Identification of novel regulatory mechanisms for Cdc42 GTPase-activating protein CdGAP/ARHGAP31, a protein involved in development and cancer

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Abstract

The small Rho GTPase proteins act as molecular switches that regulate diverse cellular processes linked mostly to the actin-cytoskeleton remodeling making them essential regulators of cell adhesion, migration and invasion. Dysregulation of their activities can result in different abnormal phenotypes particularly, tumor progression and metastasis. Hence, regulators of Rho GTPases such as Rho guanine nucleotide exchange factors (RhoGEFs) and Rho GTPase-activating proteins (RhoGAPs), are critical for normal cellular responses and are targets for subversion during oncogenic transformation.

CdGAP (Cdc42 GTPase-activating protein) is a member of a well-conserved subfamily of RhoGAP proteins and a negative regulator of the small Rho GTPases, Rac1 and Cdc42. Associated with a rare developmental disorder (AOS, Adams-Oliver Syndrome) and required for a normal angiogenesis, CdGAP plays important roles in the regulation of cell migration and proliferation during early development. In addition, recent findings characterize CdGAP as an essential synergistic component between TGFβ and HER2/Neu/ErbB-2 signaling pathways which play a positive role in cancer, particularly breast cancer. CdGAP is regulated by lipid binding, protein-protein interactions and phosphorylation, still these mechanisms are not well understood. In this work we first investigate the interaction between CdGAP and its negative regulator, the endocytic protein Intersectin. Using an *in vitro* approach, we identify a novel, atypical xKx(K/R) (SKSKK) motif in the basic rich (BR) region of CdGAP that directly interacts with the Intersectin-SH3D domain. Moreover, the well-conserved motif is required for the regulation of CdGAP activity following Intersectin binding. Next, we investigate CdGAP phosphorylation and identify two regulatory phospho-serines in the C-terminal (CT) tail, Ser-1093 and Ser-1163, that are phosphorylated by the AGC-kinase family member, RSK1. Finally, we show that 14-3-3 family members bind and regulate both the cellular localization and activity of CdGAP in a Ser-1093 and Ser-1163 phosphorylation-dependent manner. Overall, this work provides two novel CdGAP-regulatory mechanisms that can be applied in therapeutic approaches targeting this RhoGAP, particularly in breast cancers.

Résumé

Les petites protéines G de la famille Rho sont des commutateurs moléculaires qui contrôlent divers procédés cellulaires associés notamment, à la régulation du cytosquelette et jouent par conséquent, un rôle clé dans la régulation de la motilité cellulaire. La dérégulation de leur activité peut entrainer des aberrations se manifestant en particulier, par une progression du cancer et des métastases. Ainsi, les protéines régulatrices comme les facteurs d'échange de nucléotide (RhoGEFs) et les protéines activatrices des Rho GTPases (RhoGAPs) sont essentielles pour une signalisation cellulaire normale et sont en général, affectées lors des transformations oncogéniques. CdGAP (Cdc42-GTPase activating protein) est un membre d'une sous-famille de protéines RhoGAPs bien conservée qui régule négativement les Rho GTPases Cdc42 et Rac1. Associé à un trouble du développement rare, le syndrome d'Adams-Oliver ou AOS et nécessaire pour une angiogenèse normale, CdGAP joue un rôle important dans la régulation de la migration et la prolifération cellulaires au cours du développement. Récemment, CdGAP est identifié comme une composante synergique essentielle entre les voies de signalisation de TGFβ et HER2/Neu/ErbB-2 et qui joue un rôle proto-oncogénique, en particulier dans le cancer du sein. CdGAP est régulée par des mécanismes incluant les lipides, les interactions protéine-protéine et la phosphorylation, néanmoins, ces mécanismes ne sont pas bien élucidés. Dans cette étude, nous étudions en premier l'interaction entre CdGAP et son régulateur négatif, la protéine endocytique, Intersectin. En utilisant une approche *in vitro*, nous identifions un nouveau motif atypique, xKx (K/R) (SKSKK) dans la région riche en résidus basiques (BR) de CdGAP interagissant directement avec le domaine SH3D d'Intersectin. Le motif est en outre, bien conservé est requis pour la régulation de l'activité CdGAP par Intersectin. Par la suite, nous identifions deux sites de phosphorylation clés dans la région C-terminale de CdGAP, Ser-1093 et Ser-1163 qui sont phosphorylés par la protéine AGC-kinase, RSK1. Nous démontrons finalement, que les protéines adaptatrices 14-3-3 lient et régulent la localisation cellulaire et l'activité de CdGAP d'une manière dépendante de la phosphorylation des résidus, Ser-1093 et Ser-1163. La présente étude identifie deux nouveaux mécanismes de régulation de CdGAP qui peuvent être exploités dans des approches thérapeutiques ciblant cette protéine, notamment dans les cancers du sein.

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Abbreviations

3BP-1	SH3-binding protein 1
Abr	Active Bcr-related
AKT	Ak thymoma
ALS	Amyotrophic lateral sclerosis
AOS	Adams-Oliver syndrome
AP-2	Adaptor proteins complex 2
ARAP	ArfGAP and RhoGAP with ankyrin repeat and PH domains
ARHGAP	Rho GTPase-activating protein
ARNO	ARF nucleotide-binding site opener
Arp2/3	Actin-related protein 2 and 3 complexes
ATP	Adenosine triphosphate
B-A	Bcr-Abl oncoprotein oligomerisation domain
BAR	Bin/amphiphysin/Rvs
BCH	BNIP-2 and Cdc42GAP homology
Bcr	Breakpoint cluster region
BPGAP1	BCH-domain-containing, proline-rich and Cdc42 GAP-like protein 1
BR	Basic region
C1	Cysteine-rich phorbol ester binding
C2	Calcium-dependent lipid binding
CC	Coiled-coil
CAMGAP1	CIN85-associated multi-domain-containing Rho1
Cdc42	Cell division cycle 42
CdGAP	Cdc42 GTPase-activating protein
CeGAP	*Caenorhabditis elegans* GAP
CIN85	Cbl-interacting protein of 85 kDa
CIP4	Cdc42-interacting protein 4
CT	C-terminus
CLIC	Clathrin-Independent Carrier

cDNA	complementary DNA
CRIB	Cdc42 and Rac1 interactive binding
DAPI	4', 6-diamidino-2-phenylindole
DCC	Deleted in colorectal cancer
DH	Dbl homology domain
DLC	Deleted in liver cancer
DHR	Dock homology region
DMEM	Dulbecco's modified Eagle's medium
Dock	Dedicator of cytokinesis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E-cadherin	Epithelial cadherin
ECL	Enhanced chemiluminescence
Ect2	Epithelial cell transforming 2
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
F-BAR	FCH/BAR
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCH	Fes/CIP4 homology
FF	Domain with two conserved phenylalanine residues
FilGAP	Filamin A-associated RhoGAP
Fyn	Fyn proto-oncogene
FYVE	Fab 1, YOTB, Vac 1 and EEA1
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide-exchange factor

GMIP	Gem-interacting protein
GPCRs	G protein–coupled receptors
GRAF1	GTPase regulator associated with focal adhesion kinase-1
Grit	GTPase regulator interacting with TrkA
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
hLgl	H Lethal giant larvae
HMHA-1	Histocompatibility Minor HA-1
HRP	Horseradish peroxidase Ig immunoglobulin
In-SH2-D	Phosphatidylinositol 3-kinase regulatory subunit p85 inter-SH2 domain
INPP5B	Inositol polyphosphate 5-phosphatase B
IPPc	Inositol polyphosphate phosphatase, catalytic domain homologues
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IQ	Calmodulin-binding motif
JNK	c-Jun N-terminal kinase kDa kilodalton
LTCCs	L-type voltage-gated Ca(2+) channels
MAPK	Mitogen-activated protein kinase
mDia	mammalian diaphanous formins
MEKK	MAPK/ERK kinase kinase
MgcRacGAP	Male germ cell RacGAP
MLS	Microphthalmia with linear skin defect
MLC	Myosin light chain
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MYSc	Myosin complex, large ATPase
MyTH4	Myosin tail homology 4
Nadrin	Neuron-associated developmentally regulated protein
NGF	Nerve growth factor

N-WASP	Neural Wiskott-Aldrich syndrome protein
Nck	Non-catalytic region of tyrosine kinase adaptor protein
NF-Kb	Nuclear factor-kappa B
Nox5	NADPH oxidase 5
OPHN1	Oligophrenin-1
OCRL	Oculocerebrorenal syndrome of Lowe
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PAGE	Polyacrylamide gel electrophoresis
PAR	Partitioning-defective protein
PARG1	PTPL1-associated RhoGAP1
PBR	Polybasic region
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDZ	Post synaptic density protein, Drosophila disc large tumor suppressor and Zonula occludens-1 protein domain
PEI	Polyethylenimine
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase G
PLC	Phospholipase C
PLD	Phospholipase D
PRC1	Protein regulating cytokinesis 1
PRD	Proline-rich domain
PSGAP	PH- and SH3-domain-containing RhoGAP
PtdIns3P	Phosphatidylinositol 3-phosphate
PtdIns4P	Phosphatidylinositol 4-phosphate
PtdIns5P	Phosphatidylinositol 5-phosphate

PtdIns(4,5)P2, Phosphatidylinositol 4,5-bisphosphate

PtdIns(3,5)P2,	Phosphatidylinositol 3,5-bisphosphate
PtdIns(3,4,5)	P3, Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homolog
PTPL1	Protein-tyrosine-phosphatase- like protein-1
PX	Phox homology
RA	Ras association
RA-RhoGAP	Ras-associating-RhoGAP
Rab	Ras in brain
Rac	Ras-related C3 botulinum toxin substrate
RalBP1	Ral-binding protein 1
Ras	Rat sarcoma
RBD	Ral binding domain
Rho	Ras homologous
RhoGAP	GTPase-activating protein
RICH	RhoGAP interacting with CIP4 homologues
RICS	RhoGAP involved in catenin—N-cadherin and NMDA receptor signalling
RIP1	Ral-interacting protein 1
RLIP76	Ral-interacting protein 76 kDa
RNAi	RNA interference
Rnd	Resistance-nodulation-cell division
RnRacGAP	Rotund RacGAP
ROCK	Rho-associated kinase
ROBO	Homolog of drosophila roundabout
RSK	p90 ribosomal S6 kinase
SAM	Sterile α-motif
Sec14	Exocyst complex subunit 14
SH3BP1	SH3 Domain Binding Protein 1
SH2	Src homology 2
SH3	Src homology 3
siRNA	small interfering RNA
Smurf	SMAD ubiquitin regulatory factor

SNARE	SNAP receptor
SNAP	synaptosomal-associated protein
srGAP1	Slit-Robo GTPase-activating protein 1
START	StAR (steroidogenic acute regulatory)-related lipid transfer
SYD-1	Synapse-defective-1
TAGAP	T-Cell Activation RhoGTPase Activating Protein
TCGAP	TC10/Cdc42 GAP
TGN	Trans-Golgi network
tGAP1	testicular GAP1
Tiam1	T-cell lymphoma invasion and metastasis 1
Trio	Triple functional domain protein
U2OS	Human osteosarcoma cells
VAMP	Vesicle-associated membrane protein
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
VEGF-R2	Vascular endothelial growth factor receptor 2
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP-family verprolin-homologous proteins
Wrch	Wnt-responsive Cdc42 homolog
WW domain	Domain with two conserved tryptophan residues
XrGAP	*Xenopus* RhoGAP
ZEB2	Zinc Finger E-Box Binding Homeobox 2

Contribution of Authors to Manuscripts

Chapter 2:

My contribution to the manuscript entitled “Cdc42 GTPase-activating protein (CdGAP) interacts with the SH3D domain of Intersectin through a novel basic-rich motif” included the designing and performing all the experiments in Figure 2.5, which is the critical figure of the whole paper since it validates the role of the identified SKSKK motif, and helping with Figure 2.4.

