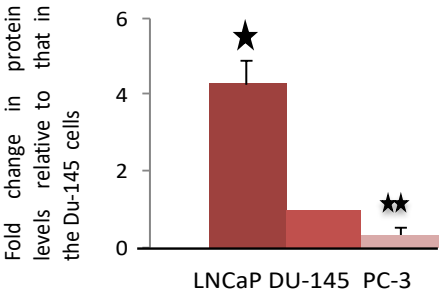

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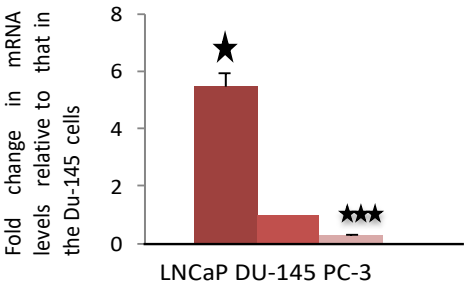

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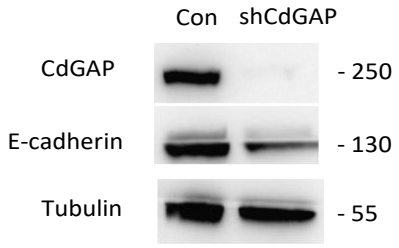

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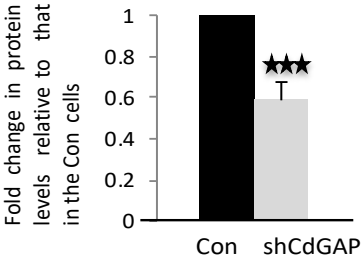

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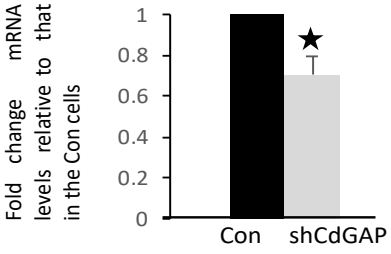

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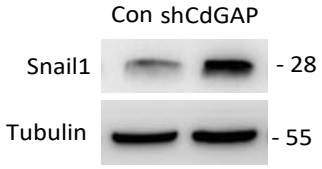

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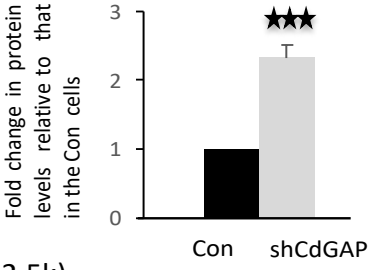

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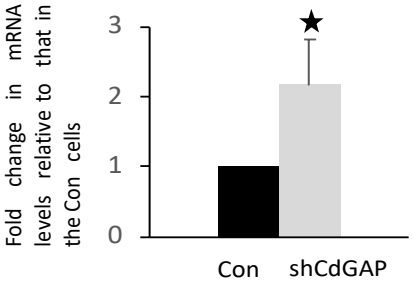

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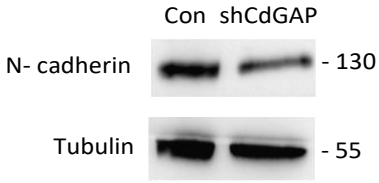

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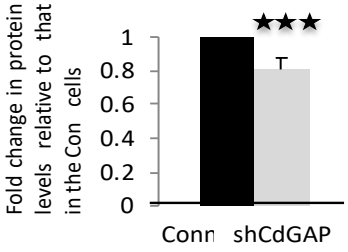

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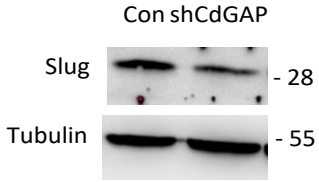

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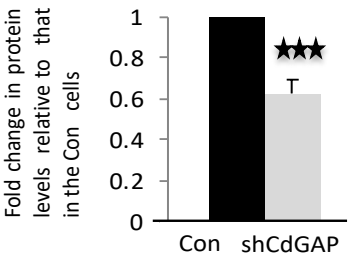
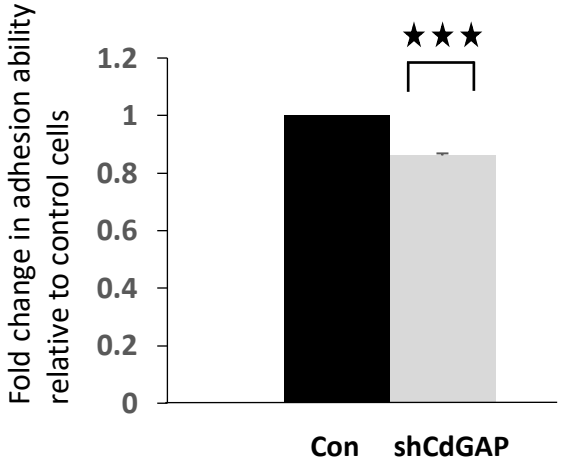


Figure 2.6: CdGAP mildly affects the adhesion of PC-3 cells when plated on fibronectin. For adhesion assay, 40 000 cells were resuspended in serum-free medium containing 0.5% bovine serum albumin and seeded on Fibronectin **(a)** or Collagen type I **(b)** coated wells, then fixed and finally stained with crystal violet. The optical density was measured for each well at 590 nm. Quantitative analysis is relative to that in shcontrol PC-3 cells. Error bars represent standard errors of mean data accumulated from three independent experiments (***P<0.01, NS= not significant).