My contribution in Figure 2.5 included:

- Generating the pEGFPC1- CdGAP(1-820)(SaSaa) plasmid containing the mutations;
- Preparing the DNA constructs expressing pRK5-mycCdGAP (1-515), pRK5-mycCdGAP(1-515)(SaSaa), pEGFPC1, pEGFPC1-CdGAP(1-820 and pEGFPC1-CdGAP(1-820)(SaSaa);
- Preparing the GST-SH3D proteins;
- Adapting, setting up and performing the GAP assays for the different conditions;
- Performing the quantitative and statistical analysis.

My contribution in Figure 2.4, which I performed in collaboration with M.P. included:

- Repeating the experiments in panel B and adding the Ponceau S blots as control;
- Helping with data quantification and analysis presented in panel C.

In addition, I reformatted the whole paper material including the Figures and the text after revision before resubmitting to FEBS Letters.

M.P. performed the remaining manuscript experiments. M.P., N.L.V. and I each wrote and prepared this manuscript.

Overall, my contribution to the work presented in this published article represents 40% of total work.

The manuscript in its final and published form is included only in the present thesis.

Chapter 3:

My contribution to the manuscript entitled “CdGAP/ARHGAP31 is regulated by RSK phosphorylation and binding to 14-3-3β adaptor protein” included designing and performing all experiments, except for figure 3.3 which were performed by V.C.. Y.H. helped with figure 3.1 G-J. H.I. helped with figure 3.6. P.P.R., R.C., and J.P.G. assisted with the identification of CdGAP phosphosites indicated in Table 3.1. N.L.V. and I each wrote this manuscript.

Original contributions to knowledge

The work presented in this thesis contributed to research knowledge by deciphering two novel molecular mechanisms involved in the regulation of activity and subcellular localization of CdGAP. Notably, the original contributions consist of:

- Identification of a novel motif, SKSKK in the basic-rich region of CdGAP, and validation of its regulatory role of CdGAP activity;
- Identification and characterization of two regulatory phospho-serines residues in the C-terminal region of CdGAP;
- Identification of a new regulatory mechanism of CdGAP activity and subcellular localization involving phosphorylation and 14-3-3 protein recruitment and binding.

Given the role of CdGAP in development and cancer, the contributions presented here will allow a better understanding of CdGAP role in small GTPase and cell motility regulation. Moreover, the mechanisms identified will impact particularly the drug discovery field by permitting novel therapeutic approaches to inhibit CdGAP in cancer.

Submitted Manuscripts

Chapter 3:

CdGAP/ARHGAP31 is regulated by RSK phosphorylation and binding to 14-3-3β adaptor protein (2016). Ali Ben Djoudi Ouadda, Viviane Calabrese, Yi He, Hidetaka Ishii, Rony Chidiac, Jean-Philippe Gratton, Philippe P. Roux and Nathalie Lamarche-Vane. **Submitted to Molecular Biology of the Cell.**

Chapter 1: Introduction and Literature Review

1.0 General introduction

The emergence of life on earth and the evolvement of complex cellular systems and enzymes allowing the use of ATP to generate energy, permitted cell movement for both prokaryotes and eukaryotes. In multicellular organisms, cell migration is a central and critical process during embryonic development and organogenesis, immune responses and wound healing, which are events required to ensure the development of the organism and to maintain its integrity. Likewise, aberrations affecting cell motility can lead to serious consequences and are at the origin of many pathological conditions such vascular diseases, mental disabilities and tumor progression and metastasis. It is therefore critical to investigate and understand the mechanisms regulating cell motility in the hope of developing better therapeutic strategies for the treatment of specific disorders.

1.1 Overview of the small GTPases

The discovery of the Ras oncogenes more than three decades ago paved the way to the discovery of dozens of similar proteins grouped under the superfamily of Ras G proteins. The Ras superfamily members are monomeric G protein enzymes of about 21kDa, which regulate many cellular processes including, cytoskeleton remodeling, cellular trafficking and cell proliferation, apoptosis, differentiation, migration and adhesion. More than 150 members have been so far characterized and can be divided into five subfamilies: Ras, Rho, Rab, Ran and Arf. They all share the guanosine triphosphate (GTP) ase function, an enzymatic activity consisting of the hydrolysis of γ-phosphate of GTP to generate guanosine diphosphate (GDP) and inorganic phosphate. These small GTPases cycle between an active GTP-bound and inactive GDP-bound forms following conformational changes at their switch 1 and 2 of their GTP-binding regions (1-4).

1.1.1 Ras GTPase regulation

Owing to their involvement in many key cellular events, the function and activity of Ras GTPases are tightly and spatiotemporally controlled by different mechanisms such as protein-protein interactions and posttranslational modifications. The GTPase

cycle is regulated by three group of proteins: the guanine nucleotide exchange factors (GEFs), which initiate the GDP to GTP exchange; the GTPase activating proteins (GAPs) that enhance the intrinsic GTP-hydrolysis activity of the GTPase; and the guanine dissociation inhibitors (GDIs) which form soluble and stable complexes with the GTPase in the cytoplasm masking its lipid moiety for plasma membrane (PM) localization (5,6).

The subcellular localization is also a contributing factor to the regulation of Ras GTPase activity and many members including Arf, Rab, Ras and Rho, are often found bound to the PM where they recruit their effectors and control thereby, their downstream signaling pathways (7,8). Most of these small GTPases, like the Rho family members, undergo posttranslational-lipid modifications (e.g. geranylgeranylation, palmitoylation and N-myristoylation) allowing the binding to negatively-charged PM phospholipids, where they translocate to carry-out their downstream activational effects (8). Typically, the addition of lipid groups is mediated via covalent bindings to a CAAX box at the C-terminal of the GTPase proteins (8,9).

However, the PM targeting requires often additional polybasic regions (PBRs) on the GTPase to be achieved (10). In fact, a significant study conducted to decipher the motifs and posttranslational modifications involved in targeting small GTPses to the PM, reported that the presence of PBRs is a requirement in 37 out of 48 studied proteins (11). The PBRs bind to specific phospholipids in the inner leaflet of the PM conferring a precise localization site for the GTPase and hence, a site-specific conduction of the GTPase function (9). The PBRs of Ras, Rab, Arf, and Rho GTPases bind either PtdIns(4,5)P (phosphatidylinositol 4,5-bisphosphate) or PtdIns(3,4,5)P3 (phosphatidylinositol 3,4,5-triphosphate) and the dissociation of the small GTPase from the PM occurs only when both these phospholipids are depleted from the PM simultaneously (11). Moreover, the presence of a PBR increases the specificity and the catalytic activity of prenylation enzymes and thus, enhances the PM localization of the small GTPase (12). The prominent role of PBRs in the PM localization makes them critical regulators of small GTPase activities as the following examples will further illustrate (Table 1.1). For instance, the C-terminal di-arginine motif (Arg-186 and Arg-

187) of Cdc42 is essential for its PM targeting and activity. The PBR binds directly to PtdIns(4,5)P2-containing membranes allowing Cdc42 to initiate oncogenic transformation of fibroblasts, whereas the mutation of this site abolished the GTPase transforming abilities (13). Similarly, the PBRs are essential for the cellular functions of several Ras family members. Indeed, many K-Ras proteins contain hypervariable C-terminal domains intended to provide various and specific PM-localization signals, whilst their PM localization usually requires both a CAAX motif and a palmitoylation site within the hypervariable region. However, in some situations such as in the absence of the CAAX box or lipid modification, the presence of a PBR is imperative for the PM localization and the GTPase activity. The PBR can achieve this role alone or in combination with lipid-modified sites or the CAAX box. As such, myristoylated K-Ras (p21K-ras) proteins require PBRs to be translocated to the PM (14). By contrast, the p21K-RasB isoform which is not palmitoylated, requires a PBR in combination with the CAAX motif for its PM localization (11,15). Interestingly, similar conserved mechanisms are found in yeast wherein PBRs mediate the lipid modification and PM targeting of the small GTPases. Actually, in an experiment looking at the Ras-dependant growth in yeast, it has been shown that the presence of a PBR at the C-terminal region of Ras2 is sufficient to drive normal growth and Ras palmitoylation (i.e., PM localization and function) despite the absence of the CAAX box, necessary for Ras2 prenylation and function (16).

1.1.2 Roles of Ras GTPases

Even though the Ras subfamilies affect many cellular events and their roles sometimes overlap in several signaling pathways, specific functions can be assigned to each subfamily according to the processes they tend to regulate the most. For instance, the Rho subfamily with its prominent members, Rac, Cdc42 and Rho is involved in gene expression regulation, actin cytoskeletal regulation and organization, as well as cell migration, adhesion and invasion (17,18). Ras subfamily proteins including the three main branches, Ras (i.e., isoforms H-Ras, N-Ras and K-Ras), Ral and Rap are central regulators of gene expression, cell survival, growth and proliferation (19-21).

Table1.1 –Examples of PBRs involved in membrane localization

Protein	Entry[a]	Cell function/ pathway	PBR	Role in membrane localization	Ref.
hLgl	Q15334	Cell polarity Tumorigenesis	652-S**R**V**K**SL**KK**SL**R**QSF**RR**I**RK**S**R**VSS**RK R**[b]	PBR phosphorylation neutralizes the positive charges and reduces PM localization.	(22)
LLTCCs	Q13936	Muscle contraction, hormone secretion, gene expression, and neuronal excitability	536-**RR**WNFC**RRK**	PBR required for PM-phosphoinositide binding and stability.	(23)
Nox5	Q96PH1	ROS production	186-APAP**R**P**R**P**RR**P**R** QLT	PBR mediates N-ter. binding to PtdIns(4,5)P2 and translocation from internal membranes to PM.	(24)
PTEN	P60484	Tumor suppressor Insulin signaling pathway	4-I**K**EIVS**R**N**KRR**	PBR is an N-ter. PtdIns(4,5)P2 - binding site responsible of membrane targeting.	(25)
G protein subunit alpha (q)	P29992	GPCRs signaling	19-**RR**INDEIE**R**QL**RR** D**KR**DA**RR**	PBR required for PM localization and protein function.	(26)
G protein subunit alpha (s)	Q5JWF2	GPCRs signaling	19-**R**EAN**KK**IE**K**QLQ**K** D**K**QVY**R**A	PBR required for PM localization and protein function.	(27)
G protein subunit alpha14	O95837	GPCRs signaling	19-Q**R**ISAEIE**R**QL**RR** D**KK**DA**RR**	PBR required for PM localization and protein function.	(28)
G protein subunit alpha16	P30679	GPCRs signaling	19-A**R**VDQEIN**R**ILLEQ **KK**QD**R**G	PBR required for PM localization and protein function.	(28)
Ras2	P01120	Ras2 dependant growth	**K**LI**KRK**	PBR allows palmitoylation and function of Ras2 in the absence of a CAAX box.	(16)
K-Ras	P01116	Ras GTPase activity	Hypervariable C-ter. region (165-185)-**KKKKK**	PBR required for PM localization and protein function.	

K-Ras	P01116	Ras GTPase activity	175-**KKKKK**	p21-Krasb is not palmitoylated but requires both the CAAX box and the PBR for its function and PM localization.	(15)
DOCK1	Q14185	RacGEF	1610-**K**QYGV**R**TMPSGL DD**RR**GS**R**P**R**	PBR required for DOCK1 translocation to the dorsal ruffles through association with phosphatidic acid.	(29)
DOCK2	Q92608	RacGEF Neutrophil chemotaxis	1619-**R**EMPDFED**RR**VG **R**P**R**SM—1695-**R**S**KKR**T	PBR required for DOCK2 translocation to the leading edge via association to phosphatidic acid.	(30)
Ect2	Q9H8V3	RhoGEF Cell division	792-ANTIC**K**ADAENLIY TADPESFEVNT**K**D MDSTLS**R**AS**R**AI**K K**T	PBR required for targeting Ect2 to the equatorial membrane during cytokenesis.	
Protein ARNO	Q99418	ArfGEF *In vitro* system	384-**RKKR**ISV**KKK**	PBR and PH domain cooperation for membrane lipid binding	(32)
Cytohesin-1	Q15438	T cell adhesion ArfGEF	128-**RKKK**VSST**KR**H	PBR and PH domain cooperation for PM localization and function of Cytohesin-1.	(33)
Cdc42	P60953	Oncogenic transformation	183-**KK**S**RR**	PBR required for Cdc42 binding at enriched-PtdIns(4,5)P2 membranes.	(13)
DLC1	Q96QB1	Tumor suppressor RhoGAP	1051-**K**HGFSWAVP**K**FM **KR**I**K**VPDY**K**D**R**	PBR required for PM recruitment and binding to PtdIns(4,5)P2. Regulation of GAP activity.	(34)
p190 RhoGAP-A	Q9NRY4	RhoGAP	1214-**RRR**NIL**R**SL**RR**NT **KK**P**K**P**K**P**R**PSIT**K**	PBR required for PM recruitment. Regulation of GAP activity.	(35)
CdGAP	Q2M1Z3	RhoGAP	2-**K**N**K**GA**K**Q**K**L**KRK**	PBR required for PM recruitment and. Regulation of GAP activity.	(36)

[a]NCBI protein entry number.
[b]Basic amino acids that make up the PBR motif are bolted.

Ran GTPases are key players in the nucleocytoplasmic protein transport (37), and finally, the Arf and Rab subfamilies are involved in vesicular transport and trafficking pathways (38-40).

Ever since Ras superfamily members are involved in many cellular events, mutations in their genes or expression dysregulation in their proteins often lead to tumor transformation. Indeed, mutations in the Ras genes occur in 30% of human tumors, mostly mutations in K-Ras and N-Ras are found with high frequency in various cancers such as pancreatic and colorectal cancers (41-44). The mutations affect generally the GAP-protein binding sites and thus prevent the GTP-hydrolysis, leading to constitutively activated GTPase and tumorigenesis (45). On the other hand, the Rho GTPase role in cancer is usually associated to expression abnormalities of these proteins or their regulators but also to the mutations in their genes, leading to increased active GTPase and oncogenic transformation (46,47).

1 .2 The Rho GTPase subfamily

Ras homologous (Rho) GTPases are highly conserved members of the Ras superfamily of small G proteins. To date, twenty mammalian Rho GTPases have been characterized from which three groups, Rho, Rac and Cdc42, have been extensively studied for their role in actin-cytoskeleton remodeling. Namely, RhoA (isoforms A, B and C) controls the formation of stress fibers (assembly of actin-myosin filaments), Rac (isoforms 1, 2 and 3) and Cdc42 regulate the formation of actin-rich lamellipodia and filopodia protrusion respectively (6,48-51).

1.2.1 Rho GTPase regulation

Similar to other Ras small GTPases, Rho proteins cycle between an active GTP-bound and inactive GDP-bound state, a switch monitored by three classes of enzymes: RhoGEFs, RhoGAPs and RhoGDIs. These enzymes ensure a precise spatiotemporal modulation of Rho GTPases in different subcellular localizations and various cellular types and environments. Moreover, many Rho GTPases undergo numerous posttranslational modifications regulating their activity and subcellular localization. Overall, the Rho GTPase activity is tightly modulated and is subjected to a complex

regulatory network for an optimal regulation outcome. In the following sections the major factors regulating Rho GTPases will be discussed with an emphasis put on RhoGAP regulation and subcellular localization (to be discussed in sections 1.3 and 1.4).

1.2.1.1 Rho Guanine nucleotide Exchange Factors (RhoGEFs)

RhoGEF proteins are the main activators of Rho GTPases by mediating their GDP to GTP substitution. So far, around 85 RhoGEF members have been identified and can be divided into two major and distinct families: DOCK and DH/PH-containing RhoGEFs (52). DOCK proteins comprise a Dock Homology Region 1 (DHR1) which binds to phospholipids and thus mediates the PM targeting, and a catalytic exchange domain, DHR1 that is in charge of the GDP to GTP exchange. DOCK proteins present a GEF activity restricted only to Cdc42 and Rac GTPases (53,54). The DH/PH-containing RhoGEF family, which is the most represented family of RhoGEFs, contains a Dbl homology (DH) domain next to a pleckstrin-homology (PH) domain (55). The former conducts the GEF activity and the latter helps the PM anchoring of the RhoGEF by interacting with the membrane phospholipids (56).

RhoGEFs, alike other GEF proteins, require generally a PM translocalization in order to conduct the GEF activity towards their Rho GTPase effectors. To do so, many RhoGEFs are endowed with lipid binding domains (LBDs) and structural motifs ensuring their recruitment to the PM. Additionally, as in many GEFs, the presence of PBRs helps the protein targeting and stability at the PM by cooperating with the LBDs. For instance, the RhoGEF Ect2 requires both its PH and PBRs to achieve translocation to the cleavage membrane during cytokinesis (Table 1.1) (31). Likewise, during neutrophil chemotaxis, Rac1 which induces the extension of the membrane protrusions at the leading edge, requires the presence of DOCK2 at the PM for its activation. DOCK2 recruitment and accumulation is achieved by a dual lipid-binding mechanism involving PtdIns(3,4,5)P3 and phosphatidic acid. The binding to phosphatidic acid which is mediated by a C-terminal PBR, stabilizes the DOCK2-PM anchoring and results in Rac1 activation and actin polymerization (Table 1.1) (30).

Because RhoGEFs are the main activators of Rho GTPases, aberrations in their expression or activity result in an unusual high activity of their target GTPases, which is

associated with malignant transformation. Indeed, abnormal overexpression levels of RhoGEFs including Tiam1, Ect2, Vav isoforms 1, 2 and 3, DOCK180 and ELMO have been reported in various cancers in which they act as oncogenes (46,57,58). As an example, the Rac1 GEF Tiam1 is overexpressed in various tumors such as breast, liver, head and neck cancers, and this is associated with a high degree of disease progression, invasion and metastasis, as well as a poor patient prognosis (59-62). Accordingly, therapeutic approaches to antagonize and lower the expression and activity of this RhoGEF are in development for cancer treatment (63).

1.2.1.2 Rho Guanine nucleotide Dissociation Inhibitors (RhoGDIs)

RhoGDI members bind the geranylgeranylated GDP-bound Rho GTPases and sequester them in the cytoplasm to prevent spontaneous activation (64). They also monitor the delivery and extraction of Rho GTPases from and to cellular membranes, where they exert their action, regulating both their cellular trafficking and activity (65,66). It appears therefore, that these proteins are important Rho GTPase regulators and dysregulation of their expression (i.e., overexpression or downregulation) is usually associated to numerous human cancers (67-69).

1.2.1.3 Posttranslational regulation

Posttranslational modifications such as ubiquitination, phosphorylation and a wide range of other modifications have been suggested to regulate many Rho GTPases, although it is unclear whether these modifications are imperative for the GTPase activity (70-74). Still, several examples display the implication of some of these modifications in human cancers. For instance, impaired ubiquitination and degradation of some Rho GTPases result in their accumulation in specific intracellular compartments and increased activity levels, which in turn promote cell migration and invasion (75,76). Likewise, the transforming capacities of Cdc42 are augmented following the phosphorylation of its Tyr-64 residue by Src-tyrosine kinase, which enhances the association with its RhoGDI, a prerequisite for the tumorigenic activity (77).

1.2.2 Role of Rho GTPases in cancer

Rho GTPases hold critical roles in many signaling pathways with prominent functions in cell cycle progression, cell adhesion, migration, survival and proliferation.

Beyond their direct effects on the regulation of cytoskeletal dynamics, Rho GTPases are involved in several essential physiological processes including neurogenesis and early embryogenesis. Their action results mainly from their ability to bind and regulate an extensive range of effectors involved particularly in actin cytoskeleton and gene transcription regulation (6,48,51).

Dysregulations occurring at the level of Rho GTPase-affected pathways are generally associated with oncogenic transformation and several reports increasingly highlight the crucial role of Rho GTPases in cancer onset, progression and metastasis (46,47,78). In contrast to cancers initiated by point mutations in Ras GTPases, the majority of tumors promoted by Rho members seems to be a consequence of altered expression (i.e., overexpression) and increased GTPase activity of these proteins (47,79-81). For instance, high expression levels of Rac1 and Cdc42 are found in several tumors such as breast and testicular cancers (82,83). Also, Rac1 is overexpressed in colon and lung carcinomas, whereas Cdc42 is overexpressed in various other cancers including melanoma and colorectal adenocarcinoma (84,85). RhoA is overexpressed in a wide range of tumors including breast, liver, lung and colon cancers and, in some cases, the expression positively correlated with increased RhoA activity and cancer progression (82,83,86,87). Sustained Rho GTPase activity in cancer is often a consequence of abnormalities in the Rho GTPase regulators with frequently higher RhoGEF and reduced RhoGAP activity and/or expression (47,88). In addition to aberrant expression and GTPase activity misregulation, recent findings reported mutations in Rho GTPases associated to cancer such as the one discovered in Rac1, Rac (Pro29Ser), which is associated with high prevalence in melanomas, making Rac1 the most commonly mutated Rho GTPase (89,90). Conversely, some Rho GTPases can stimulate tumor-suppressing activities as for example in the case of Rac1-dependent regulation of apoptosis and the anti-tumorigenic function of RhoB in lung cancer (91,92).

1.3 Rho GTPase-Activating Proteins (RhoGAPs)

Given that the intrinsic GTPase activity of Rho GTPases is very slow and inefficient, the hydrolysis of GTP to GDP is enhanced by the RhoGAP class of enzymes leading to the deactivation of the Rho GTPase (93,94). To date more than 70 genes have been identified to encode a RhoGAP-domain containing protein, and nearly all these proteins share a hallmark GAP domain containing the "arginine finger" motif characterized by a conserved arginine residue, critical for the catalytic activity (95-97). Indeed, the positively charged residue enhances the intrinsic-GTP hydrolysis by neutralizing the negative charges of the γ-phosphate of GTP during the transition state, and by stabilizing and positioning different residues such as the catalytic glutamine residue of the GTPase protein involved in the enzymatic reaction (98,99).

1.3.1 RhoGAP regulation by phosphorylation

RhoGAP proteins are regulated by different mechanisms including protein-protein and lipid-protein interactions, and posttranslational modifications such as phosphorylation. Indeed, most RhoGAPs are phosphoproteins and the regulation by phosphorylation could either stimulate or inhibit the RhoGAP activity. For example, phosphorylation of FilGAP (filamin A-associated RhoGAP) downstream of Rho/ROCK pathway triggers its PM translocation and increases its activity towards Rac (100,101). Similarly, phosphorylation of ArhGAP22 by Akt/PKB generates 14-3-3 protein-binding sites, and the subsequent recruitment of these proteins stimulates its RhoGAP activity for Rac (102). Equally, a Src kinase-mediated phosphorylation of Nadrin (neuron-associated developmentally regulated protein) stimulates its GAP activity (103-106). Conversely, phosphorylation of ArhGAP33 at Tyr-406 residue by the protein-tyrosine kinase Fyn inhibits its GAP activity (107). Finally, a negative regulation of CdGAP (Cdc42 GTPase-activating protein) activity is noticed following phosphorylation of Thr-776 residue by both Erk1/2 and GSK3 kinases (108,109).