2.6a) Fibronectin



2.6b) Collagen I

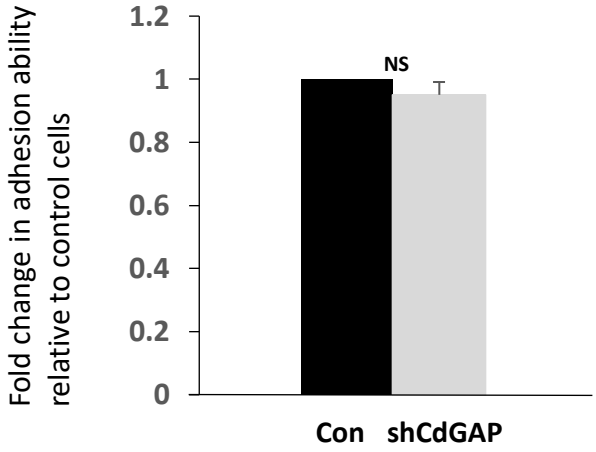
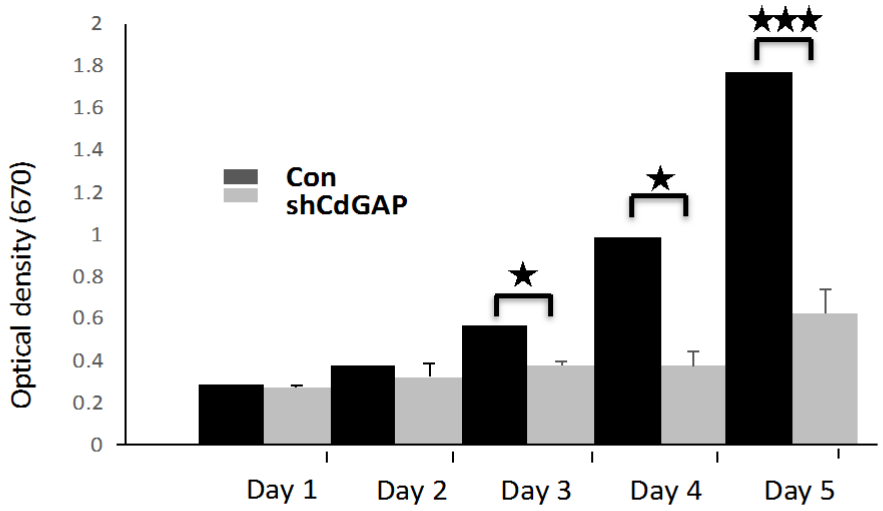


Figure 2.7: Downregulation of CdGAP suppresses cell proliferation, induces cell cycle arrest and increases apoptosis in PC-3 cells. (a) 250 cells from the shcontrol or shCdGAP PC-3 cells were seeded onto 96-well plates for 5 days, followed by addition of MTT solution to each well for the last 4 h of treatment on each day. Quantitative analysis represents optical density at 670 nm on the y axis while the x-axis represents days after plating the cells. **(b)** An *in vitro* colony formation assay was performed by seeding 250 cells representing either shcontrol and shCdGAP PC-3 cells, in 6-well plates. Colonies were fixed with methanol, stained with crystal violet and counted 10 days after plating. **(c)** Images representative of the quantification in (b). **(d)** cellular DNA of shcontrol and shCdGAP PC-3 cells was stained with propidium iodide and cell-cycle phases were assessed through flow cytometry analysis. **(e)** Statistical results for cell cycle phases in (d). **(f)** Representative images determined by Annexin V-labelled flow cytometry assay of cell apoptosis in PC-3 cells with or without CdGAP depletion. **(g)** Statistical results suggesting increased apoptosis via the apoptosis assay. FITC, fluorescein isothiocyanate. All quantitative analysis is relative to that in shcontrol PC-3 cells. Error bars represent SEM. n=3 (**P<0.01, *P<0.05).