1.3.2 RhoGAP membrane localization

Membrane binding is a common characteristic of many proteins, which exert their function at the level of cellular membranes. The targeting to PM is achieved by different mechanisms including direct binding through LBDs (e.g., PH, C1, C2, BAR, FYVE,

FERM and ENTH) and indirect recruitment through interaction with protein partners. Positively charged PBRs found in many membrane-associated proteins, which are very well conserved throughout evolution, have been documented to directly interact with the negatively charged membrane phospholipids, ensuring therefore the protein targeting to the membrane (110-112).

The primary function of RhoGAPs is the stimulation of the intrinsic GTPase activity of their target Rho GTPases. Since most of these small GTPases in their active, GTP-bound form localize to cellular membranes, the RhoGAPs need to be targeted to the membranes in order to interact with them (113,114). Accordingly, several membrane targeting mechanisms evolved throughout evolution to regulate the subcellular localization of RhoGAPs. Among these mechanisms, some involve targeting through protein-protein interactions, whereas the majority involves protein-lipid regulation. Thus, to permit the interaction with the negatively-charged membrane phospholipids, many RhoGAPs harbor LBDs, such as PH, C1, PX (phox homology) and BAR (Bin-Amphiphysin-Rvs) domains. These domains, which are abundant in various types of proteins, are usually enriched in basic positively-charged amino acids including arginines and lysines (115,116). However, besides few examples in which LBDs target efficiently the protein to the membrane, generally additional mechanisms are required for an efficient, precise and stable RhoGAP recruitment to the membranes. Recently, many studies report membrane-targeting roles, RhoGAP-activity and substrate-specificity regulation by PBRs from numerous RhoGAPs including DLC1, CdGAP and p190RhoGAPs (Table 1.1) (34-36,112,117). These PBRs are present in most RhoGAPs, both N-terminal and/or C-terminal to the RhoGAP domain. Moreover, they are well conserved within members of the same RhoGAP subfamily, suggesting conserved regulatory mechanisms (Table 1.2).

1.3.3 RhoGAP role in cancer

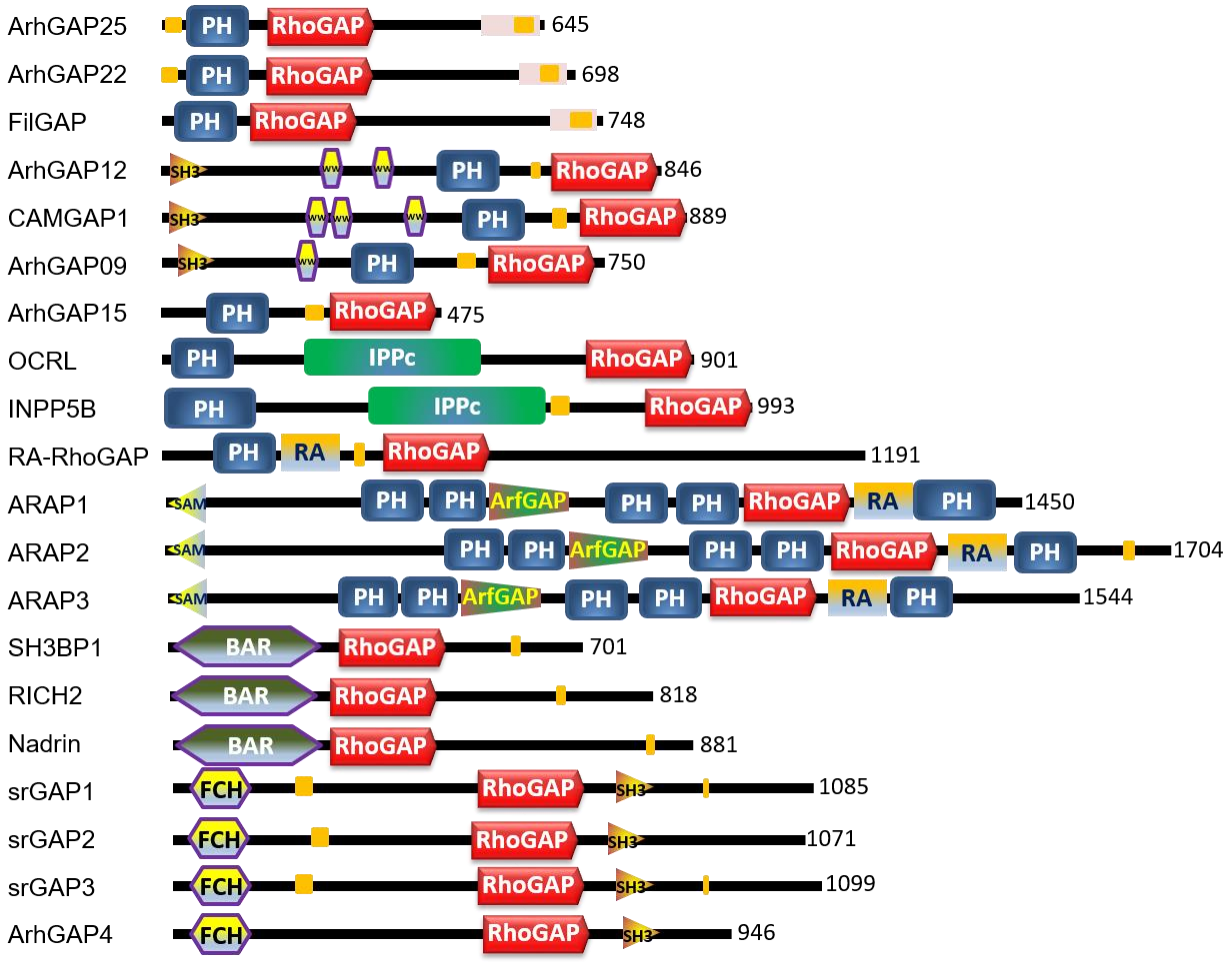
In addition to the termination of Rho GTPase signalings, several RhoGAPs are involved in other cellular functions and in a variety of intermolecular interactions leading to the regulation of a wide spectrum of cellular events (95). Accordingly, abnormalities in their activity and/or protein expression lead frequently to dysregulation of Rho GTPase

activities and diseases, such as tumorigenesis. For a long time, owing to their negative regulation of Rho GTPases, many RhoGAPs have been classified in the category of tumor suppressors. Notably, DLC (deleted in liver cancer) family members have been well characterized for their tumor-suppressing roles and they are found typically depleted in various cancers (118-120). Also, decreased expression of ArhGAP18 is seen as a hallmark of many human tumors such as breast and lung cancers (44,121,122). On the other hand, recent reports assign to some RhoGAPs proto-oncogenic roles in various cancers and show that they can promote tumorigenesis. As such, p190RhoGAP-B exerts a pro-tumorigenic role during tumor progression and metastasis in breast cancer (123). Similarly, RacGAP1, which is overexpressed in cancer cell lines, displays a positive role in many cancers like in human hepatocellular carcinoma where it cooperates with the RhoGEF Ect2 to promote tumor growth and metastasis, while its depletion blocks the tumorigenic process (124-126). Furthermore, CdGAP exhibits elevated expression levels in various cancer cell lines and in cancer patients, and this is associated with poor survival prognosis, particularly in breast cancer (127,128). Notably, CdGAP has been shown to mediate the TGBβ-induced tumorigenic effects (i.e., cell migration and invasion) in a mammary breast cancer cell line by transcriptionally repressing the expression of E-cadherin and thereby, triggering epithelial-mesenchymal transition (EMT)(127,128).

1.4 RhoGAP protein subfamilies

In the following sections, recent findings regarding the regulation and the function of individual RhoGAP proteins will be addressed. In addition, the molecular mechanisms directing their membrane recruitment will be discussed with an emphasis placed on the role of LBDs and PBRs. According to their LBD and general domain structure and function, RhoGAPs were classified into seven groups: PH-, BAR/FCH (Bin-Amphiphysin-Rvs/ Fes/CIP4 Homology)-, C1-, SEC14-, START (StArR-related lipid transfer)-domain containing RhoGAPs, multiple LBDs- and PBR (LBD-lacking-RhoGAPs)- containing RhoGAPs (Figure 1.1).

A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FilGAP
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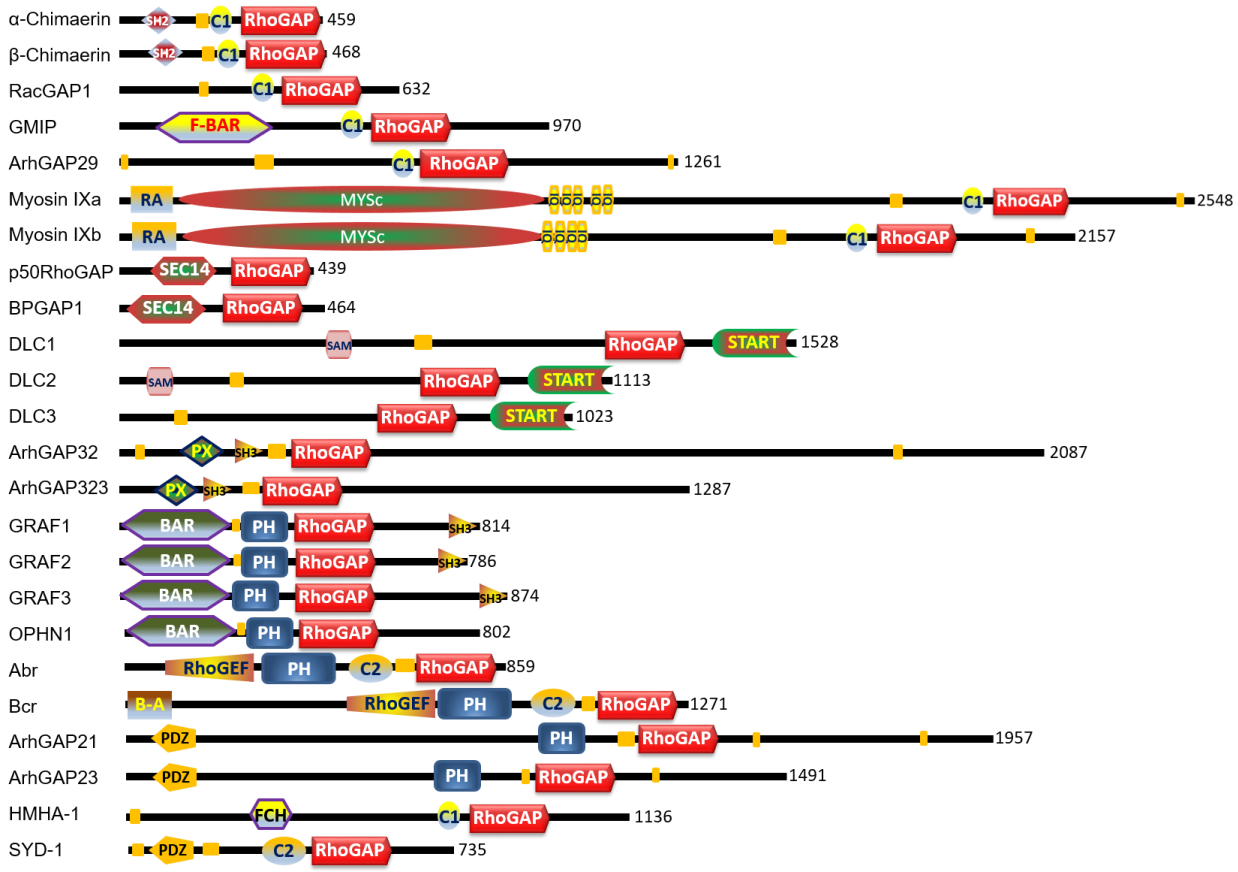
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RhoGAP
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β-Chimaerin
SH2
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RacGAP1
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632
GMIP
F-BAR
C1
RhoGAP
970
ArhGAP29
C1
RhoGAP
1261
Myosin IXa
RA
MYSc
C1
RhoGAP
2548
Myosin IXb
RA
MYSc
C1
RhoGAP
2157
p50RhoGAP
SEC14
RhoGAP
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BPGAP1
SEC14
RhoGAP
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DLC1
SAM
RhoGAP
START
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SAM
RhoGAP
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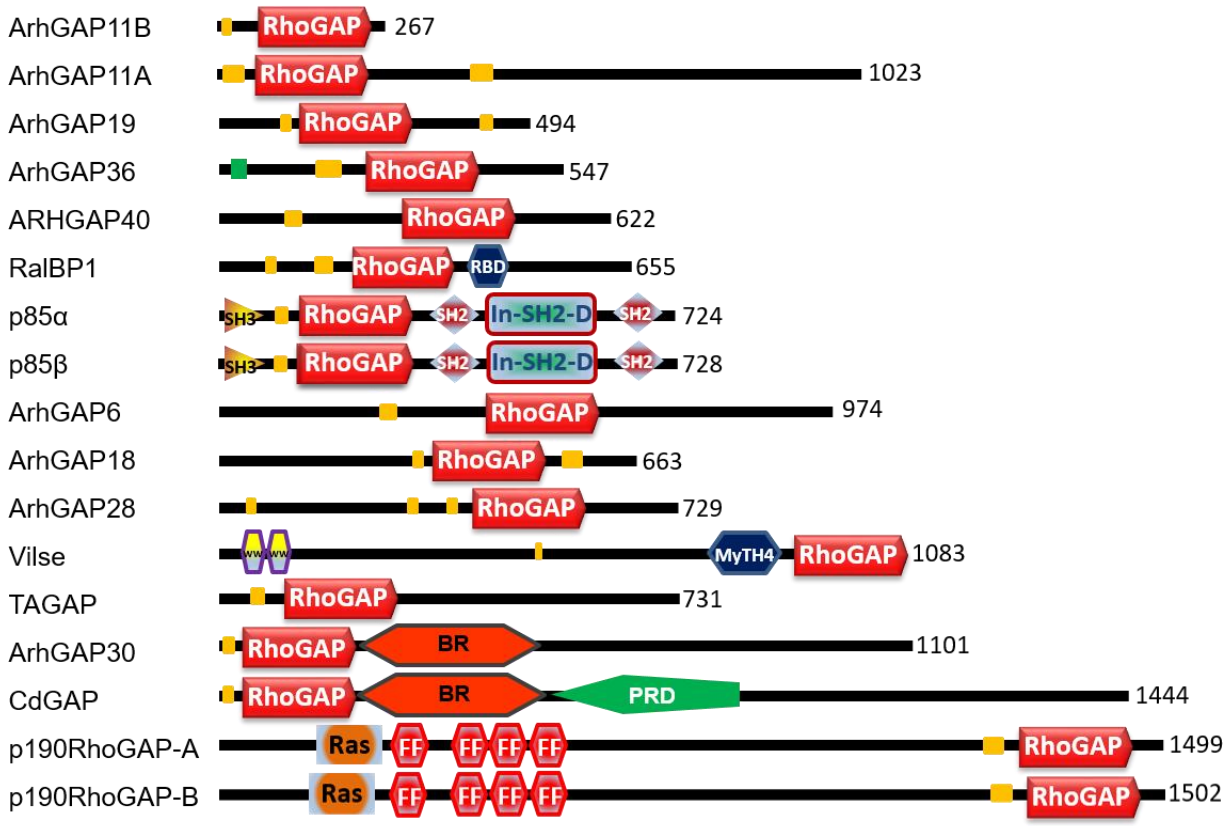
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FF
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FF
RhoGAP
1499
p190RhoGAP-B
Ras
FF
FF
FF
FF
RhoGAP
1502

Figure 1.1– Domain organization of RhoGAP proteins. Many RhoGAPs are mutlidomain proteins and are involved through their functional domains in several cellular processes. They exert their role at different subcellular locations notably at the plasma membrane which requires for that a membrane anchoring mechanism. As such, the presence of polybasic regions (PBRs) is thought to contribute to the membrane targeting and hence, to the regulation of RhoGAPs. Amino acid sequences of *H.Sapiens* (human) RhoGAPs were analyzed for domain prediction using the following online software: SMART (http://smart.embl.de/)(129), Pfam 30.0 (http://pfam.xfam.org/)(130) and InterPro (https://www.ebi.ac.uk/interpro/)(131). In addition, domain annotations were verified according to the literature for each individual RhoGAP protein. (A) PH and FCH/BAR lipid binding domain (LBD)-containing RhoGAPs. (B) Other LBD-containing RhoGAPs. (C) PBR (LBD-lacking-RhoGAPs)-containing RhoGAPs. Abbreviations for domains are as follows: B-A, Bcr-Abl oncoprotein oligomerisation domain; BAR, Bin/amphiphysin/Rvs; BR, basic region; C1, cysteine-rich phorbol ester binding; C2, calcium-dependent lipid binding; CC, coiled-coil; F-BAR, FCH/BAR; FF, domain with two conserved phenylalanine residues; FCH, Fes/CIP4 homology; In-SH2-D, phosphatidylinositol 3-kinase regulatory subunit p85 inter-SH2 domain; IQ, calmodulin-binding motif; IPPc, Inositol polyphosphate phosphatase, catalytic domain homologues; MYSc, myosin complex, large ATPase; MyTH4, myosin tail homology 4; PDZ, Post synaptic density protein, Drosophila disc large tumor suppressor and Zonula occludens-1 protein domain; PH, pleckstrin homology; PRD, proline-rich domain; PX, Phox homology; RA, Ras association domain; RBD, Ral binding domain; Ras, Small GTPase binding domain; SAM, sterile α-motif; Sec14, Sec14-like; START, StAR (steroidogenic acute regulatory)-related lipid transfer; WW, domain with two conserved tryptophan residues. Yellow band, PBR; green band, transmembrane motif; beige band, coiled coil (CC) domain.

1.4.1 PH-domain containing RhoGAPs

PH domains are abundant LBDs and important motifs helping to direct their host protein to cellular membranes by binding to the negatively-charged membrane phospholipids. Yet, less than 10% of PH domains display, at the same time, high binding affinity and specificity to membrane-enriched phosphoinositides including PtdIns3P- (phosphatidylinositol 3-phosphate), PtdIns5P- (phosphatidylinositol 5-phosphate) and PtdIns(3,5)P2-enriched membranes, whereas more than 90% show weak interaction with these phospholipids. As a result, the majority of PH domains are inefficient for a stable targeting of the protein to the cellular membranes and additional LBD motifs, such as PBRs are required for a stable and specific recruitment (132).

FilGAP (ArhGAP24), ArhGAP22 and ArhGAP25

FilGAP subfamily proteins are important regulators of the actin cytoskeleton and key coordinators of the Rho and Rac GTPase signaling crosstalk (101). They contain in their N-terminus a PH domain next to a central RhoGAP domain, and a coiled coil (CC) domain in the C-terminal region rich in basic residues (Figure 1.1A). All three proteins and their splice variants display a RhoGAP activity towards Rac, which seems to be regulated by their subcellular localization and PM targeting. FilGAP is sequestrated and inactivated in the F-actin rich structures such as focal adhesions, lamellae or membrane ruffles as a result of its binding to filamin A, a cytoskeleton protein through the CC domain. A Rho/ROCK-mediated phosphorylation of FilGAP likely releases it from the filamin A and induces its translocation to the PM, where it suppresses Rac activity (100,101). The PM recruitment is achieved through a synergic cooperation between the FilGAP's PH domain, which binds both PtdIns(3,4,5)P3 and activated Arf6, and the CC domain which harbors a PBR (133).

ArhGAP22, which does not bind to filamin A, localizes to endosomes from where it translocates to membranes ruffles to inhibit Rac activity, lamellipodia formation and cell spreading (134). Its RacGAP activity is promoted by an Akt/PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)-dependent phosphorylation and a subsequent 14-3-3 protein recruitment, in a process requiring the PH domain (102). Moreover, the CC domain seems to regulate the RacGAP activity since its absence