2.7a)



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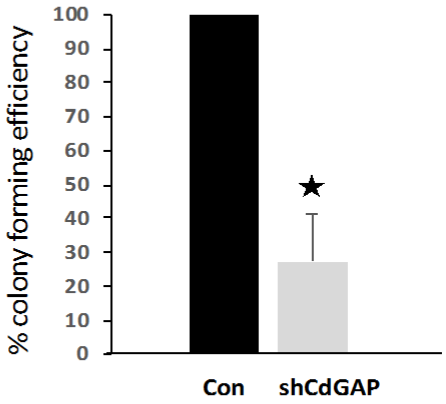

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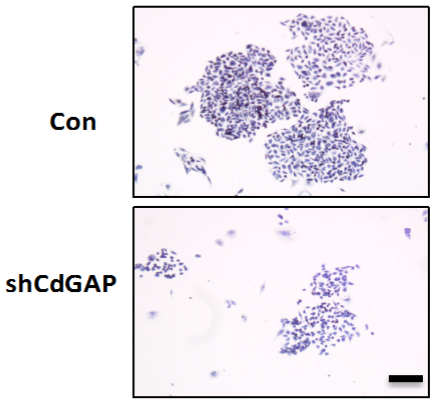

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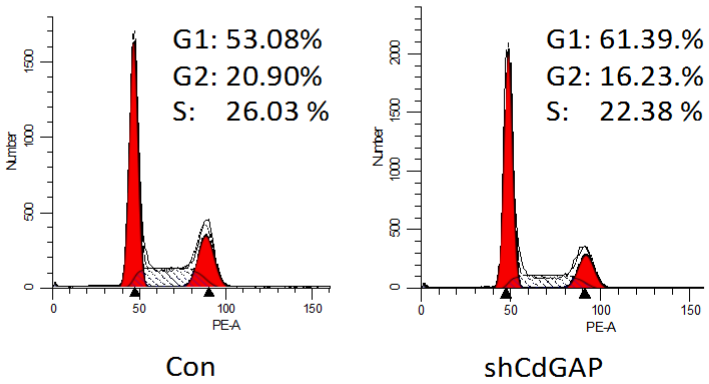

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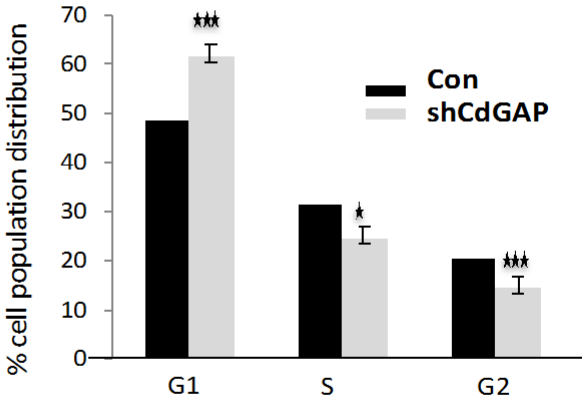

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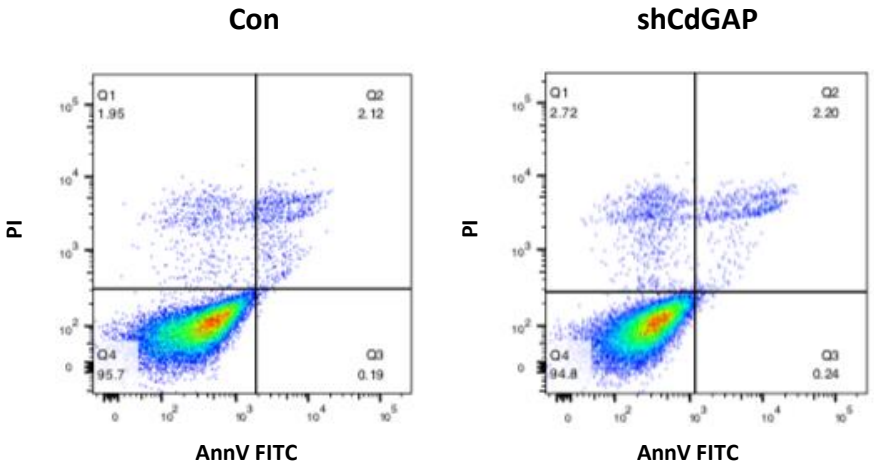

Con
shCdGAP
PI
AnnV FITC
Q1
1.95
Q2
2.12
Q4
95.7
Q3
0.19
Q1
2.72
Q2
2.20
Q4
94.8
Q3
0.24

2.7g)

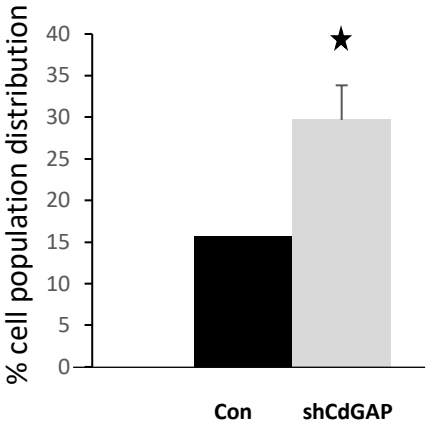
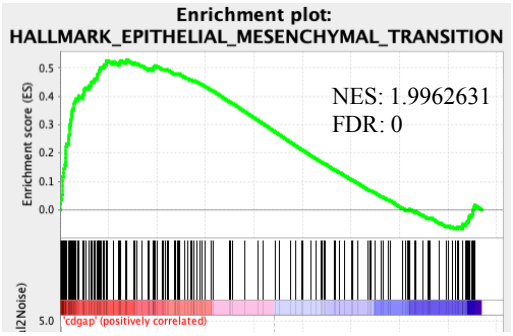

% cell population distribution
40
35
30
25
20
15
10
5
0
Con
shCdGAP

Figure 2.8: Global gene expression analysis of CdGAP-depleted PC-3 cells reveals the biological functions associated with CdGAP (a-d) Gene expression profiles analyzed by GSEA analysis of significantly modulated genes related to EMT, cell-cycle processes, apoptosis and p53 pathway between shcontrol and shCdGAP PC-3 cells. **(e)** Heat map depicts the difference in cell-cycle checkpoint genes with p21 (CDKN1A) as the top hit in this pathway (Indicated with an arrow). **(f)** p21 mRNA levels were measured by Q-PCR. 18S ribosomal RNA was used as loading control. Error bars represent SEM. n=3 (*P<0.05).