Table1.2 – PBRs from lipid binding domain-containing RhoGAPs

RhoGAP	Target	Organism	PBR at the N-terminus of the GAP domain	Lipid binding domain	Entry[a]
ArhGAP22	Rac (134)	*H. sapiens* *M. musculus*	12-A**R**S**K**SLVMGEQS**R**SPG**R**MPCPH**R** 17-T**R**S**K**SLVMGEQS**R**SPG**R**PLVPH**K**[b]	PH	Q7Z5H3 Q8BL80
ARhGAP25	Rac (135)	*H. sapiens* *M. musculus*	4-**K**LP**R**NWDFNL**K**VEAA**K**IA**R**S**R** 4-**K**LP**R**NWDFNL**K**AEAS**K**IA**R**S**R**	PH	P42331 Q8BYW1
ArhGAP09	Rac1 and Cdc42 (136)	*H. sapiens* *M. musculus*	497-**RR**SSI**R**GPEGTEQN**R**V**R**N**K**L**KR**LIA**KR** 394-**RR**TSS**R**CAEGTDQ**K**N**R**V**R**N**K**L**KR**LIA**KR**	PH	Q9BRR9 Q1HDU4
ArhGAP12	Rac1 (137)	*H. sapiens* *M. musculus*	624-**KK**T**KK**NL**KK**FLT**RR** 616-**KK**T**KK**NL**KK**FLT**RR**	PH	Q8IWW6 Q8C0D4
ArhGAP15	Rac1 (138)	*H. sapiens* *M. musculus*	248-**K**N**R**V**K**S**R**L**KK**FIT**RR**PSL**K**TLQE**K**GLI**K** 254-**K**N**R**V**K**S**R**L**KK**FIS**RR**PSL**K**TLQE**K**GLI**K**	PH	Q53QZ3 Q811M1
CAMGAP1	Rac1 and Cdc42 (139)	*H. sapiens* *M. musculus*	666-**K**V**R**H**K**L**RK**FLQ**RR** 646-**R**V**R**H**K**L**RK**FLQ**RR**	PH	Q6ZUM4 A2AB59
RA-RhoGAP	RhoA (140,141)	*H. sapiens* *M. musculus*	326-**K**TF**KRRR**SIINWAFW**R** 326-**K**TF**KRRR**SIINWAFW**R**	PH	Q9P2F6 Q6IFT4
srGAP1	Cdc42 and RhoA (142)	*H. sapiens* *M. musculus*	211-**RR**SSV**KK**IE**K**M**K**E**KR**QA**K**YSEN**K**L**K**SI**K**A**R** 211-**RR**SSV**KK**IE**K**M**K**E**KR**QA**K**YSEN**K**L**K**SI**K**A**R**	BAR/FCH	Q7Z6B7 Q91Z69
srGAP2	Rac1 (143)	*H. sapiens* *M. musculus*	226-**KK**IE**K**M**K**E**KR**QA**K**YTEN**K**L**K**AI**K**A**R** 226-**KK**IE**K**M**K**E**KR**QA**K**YTEN**K**L**K**AI**K**A**R**	BAR/FCH	O75044 Q91Z67
srGAP3	Rac1 (144)	*H. sapiens* *M. musculus*	211-**RR**SSV**KK**IE**K**M**K**E**KR**QA**K**YSEN**K**L**K**CT**K**A**R** 211-**RR**SSV**KK**IE**K**M**K**E**KR**QA**K**YSEN**K**L**K**CT**K**A**R**	BAR/FCH	O43295 Q812A2
α-Chimaerin	Rac1 (145-147)	*H. sapiens* *M. musculus*	182-**KR**LTSLV**RR**ATL**K**ENEQIP**K**YE**K** 182-**KR**LTSLV**RR**ATL**K**ENEQIP**K**YE**K**	C1	P15882 Q91V57
β-Chimaerin	Rac1 (145-147)	*H. sapiens* *M. musculus*	163-**R**E**K**VS**RR**LS**R**S**K**NEP**RK** -	C1	P52757 -
RacGAP1	Rac1, Cdc42 and RhoA (126,148,149)	*H. sapiens* *M. musculus*	173-**K**TF**K**L**KKR**E**KRR**STS**R** 174-**K**NF**K**M**KKR**E**KRR**SNS**R**	C1	Q9H0H5 Q9WVM1
Myosin IXa	RhoA (150)	*H. sapiens* *M. musculus*	1819-**RK**EF**K**EN**K**EPSP**K**A**KRKR** 1822-**RK**EF**K**EN**K**EPSP**K**A**KRKR**	C1	B2RTY4 Q8C170
Myosin IXb	RhoA (151)	*H. sapiens* *M. musculus*	1480-**K**G**KK**N**R**NV**K**IG**K**ITVSE**K**W**R** 1438-**K**G**KK**N**R**N**RK**VGQITVSE**K**W**R**	C1	Q13459 Q9QY06
DCL1	RhoA, RhoB and RhoC (117)	*H. sapiens* *M. musculus*	1051-**K**HGFSWAVP**K**FM**KR**I**K**VPDY**K**D**R** 615-**K**HGFSWAVP**K**FM**KR**I**K**VPDY**K**D**R**	START	Q96QB1 Q9R0Z9
DLC2	RhoA, RhoB and RhoC (117)	*H. sapiens* *M. musculus*	636-**K**HGWTWSVP**K**FM**KR**M**K**VPDY**K**D**K** 636-**K**HGWTWSVP**K**FM**KR**I**K**APDY**R**D**K**	START	Q9Y3M8 Q923Q2
DLC3	RhoA, RhoB and RhoC	*H. sapiens* *M. musculus*	545-**K**QGWVWSMP**K**FM**RR**N**K**TPDY**R**GQ 541-**K**QAWVWSMP**K**FM**KR**N**K**TPDY**R**GH	START	Q92502 Q8K031

ArhGAP32	Cdc42, Rac1 and RhoA (152,153)	*H. sapiens* *M. musculus*	333-**K**PVS**KK**HG**K**LITFL**R**TFM**K**S**R**PT**K**Q**K**L**K**Q**R** 333-**K**PVS**KK**HG**K**LITFL**R**TFM**K**S**R**PT**K**Q**K**L**K**Q**R**	PX	A7KAX9 Q811P8
ArhGAP33	TC10 and Cdc42 (154)	*H. sapiens* *M. musculus*	280-**R**P**R**G**K**LAGLL**R**TFM**R**S**R**PS**R**Q**R**L**R**Q**R** 304-**R**P**R**G**K**LAGLL**R**TFM**R**S**R**PS**R**Q**R**L**R**Q**R**	PX	O14559 Q80YF9
GRAF1	Cdc42 and RhoA (155-158)	*H. sapiens* *M. musculus*	251-**KK**M**K**ENPLEH**K** 251-**KK**M**K**ENPLEH**K**	BAR/PH	Q9UNA1 Q6ZQ82
GRAF2	RhoA and Cdc2 (159)	*H. sapiens* *M. musculus*	251-**K**I**R**QNP**K**DH**KR** 251-**K**I**R**QNP**K**DQ**KR**	BAR/PH	A1A4S6 Q6Y5D8
OPHN1	Rho (160,161)	*H. sapiens* *M. musculus*	250-**KKR**M**K**EAPQTC**K**-261 250-**KKR**M**K**EAPQTC**K**-261	BAR/PH	O60890 Q99J31
Abr	Cdc2 and Rac (162,163)	*H. sapiens* *M. musculus*	619-**K**VEFSM**K**FTS**R**DMSL**KR**TPS**KK** 619-**K**VEFSM**K**FTS**R**DMSL**KR**TPS**KK**	C2/PH	Q12979 Q5SSL4
Bcr	Cdc2 and Rac (162,163)	*H. sapiens* *M. musculus*	1041-**KR**MPS**RK**QTGVFGV**K**IAVVT**KR** 1040-**KR**MPS**RK**QTGVFGV**K**IAVVT**KR**	C2/PH	P11274 Q6PAJ1
ArhGAP21	Cdc42 (164,165)	*H. sapiens* *M. musculus*	1105-**RK**LLS**K**DDTSPP**K**D**K**GTW**RK**GIPSIM**RK** 1099-**RK**LLS**K**DDTSPP**K**D**K**GTW**RR**GIPSIV**RK**	PDZ/PH	Q5T5U3 Q6DFV3
ArhGAP23	-	*H. sapiens* *M. musculus*	893-**KK**N**KK**AAP**R**AFGV**R** 889-**KK**N**KK**AAP**R**AFGI**R**	PDZ/PH	Q9P227 Q69ZH9
SYD-1	-	*H. sapiens* *M. musculus*	7-**RK**TFS**R**L**R**G**R**E**K**LP**RKK**SDA**K**E**R** 7-**RK**TFS**R**L**R**G**R**E**K**LP**RKK**SEA**K**D**R**	PDZ/C2	Q6ZW31 Q9DBZ9

[a]NCBI protein entry number.

[b]Basic amino acids that make up the PBR are bolted.

impairs the PM localization and the ArhGAP22-induced cell spreading suppression in human melanoma A7 cells (134).

ArhGAP25 is a newly discovered RacGAP involved in cell trafficking and a negative regulator of phagocytosis with expression restricted to hematopoietic cells such as leukocytes, where it is found both in the cytosol and the PM (135). The domain structure of this RhoGAP is very similar to that of FilGAP and ArhGAP22, and comprises PBRs in both its N- and C-termini (within the CC domain), suggesting a conserved and shared mechanism within this subfamily governing the PM recruitment and the GAP activity.

ArhGAP09, ArhGAP12, ArhGAP15 and CAMGAP1 (CIN85-associated multi-domain-containing Rho1, ArhGAP27)

Members of this group share a domain structure consisting of a C-terminal RhoGAP, a central PH domain and, except for ArhGAP15, an N-terminal SH3 domain, as well as several repeated tryptophan residue (WW) motifs in the central region (Figure 1.1A).

ArhGAP09 displays a RhoGAP activity towards Cdc42 and Rac1 and to a lesser extent RhoA. It is expressed predominantly in peripheral blood leukocytes, spleen, and thymus and belongs to a family of PH domains characterized by binding to the phosphoinositides in a non-canonical fashion (136). Also, ArhGAP09 mediates the crosstalk between the Rho GTPase and MAP kinase signaling by preventing Erk1/2 and p38 activation through binding to and sequestrating them (166).

ArhGAP12, which is a RhoGAP for Rac1, is ubiquitously expressed with regulatory roles notably in the developing brain wherein it regulates the excitatory synaptic structure and function (167). In NK2R-HEK cells, this RhoGAP suppresses the Rac1 activity at the blebbing membranes where it translocates, though the translocation mechanism is unknown (137). The inhibition of Rac1 activity accounts for the tumor-suppressing activity of ArhGAP12 which is frequently targeted for transcriptional repression downstream of growth factors promoting tumorigenesis such as the hepatocyte growth factor (HGF) (168).

ArhGAP15 was first characterized for its specific Rac1 activity *in vitro,* whereas its overexpression induced cell rounding and actin stress fiber formation, and increased cell contraction (138). The events are achieved at the cell periphery and are mediated by a PH domain-dependent translocation of ArhGAP15. As such, partial deletion of the PH domain disturbed the PM localization of ArhGAP15 and abolished its effects (138). Additionally, the PH domain is required for the binding between ArhGAP15 and Rac effectors, Pak1 and 2 kinases, which results in a mutual inhibition of both GAP and kinase activities (169).

Finally, CAMGAP1 is a ubiquitously expressed RhoGAP displaying an *in vitro* GAP activity towards both Rac1 and Cdc42 (139). It binds and regulates the endocytic and adaptor protein CIN85 suggesting its involvement in endocytosis, although the underlying mechanism is unknown (139).

OCRL (Oculocerebrorenal syndrome of Lowe) and INPP5B (inositol polyphosphate 5-phosphatase B)

OCRL (also known as INPP5F, inositol polyphosphate 5-phosphatase F, and OCRL1) and its homologous INPP5B (OCRL2) are members of the inositol 5-phosphatase family of enzymes characterized by dephosphorylating selectively the PtdIns(4,5)P2 and/or PtdIns(3,4,5)P3 at the position 5 of the inositol ring (170). As distinct members, OCRL and INPP5B contain in addition to the IPPc (Inositol polyphosphate phosphatase catalytic) domain characteristic of the 5-phosphatases, additional N-terminal PH, C-terminal ASH (ASPM–SPD2– Hydin), and RhoGAP domains. Mutations in OCRL are associated with Lowe syndrome (X-linked human disease) and Dent's disease which are developmental disorders affecting eyes, kidney and brain (170). The mutations generally impairs the phosphatase activity leading to the accumulation of PtdIns(4,5)P2 in different cell compartments affecting thereby many cellular processes (171).

OCRL is positioned primarily to the Golgi/TGN, but it is also found in most cellular membranes where it oversees the protein recruitment (172). The membrane localization of OCRL is achieved through the module ASH/RhoGAP domain, as the PH domains of both OCRL and INPP5B lack the basic clusters necessary for

phosphoinositide binding (173). The mechanism involves a formation and a cooperation of two complexes: the first is formed between the ASH domain and the adaptor protein APPL1 and Rab5; the second between the catalytically inactive RhoGAP domain and Rac1 and Cdc42 in a GTP-dependent manner, together the complexes coordinate the PM recruitment of OCRL (174). On the other hand, INPP5B contains in addition to the PM anchoring domains found in OCRL, a C-terminal CAAX motif that may help with its PM recruitment (173).

RA-RhoGAP (Ras-associating-RhoGAP)

RA-RhoGAP (ArhGAP20) was initially characterized for its role in the Rap1-dependent neurite outgrowth and for mediating the Rho and Rap crosstalk. This RhoGAP contains in addition to its N-terminal PH domain, a RhoGAP domain which selectively represses RhoA activity, and an RA domain mediating the binding to Rap1 (140,141). The RhoGAP activity is modulated and enhanced by Rap1 binding which lead as a result, to RhoA inhibition and neurite extension (141). The PM recruitment of RA-RhoGAP is conducted via the PH domain which interacts with phosphatidic acid (PA) for stability. Indeed, an RA-RhoGAP mutant harboring a PH-domain lacking the PA-binding ability is still able to localize to the PM but in a much lower proportion than the wild-type protein, stressing the need for a PH/PA association to ensure a strong and stable PM binding (175).

ARAP (ArfGAP with RhoGAP domain, Ankyrin repeat and PH domain) 1, ARAP2 and ARAP3

Members of this subfamily regulate both Arf and Rho GTPases through their ArfGAP and RhoGAP domains respectively, making them the convergence point of these small GTPase signaling pathways. Their multidomain structure comprises in addition to five PH domains, a SAM and an RA domain in the N- and C-termini, respectively. Through their complex structure, they are involved in a wide range of cellular functions including the regulation of cell shape and motility, which is carried out mainly through the modulation of Arf6, Rac1, Cdc42 and RhoA GTPase activities (176,177). As well, the presence of several functional domains implies a tight spatiotemporal control of their activities and effector selection, and also involves

intramolecular interactions and coordination between their different domains. For instance, ARAP1 targeted to the Golgi, has its Arf- and RhoGAP activities collaborate to induce cell rounding and inhibit cell spreading (177). Also, ARAP1 activated by Rap1 binding to the RA domain, modulates Rac1 and RhoA activities and contributes thereby to the PDGF-induced structure formation at the leading edge of migrating NIH3T3 cells (178). Similarly, ARAP2 participates in cell migration and adhesion by regulating Arf6 activity, integrin β1 trafficking and focal adhesion formation (179). Likewise, ARAP3 by inhibiting RhoA and mediating the cycling of Arf6, stimulates the growth factor-induced lamellipodia expansion, while ARAP3-deficient cells display round-cell shape, fine stress fibers and reduced lamellipodia (180).

The membrane phospholipid composition and concentration are the critical parameters weighing on ARAPs activity and substrate selection. ARAPs bind preferentially to the PtdIns(3,4,5)P3-enriched cellular membranes principally through their N-terminal PH-domain tandem (Figure 1.1A) (181). However, basic residue clusters from other PH domains and/or regions elsewhere in the protein are necessary for a strong binding and activity stimulation, since deletion of any PH domain (i.e., in ARAP3) prevented the interaction with PtdIns(3,4,5)P3 (181). Still, the recruitment to the PM is not always mediated by the PH domain/PtdIns(3,4,5)P3 binding. Indeed, ARAP1, in mediating EGFR-regulated endocytic trafficking, is recruited to the PM independently of the PH domain/PtdIns(3,4,5)P3 association, suggesting additional/alternative mechanisms to conduct the PM recruitment (182). Yet, the PH domain/PtdIns(3,4,5)P3 binding is still required for a full GAP activity and improved endocytic function (182).

1.4.2 BAR/FCH-domain containing RhoGAPs

BAR domains are highly-conserved motifs of amphiphysins which sense and bind to the negatively-charged membranes. They are also able to induce morphological changes such as membrane deformation/curvature and tubulation, making them essential domains for protein-membrane interactions (183,184). FCH/F-BAR domains are a subclass of BAR domains and are also centerpieces of membrane-deforming proteins, mediating the crosstalk between the actin cytoskeleton and the PM in various processes such as lamellipodia and filopodia formation during endocytosis, chemotaxis

or polarity (184-186). Furthermore, these domains engage in keys regulatory roles notably for Rho GTPases, by controlling the cellular localization and the GAP activity of many RhoGAPs [reviewed in (187)].

SH3BP1 (SH3 Domain Binding Protein 1), **Nadrin (RhoGAP interacting with CIP4 homologues, RICH1) and RICH2**

The three RhoGAPs of this subfamily share a domain structure consisting of an N-terminal BAR domain, a central RhoGAP domain and a C-terminal proline-rich region. SH3BP1 (also known as 3BP1) displays a GAP activity toward Rac1 and Cdc42 and modulates the Rho GTPase/actin cytoskeleton-related functions such as cell migration, epithelial cell-cell adhesion and morphogenesis (188,189).

Nadrin (also called, RICH1 and ArhGAP17) is well characterized for its actin cytoskeleton regulation, specifically in the nervous system, and for its roles in exocytosis, platelet adhesion and aggregation, as well as epithelial-cell polarity. Nadrin and its five isoforms display a RhoGAP activity towards RhoA, Cdc42 and Rac1 in an isoform-specific fashion (103,190).The GAP activity is regulated at least by Src-mediated tyrosine phosphorylation and by BAR domain-mediated subcellular localization (103-106). Indeed, the BAR domain is actively involved in the membrane-curvature sensing /formation and in the recruitment to the PM, guiding and positioning therefore the RhoGAP activity of Nadrin next to its Rho GTPase targets (191,192).

Rich2 (also known as, Nadrin1 and ArhGAP44) regulates Rac1 and Cdc42 GTPase activities and cytoskeletal dynamics mostly in epithelial-cell polarity and in the central nervous system during spine morphogenesis (193-195). For instance, in dendritic spines of cultured hippocampal neurons, Rich2 is involved in synaptic plasticity and in synaptic long-term potentiation (LTP) regulation. The mechanism involves association of the RhoGAP to postsynaptic scaffolding protein, Shank3 and its translocalization to Rab11-positive recycling endosomes wherein it controls the exocytosis and the recycling of the GluA1 AMPA-receptor subunit (193). The events are mediated by the BAR domain, which directs Rich2 to the endosomal recycling compartment and ensures its implication in the AMPA receptor/Rich2-dependent recycling (193).

srGAP1 (Slit-Robo GTPase-activating protein 1), srGAP2, srGAP3 and ArhGAP4

The RhoGAPs of this subfamily are key molecular integrators of the external-guidance cues in the Slit-Robo repulsive system of axon guidance and neuronal development (142,196). Disruptions of these well-conserved srGAP-regulated functions are often associated with brain developmental disorders such as mental retardation, learning and memory deficiencies (144,196-198). srGAP and ArhGAP4 consist of an N-terminal FCH/F-BAR domain, a central RhoGAP domain, and a C-terminal SH3 domain mediating the binding to the intracellular domain of the receptor Robo (142,197). While srGAP2 and srGAP3 downregulate preferentially Rac1 activity, srGAP1 in a Slit dose-dependent manner, targets both Cdc42 and RhoA, and ArhGAP4 is active towards Cdc42 and Rac1 (142,143,199,200).

The biological functions of srGAP1, 2 and 3 are frequently mediated by their FCH/F-BAR domains (hereby designed as, F-BAR1, F-BAR2 and F-BAR3 respectively), which can interact with each other to form functional heterodimers. The domains display also differential binding affinities to the PM-negatively charged phospholipids, with F-BAR2 showing the highest binding affinity (201). These features allow complementary and/or opposite regulatory effects of these RhoGAPs in given cellular processes such as, cell-morphogenesis which they regulate synergically by their differential modulation of the membrane deformations. For instance, srGAP2 (F-BAR2) and srGAP3 (F-BAR3) stimulate filopodia formation, whereas srGAP1 (F-BAR1) prevents it in both cortical neurons and non-neuronal cells (198,201). The targeting of srGAP2 to the contact-protrusions by its F-BAR2 domain, permits the spatiotemporal inhibition of Rac1 and protrusion extension, leading to contact inhibition of locomotion (CIL) of migrating fibroblasts (143). Finally, the F-BAR3 domain-mediated srGAP3 translocation to the PM at the leading edge leads to the inhibition of lamellipodin, a key protein in actin dynamics and cell protrusion formation, and subsequently to the suppression of the Rac-WAVE signaling and lamellipodia formation (202).

1.4.3 C1-domain containing RhoGAPs

C1 and C2 LBDs have been first characterized as cysteine-rich conserved regions among protein kinase C (PKC) isoforms (203,204). Later, structural studies

provided more information about the lipids they bind and the mechanisms of recognition (204,205). C1 domains are motifs with approximatively 50 amino acids, with a zinc finger cysteine-rich and compact structures well characterized for binding to lipid second messenger n-1,2-diacylglycerol (DAG) and phorbol esters (204,206,207). Yet, not all C1 domains bind DAG and those which do not are classified as atypical C1 domains. The C1-DAG/phorbol ester binding mediates the regulation of subcellular localization of numerous proteins and allows their recruitment to the cellular membranes (206,207)

α and β-chimaerins

Chimaerin subfamily members better known for their RhoGAP activity towards Rac1, are involved in numerous cellular events, including neuritogenesis and development, and in diseases such as cancer progression and Alzheimer's (145-147,208-211). This subfamily is composed of five proteins namely, α1-, α2-, β1-, β2- and β3- chimaerins, all originated from alternative splicing of two genes: CHN1 (ARHGAP2, CHN) and CHN2 (ARHGAP3, BCH)(212). They all harbor a C-terminal RhoGAP domain and an N-terminal SH2 domain mediating the binding to receptor tyrosine kinases (RTKs), as well as a central C1 domain which binds with high affinity to DAG and phorbol esters.

The RhoGAP activity and subcellular localization are regulated basically by phorbol esters and DAG through binding to the C1 domain (212). For instance, activation of growth factor receptors such as EGFR, which induces DAG production by phospholipase C (PLC), stimulates both activity and PM relocalization of chimaerins (212). Interestingly, both α2- and β2-chimaerins present an auto-inhibitory mechanism involving intramolecular contacts, which mask and prevent the C1 and the RhoGAP domains from interacting with membrane lipids and GTP-Rac1 respectively (213). Eventually, the binding of phorbol esters/DAG disturbs the inhibitory contacts and induces conformation changes allowing the exposure of both the C1 and RhoGAP domains leading subsequently, to PM translocation of the chimaerin and stimulation of its GAP activity (214).

Moreover, PM localization implicates other motifs on the RhoGAP which bind various partners in a DAG-dependent fashion. As an example, the PM translocation of

β2-chimaerin and the inhibition of Rac1 activity induced by PMA (phorbol 12-myristate 13-acetate) stimulation requires a simultaneous coordination between the DAG/C1 binding and the association to protein adaptor Nck1, which is achieved through an N-terminal, atypical proline-rich motif within the RhoGAP domain (215). Furthermore, the C1 domain can mediate the subcellular compartmentalization of chimaerins through protein-protein interactions. As such, the perinuclear translocation of β2- chimaerin is mediated by an interaction with an endoplasmic reticulum/Golgi protein, p23/Tmp21 which serves as a C1-docking site (216).

RacGAP1

RacGAP1 [also referred to as Male germ cell Rac GAP (MgcRacGAP)/CYK-4)] consists of a C-terminal RhoGAP domain flanked N-terminally by a C1 domain, and a long N-terminal region with a PBR. It is active towards Rac1, Cdc42 and RhoA and is an important regulator of cell cycle and mitosis, although whether the GAP activity is relevant to this regulation is still unclear (126,148,149). During mitosis, RacGAP1 localizes to the mitotic spindle where it forms complexes with key cytokinesis regulators, such as the kinesin-like protein, ZEN-4/CeMKLP1, Ect2, MKLP1, Aurora B and PRC1 (148,217). The protein complexes are crucial for a precise spatiotemporal localization of the different binding partners, emphasizing the significance of the RacGAP1 role for a normal completion of the cytokinetic process (125,149,217).

In addition to its physiological function in modulating cytokinesis, dysregulation of RacGAP1 has been associated to tumorigenesis in many cancers. For instance, it has been shown to collaborate with Ect2 to promote tumor recurrence, growth and metastasis in human hepatocellular carcinoma through regulation of the Rho/ERK signaling axis (124,125). Similarly, a tumor promoting role of this protein has been documented in breast cancer (126). Indeed, high RacGAP1 expression levels have been detected in many breast-cancer cell lines, whereas its depletion resulted in increased RhoA activity and cell spreading, and induced cell proliferation defects coupled to cell growth inhibition as a result of cytokinesis failure (126).