2.8a)



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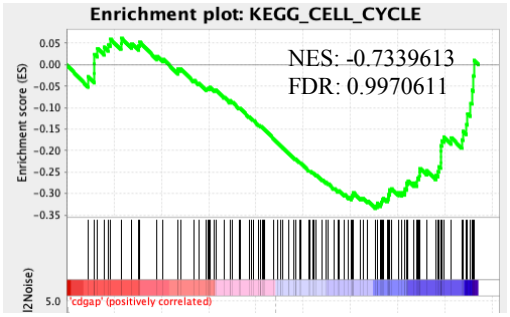

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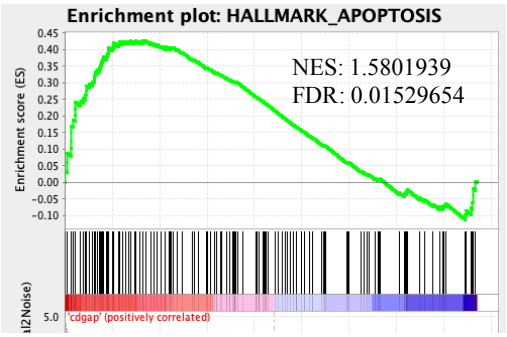

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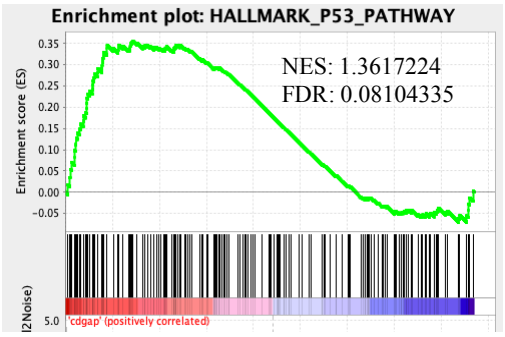

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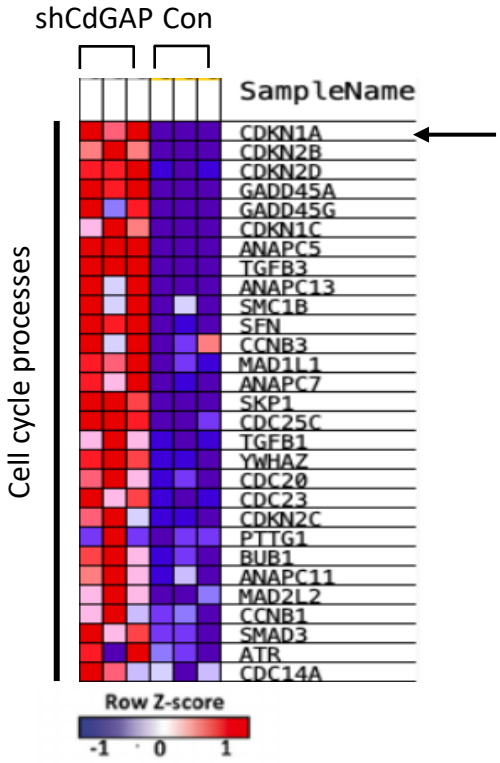

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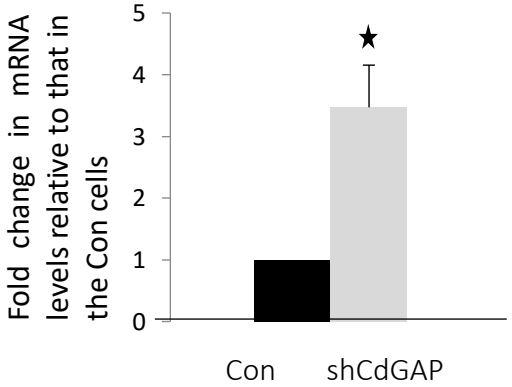
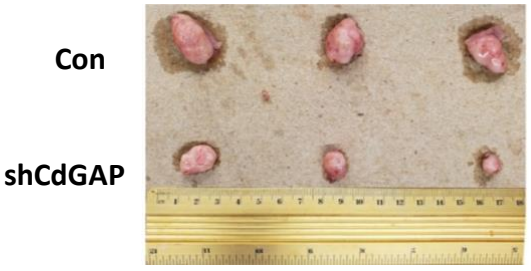


Figure 2.9: The loss of CdGAP delays tumor formation and attenuates tumorigenesis induced by prostate cancer PC-3 cells *in vivo*. (a) PC-3 cells with (shcontrol= 12 mice) or without CdGAP (shCdGAP = 11 mice) were injected in the right flanks of 7 weeks-old nude mice. Images are representative of endpoint tumors that formed in the shcontrol and shCdGAP groups. **(b)** Tumor volume was measured three times a week upto 34 days and is described as the mean volume of each group. (*** $P < 0.01$). **(c)** Date of tumor initiation was assigned when tumors reached a volume of 20 mm^3. The data has been presented as a Kaplan- Meier tumor-free survival curve. **(d)** Weight of xenografts were measured and represented as mean tumor weight in each group. Error bars represent standard errors of the mean (**$P<0.01$, *$P<0.05$).

2.9a)



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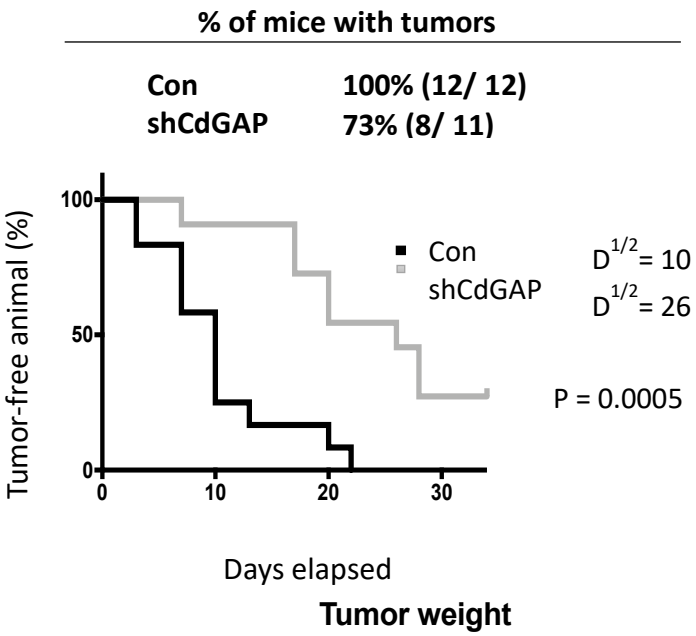

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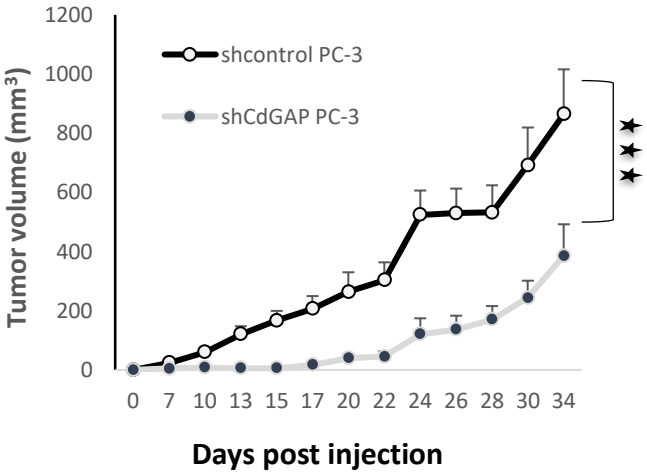


2.9d)

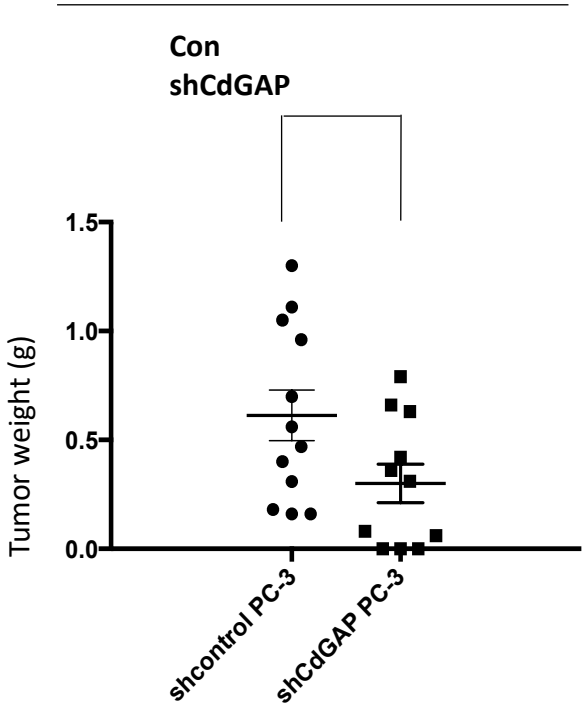


Table 2.1: List of primers for Q-PCR

Gene Name	Forward Primer	Reverse Primer
Snail1	CCCTCAAGATGCACATCCGAA	GACTCTTGGTGCTTGTGGAGCA
CDH1	CCCGCCTTATGATTCTCTGCTCGTG	TCCGTACATGTCAGCCAGCTTCTTG
P21	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG

Chapter 3

SUMMARY AND FINAL CONCLUSIONS

3.0 Summary of original findings

The work presented in this thesis reveals a novel regulation of CdGAP as a promoter of prostate cancer. These roles of CdGAP were characterized through four experimental setups: First, we examined whether CdGAP is expressed in human prostate cancer cells and tissues; Second, by generating stable CdGAP knockout prostate cancer PC-3 cell line we investigated a role of CdGAP in prostate cancer cell proliferation, adhesion, migration and invasion; Third, we demonstrated a role of CdGAP in prostate cancer *in vivo* by performing a subcutaneous injection to assess tumor formation; Fourth, global gene analysis through RNA- sequencing was used to determine pathways under CdGAP influence in prostate cancer. The findings are outlined below:

3.0.1 CdGAP is expressed in a cohort of human prostate cancer cell lines and prostate cancer tissue

The work presented in this thesis is the first study to reveal the expression of CdGAP in the context of prostate cancer. Before this study, CdGAP has been reported as the major GAP expressed in ErbB2-induced mouse breast cancers [180]. Therefore, first, we obtained normal human prostate and prostate cancer tissues from 285 patients and performed tissue microarray analysis immunohistochemistry (IHC) staining. These samples exhibited that strong nuclear localization of CdGAP correlated with early biochemical relapse in patients. Furthermore, alterations in the CdGAP/ARHGAP31 inversely correlated with disease-free survival in prostate cancer patients (n=485). On this basis, we decided to examine the levels of CdGAP in a cohort of human prostate cancer cell lines. Western blot and Q-PCR analysis confirmed high levels of CdGAP in the castration resistant and metastatic human prostate cancer PC-3 cells when compared to the androgen receptor responsive LNCaP cells. Thus, we have presented evidence of the expression of CdGAP in both human prostate cancer cell lines and prostate cancer tissues.

3.0.2 CdGAP regulates prostate cancer cell migration, invasion, proliferation and apoptosis

It is well known that prostate cells require androgens to proliferate during development. Similarly, in prostate cancer, tumor cells rely on androgens for growth [11]. However, the aggressive form of this disease defined as castration-resistance prostate cancer (CRPC) is a state characterized as not dependent on androgens for survival and thus the use of androgen deprivation therapy to treat

patients is ineffective here [9, 11]. Thus, identification of new therapeutic strategies to treat patients diagnosed with CRPC is a dire need. Hence, to test our hypothesis that CdGAP is a promoter of prostate cancer we decided to conduct the majority of our experiments using the androgen-independent metastatic castration resistant PC-3 cells. By using shRNA targeting CdGAP expression we created CdGAP-depleted PC-3 cells. We next performed migration and invasion assays which revealed that downregulation of CdGAP strongly inhibited these processes in PC-3 cells. Concurrently, overexpression of CdGAP in another metastatic and castration resistant cell line DU-145, increased the motility and invasion of these cells. Thus, confirming a prominent role of CdGAP in prostate cancer cell migration and invasion. Furthermore, we analyzed cell proliferation, via anchorage independent growth assays and demonstrated suppression of cell proliferation upon CdGAP-depletion. An extension of these studies revealed that PC-3 cells were arrested in the G0/G1 phase and this was accompanied by increased apoptosis in these cells, which may explain the inhibition of cell proliferation. Together, the work presented in this thesis demonstrated a novel role of CdGAP in prostate cancer cell proliferation, apoptosis, migration and invasion.

3.0.3 CdGAP is a promotor of prostate cancer progression

We examined whether CdGAP was required for tumor growth in xenograft assays using CdGAP-depleted PC-3 cells. Either control or CdGAP-depleted PC-3 were injected subcutaneously into the right flanks of nude mice and these mice were monitored until 34 days. Our analysis revealed that downregulation of CdGAP attenuated tumor initiation, decreased tumor volume and weight by half when compared to control PC-3 cells. Collectively, these results extended the tumor-promoting potential of CdGAP characterized in the cellular models to a biological level with potential clinical implications.

3.0.4 Identification of CdGAP-associated pathways in prostate cancer

To probe CdGAP associated pathways in prostate cancer, we performed RNA-deep sequencing. Such a global gene expression approach allowed us to determine the expression of genes in an unbiased manner. Our analysis revealed that cell migration, cell-cycle processes, cell apoptosis and the p53 pathway gene networks were most significantly differentially regulated upon CdGAP-

depletion in these cells. All these pathways were in corroboration with our experimental results obtained through the *in vitro* and *in vivo* experiments reported in chapter 2. Furthermore, one of the targets, p21 was significantly increased in CdGAP-depleted PC-3 cells. Our Q-PCR results confirmed this pattern of expression. This agrees with the cell cycle analysis and the observed cell-cycle arrest in the G0/G1 phase, thus confirming a robust regulation of cell proliferation by CdGAP in PC-3 cells. Previously, CdGAP was shown to promote TGFβ-dependent cell motility and invasion of breast cancer cells. Furthermore, a novel nuclear function for CdGAP in the regulation of expression of genes involved in epithelial-to-mesenchymal transition (EMT) highlighted its role in the tumorigenesis and metastasis of breast cancer [179, 180]. Together with the reports from breast cancer, our studies have highlighted the pro-oncogenic roles of CdGAP at a global level.

3.1 Future Directions

The cell lines chosen in this study can be distinguished based on a multitude of prostate markers that they express, and one is the androgen receptor [218]. Examination of CdGAP protein and mRNA expression revealed that hormone insensitive PC-3 and DU-145 cells, both of which do not express the androgen receptor, exhibited higher levels of CdGAP when compared with the androgen- responsive cell line LNCaP [218]. The androgen receptor signaling pathway is crucial to maintain normal prostate homeostasis. Aberrant activity of androgen receptor—due to loss-of-function or constitutively activating mutations—results in dysregulation of this signaling pathway which, is often implicated in prostate cancer [11]. As LNCaP cells expressed the lowest levels of CdGAP, it may of interest to assess the effects of hormone signalling on CdGAP expression. Therefore, to test whether androgen signalling inhibits CdGAP expression, LNCaP cells can be cultured in an environment that mimics androgen deprivation using charcoal stripped FBS. This can also be achieved by administering the anti-androgen drug casodex, a drug that binds with high affinity to androgen receptors and inhibits androgen signaling [227].

A role of CdGAP in the regulation of prostate cancer cell proliferation, apoptosis, migration, invasion and tumorigenesis has been established. However, it remains to be determined whether these roles of CdGAP are GAP dependent or independent in order to further investigate how CdGAP exerts its functions in prostate cancer. To achieve this, we could transfect different domains of CdGAP and perform rescue experiments in the CdGAP-depleted PC-3 cells. Alternatively, to demonstrate this, the different domains of CdGAP can be transfected in DU-145 cells and this will identify the domains responsible for the pro-cancer roles of CdGAP in prostate cancer.

The results obtained from the subcutaneous injection confirmed a positive role of CdGAP in prostate cancer initiation and progression. However, we need to further characterize a role of CdGAP in the tumorigenesis and metastasis of prostate cancer which will validate it as a novel biomarker in prostate cancer. This can be achieved by two experimental set-ups both of which will provide greater insight into the aforementioned properties: 1) orthotopic injection of CdGAP-depleted PC-3 cells and control PC-3 cells in the mouse prostate [228]. After the injection, tumor growth will be assessed and analyzed as was the case with the subcutaneous injection.

Furthermore, since the orthopic injection is directly in the prostate it is more representative of the microenvironment of the human tumor and is also a more realistic readout of the tumor stages and progression [228]. Lastly, this experiment will provide valuable information about the regulation of metastasis of PC-3 cells upon CdGAP-depletion; 2) The phosphatase and tensin deleted on chromosome ten (PTEN) conditional prostate deletion mouse model is a well-known experimental setup to investigate the molecular mechanisms underlying prostate cancer progression [229]. Therefore, genetic ablation of PTEN combined with a conditional inactivation of CdGAP expression in the prostate epithelium will be another tool to provide further insight into the regulation of CdGAP in prostate tumorigenesis. This *in vivo* mouse model will specifically help further understand the biological implication of CdGAP in prostate cancer and may lead to potential therapeutic strategies.

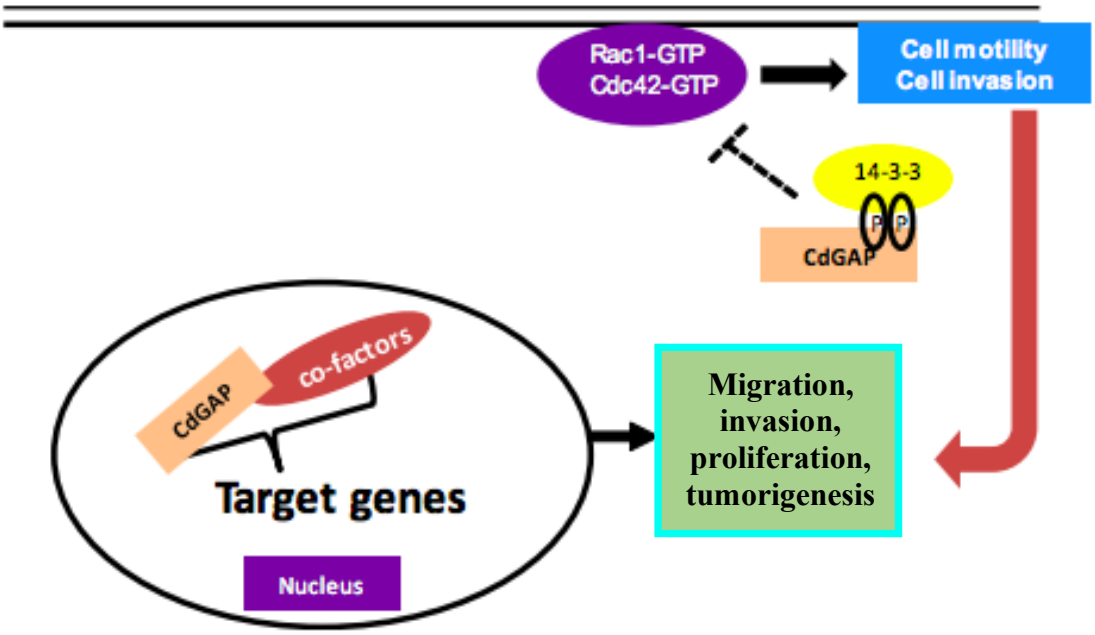
Overall, the data presented in this thesis have provided robust evidence of a role of CdGAP in prostate cancer. However, a limitation of our current study is the lack of investigation of the downstream targets through which these effects are observed and more studies to this end will enable the characterization of the mechanism of CdGAP in prostate cancer (Figure 3.1). The effects of CdGAP in prostate cancer differ from that in breast cancer as we do not observe a similar mechanism of increased E-cadherin expression upon CdGAP depletion. Interestingly, we do report 10% nuclear localization of CdGAP in PC-3 cells which proposes a potential pro-oncogenic nuclear function of CdGAP in prostate cancer as well. However, this remains to be investigated. Thus, in line with the transcriptional role of CdGAP in breast cancer and the observed nuclear localization of CdGAP in prostate cancer, in order to find novel transcriptional targets in prostate cancer we could perform chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq). We already present preliminary information about differential regulation of some genes such as p21, N-cadherin and Slug through the RNA-sequencing results. However, several interesting questions remain unanswered about the downstream mechanisms and whether the aforementioned genes are a direct target of CdGAP in prostate cancer—all of which can be addressed with the ChIP-seq approach. Also, since we will be looking at large-scale data this approach will be unbiased and will highlight key biological processes influenced by CdGAP in prostate cancer cells. This data combined with the existing data from breast cancer can then be analyzed to broaden our knowledge of CdGAP in cancer at a global level. Altogether, this work will confirm the role of

CdGAP as a positive regulator of prostate cancer tumorigenesis and elicit it as a prominent therapeutic target to improve treatments of prostate cancer.

3.2 Figure for chapter 3

Figure 3.1: Model of Rac1/ Cdc42 regulator CdGAP as a novel promoter of prostate cancer: The interaction of CdGAP with 14-3-3 family of adaptor proteins inhibits the nucleocytoplasmic shuttling of CdGAP resulting in sequestration of CdGAP in the cytoplasm and inhibition of its GAP activity [206]. On the other hand, CdGAP can translocate to the nucleus, and through its effect on target genes it can promote migration, invasion, proliferation and tumorigenesis in prostate cancer.

Figure 3.1: Model of Rac1/ Cdc42 regulator CdGAP as a novel promoter of prostate cancer



3.3 General Conclusions

Ras oncogenes were discovered more than three decades ago and mutations in them are identified in over 30% of human cancers [230]. Furthermore, these mutations are typically gain-of-function mutations occurring in the GAP-protein binding sites resulting in defective GTP-hydrolysis and rendering the GTPases as constitutively active [28]. Our knowledge of the role of Rho GTPases and its regulators in cancer has started gaining momentum only recently. Unlike their Ras counterparts, mutations in Rho GTPases have been reported; however, mostly Rho GTPases are implicated in cancer due to aberrant activity or altered activation. Both of these can occur as a result of dysregulation in the activity or expression of the effectors or regulators—GAPs and GEFs—of Rho GTPases [31, 46, 50]. Consequently, more data is being reported that is drawing connections between the regulators of Rho GTPases and various malignancies, which includes cancer.

RhoGEFs activate the GTPases while RhoGAPs inactivate their functions and have thus been traditionally viewed as oncogenes and tumor-suppressors, respectively [46, 50]. It is intriguing that 20 Rho GTPases have been identified whereas 80 RhoGAPs and 82 RhoGEFs have been reported. Thus, the family of Rho regulators outnumbers their GTPases by almost a 4:1 ratio [40]. Hence, it is not surprising that the paradigm assigning these regulators specific roles is now being increasingly challenged. Interestingly, apart from their functions to inactive GTPases and act as tumor-suppressors, recent evidence has associated RhoGAPs in cancer with scaffold protein functions, transcriptional regulation of genes and as tumor-promoters—all of which are GAP-independent functions [134, 147, 177, 179, 180, 226, 231]. To this end, the work presented in this thesis challenges the claim that conventionally views RhoGAPs as tumor-suppressors and supports a novel hypothesis whereby the expression of a RhoGAP has been implicated as an oncoprotein in cancer. In particular, we have investigated the role of a regulator of CdGAP in prostate cancer and demonstrated it as a promoter of prostate cancer cell proliferation, migration, invasion and tumorigenesis. There exists other evidence that has ascribed pro-cancer functions to CdGAP mainly through studies conducted in breast cancer [179, 180]. Collectively, the work presented in this thesis and previously established about CdGAP in the context of cancer highlights a novel regulation of a RhoGAP in human health and disease. Further studies are required to characterize the mechanism of CdGAP in prostate cancer. Ultimately, we hope that more research to this end

will contribute towards enhancing our understanding of the regulation of RhoGAPs and subsequently result in the development of new targets in the treatment of cancer.

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