GMIP(GEM Interacting Protein) and ArhGAP29

GMIP and ArhGAP29 are ubiquitously expressed and share a central C1 domain and a RhoGAP domain, that directs a GAP activity towards RhoA (218,219). ArhGAP29 [also referred to as PTPL1-Associated RhoGAP 1 (PARG1)] has been characterized first as a RhoGAP interacting with the phosphatase PTPL1 by binding to its PDZ domain, and the complex was proposed to collaborate to inhibit RhoA activity (219). Although ArhGAP 29 contains a C1 domain, the translocation to the PM seems to be mediated by an unconventional C-terminal motif (PQFS) that binds to PDZ domains as the following example will illustrate (219,220) .

Indeed, it has been shown recently that ArhGAP29 binds to the PDZ domain of Radil [ras-association (RA) and dilute domain-containing protein], an effector of the small GTPase Rap1, and to form a complex with target proteins at the PM to regulate junctional tightening and endothelial barrier functions. Upon its activation, Rap1 translocates to the PM Radil and its close relative, adaptor protein Rasip1 (ras-interacting protein 1) by binding their RA domains, while ArhGAP29 is translocated to the PM through binding to Radil. At the PM, the three proteins Radil, Rasip1 and ArhGAP29 form a regulatory-trimeric complex which cooperates to inhibit RhoA activity and stress fiber formation, leading to increased junctional tightening and improved endothelial barrier function (220,221).

GMIP has been identified in a yeast two-hybrid screen using Gem, a Ras-related protein (218). GMIP, which has been associated to major depressive disorder (MDD), is imperative in neurite growth, axonal guidance and neuronal migration, a function that it exerts by antagonizing the RhoA signaling pathway (222,223). GMIP is enriched at the PM where it inhibits RhoA activity but the mechanism of the translocation is unclear and may involve a recruitment via active Gem (218,224).

Myosin IXa and b

The single-headed actin motors proteins, Myosin IXa (Myr7) and Myosin IXb (Myr5) are a class out of twenty-four classes forming the superfamily of myosins, a diverse group of molecular motors implicated in cellular transport of different biological molecules such as vesicles and organelles (225). The multidomain proteins, which are

the largest characterized RhoGAPs, share a domain structure including an N-terminal RA domain next to a large motor domain (MYSc, the head), several calmodulin-binding motifs (IQ, the neck), an atypical C1 domain and a C-terminal RhoGAP domain (the tail) that displays a specific activity toward RhoA (151,225,226).

The subcellular localization of these RhoGAPs is determinant for their RhoGAP function. They are frequently localized to regions with active F-actin polymerization, wherein they can modulate RhoA activity and the RhoA-related actin-cytoskeletal remodeling (226,227). Likewise, Myosin IXb is targeted to extending ruffles, filopodia and lamellipodia, and its recruitment is achieved by the Myosin IXb-motor activity, through a conserved polylysine cluster (KKKK) within the ATPase domain (227). Finally, Myosin IXa has been shown to play key roles in the complex process of collective-cell migration by regulating Rho activity in cell-cell contacts at the PM (226).

1.4.4 SEC14-domain containing RhoGAPs

The phosphatidylinositol-transfer protein (Sec14) from yeast, which mediates the exchange of phosphatidylinositol and phosphatidylcholine at the cellular membranes, is an LBD involved in many cellular processes such as, cellular trafficking and vesicle budding from the Golgi complex (228). A Sec14-homology domain is found several mammal proteins including RhoGEF and RhoGAPs, and is involved in many signaling events by mediating interactions with the negatively-charged membrane phospholipids (229,230).

p50RhoGAP and BPGAP1 [(BNIP-2 and Cdc42GAP homology)-domain-containing, proline-rich and Cdc42 GAP-like protein 1]

p50RhoGAP (also known as, ArhGAP1, Cdc42GAP and RhoGAP1), which is among the first RhoGAPs to be characterized, shares 54% sequence identity with its homologous BPGAP1 and are both ubiquitously expressed (147,230). They share a domain structure consisting of an N-terminal SEC14/BCH (BNIP-2 and Cdc42GAP homology) and a C-terminal RhoGAP domains. Through their functional GAP domain and GAP activity, they regulate a wide range of signaling pathways including the actin-cytoskeleton remodelling and cell motility (231-233). The RhoGAP activity of p50RhoGAP is directed toward RhoA, Cdc42 and Rac1. BPGAP1, on the other hand,

although it interacts with all three Rho GTPases, its GAP activity is directed preferentially towards RhoA. Of note, many factors may affect their GAP activity including for instance, the prenylation of the small GTPase effector and the translocation to the PM (234,235). The SEC14/BCH domain of both RhoGAPs mediates significant regulatory functions such as, conduction of intra and intermolecular protein-protein interactions, interaction with the membrane phospholipids and regulation of the GAP activity (232,236-238).

Notably, the p50RohGAP activity towards non-prenylated Rac is auto-inhibited by intramolecular interactions between residues in the C-terminal GAP domain and amino acids (1-48 and 169-197) from the SEC14/BCH domain. However, this effect is prevented and the GAP activity towards Rac restored following expression of prenylated-Rac or p50RohGAP-deletion mutants lacking the indicated N-terminal residues (236).

Remarkably, recent findings unveiled an exclusive and interesting regulation mechanism of RhoA by p50RhoGAP involving the SEC14/BCH domain. Indeed, this domain has been shown to bind and sequester RhoA both in its GTP- or GDP-bound states, preventing thus its inactivation by the adjacent RhoGAP domain (232). Additionally, the SEC14/BCH domain is involved in targeting p50RhoGAP to the endosomal membrane, wherein it colocalizes with the small GTPases, Rab11 and Rab5, and where it might be involved in the endocytic process by mediating the crosstalk between Rap and Rho GTPases (237). Finally, BPGAP1 through a versatile SEC14/BCH domain, has been shown to mediate the crosstalk between Ras and Rho signaling in cell differentiation and morphogenesis (239).

1.4.5 START-domain containing RhoGAPs

The START domain is an LBD found in several signaling proteins such as StAR and HD-ZIP and engages in a variety of protein/lipid interactions notably with membrane phospholipids (240). It is found also in large-multidomain proteins where it contributes often to the regulation of the adjacent domains on the same protein such as the RhoGAP domain and the homeodomain (240,241).

DLC (Deleted in liver cancer) RhoGAPs

The RhoGAP subfamily of DLC proteins comprises three members: DLC1, DLC2 and DLC3, and several alternative splicing isoforms. They are ubiquitously expressed and composed of a C-terminal START domain, a RhoGAP domain, a functional PBR and finally, except for DLC3, a sterile alpha motif (SAM) domain. They all display a RhoGAP activity towards RhoA and by extent, RhoB and C (117,119,120). The DLC proteins are well characterized both *in vitro* and *in vivo* for their tumor-suppressing activity and as such, they are involved in the inhibition of cell growth, proliferation, migration and invasion, as well as tumor progression and metastasis (118-120). Many of the above functions are carried out through the control of the actin cytoskeleton by spatiotemporal inhibition of Rho signaling at the membranes. DLC1, a potent tumor-suppressor which is deleted in various cancers and the most studied member of this family, has been shown to be recruited to different cellular compartments, notably to focal adhesions and to the leading edge of migrating cells wherein it suppresses the Rho signaling (118,119). Interestingly, a novel PBR (**K**HGFSWAVP**K**FM**KR**I**K**VPDY**K**D**R**) (Tables 1.1 and 1.2), adjacent to the RhoGAP domain has been reported to modulate DLC1 activity *in vitro* and *in vivo*, and to regulate its effects on Rho signaling *in vivo* (34). Moreover, the PBR is sufficient to carry out the DLC1 recruitment and binding to PtdIns(4,5)P2-enriched membranes *in vitro* (34). However, DLC1 recruitment to the membranes *in vivo* seems to require the cooperation of the PBR with other LBD domains such as, the SAM and START domains, and/or other DLC1 motifs mediating interactions with proteins at the translocation site similar to tensin in focal adhesions (34,242-244). This is likely due to the low binding affinity of PBRs for phospholipids making them inadequate to ensure alone the PM recruitment *in vivo* (7,8,11).

1.4.6 Multiple LBD-containing RhoGAPs

GRAF1 (GTPase regulator associated with focal adhesion kinase-1), GRAF2, GRAF3 and OPHN1 (Oligophrenin-1)

The RhoGAPs of this group present a domain organization consisting of two LBDs, an N-terminal BAR and a central PH domains, a central RhoGAP domain, and except for OPHN1, a C-terminal-SH3 domain.

GRAF1, the founding member of this subfamily is found as a dimer with a RhoGAP activity for Cdc42 and RhoA. In addition, it is a key regulator of actin dynamics and is involved in a variety of cellular signalings such as endocytosis, cell migration and adhesion, and membrane fusion (155-158). Most of this functions are dependent of the GRAF1 binding to and induction of membrane curvatures mediated by the PH and BAR domains (245). To illustrate, the GRAP1 role in the small GTPase-controlled endocytic pathway, the clathrin-independent carrier (CLIC), is mediated by the PH and BAR domains which target the RhoGAP to these tubular, PtdIns(4,5)P2-enriched membrane carriers (158). Consequently, GRAF1 localization to and coating of the CLICs permits the endocytic-membrane turnover at the leading edge and at the cell/matrix contact sites. In addition, the RhoGAP activity towards Cdc42 at the leading edge of the migrating cells facilitates the control of cell migration, adhesion and spreading (155,157). Besides, GRAF1 is a key element in muscle differentiation, fusion and repair after injury, which are driven in a BAR domain-dependent manner. As such, it has been shown that during myogenesis GRAF1, which is transiently upregulated in myoblasts, is recruited to the PM via the BAR domain wherein it downregulates the RhoA activity and induces muscle differentiation (246). Similarly, GRAF1 and GRAF2 are required for myotube formation and myoblast fusion, which they regulate by promoting the translocation to PM of the fusogenic ferlin proteins, in a BAR domain-mediated manner (247). Furthermore, GRAF1 contributes to the fusion/repair of damaged muscle PM by targeting to the injury site, dysferlin, a protein involved in rapid membrane repair (248). GRAF2 inhibits both the RhoA and Cdc2 GTPase activities and is ubiquitously expressed with high expression levels detected in the skeletal muscle (159). GRAF3, a newly characterized RhoGAP with expression restrained to smooth muscle cells

(SMCs), downregulates both the RhoA activity and signaling in SMCs to ensure a normal blood pressure homeostasis (156). OPHN1 is expressed abundantly in the brain and is one of the proteins associated to XLID (X-linked intellectual disability) syndrome (249). OPHN1 regulates the clathrin-mediated endocytosis (CME) and localizes to endocytic sites where its RhoGAP activity suppresses the Rho signaling pathway and thereby, inhibits endocytosis (160,161). The BAR domain plays a major role in the OPHN1 regulation, likely by directing its localization to the cellular membranes. For instance, genetic aberrations such as deletions or insertions affecting this domain have been documented in patients suffering from OPHN1-related mental retardation or XLID syndrome (249,250).

Bcr (Breakpoint cluster region) and Abr (Active Bcr-related)

BCR was first characterized as a part of the fused BCR-ABL gene which resulted from a translocation between the chromosome 9 and 22. The fusion product, the Bcr-Abl protein, is a functional oncogene that causes chronic myelogenous leukemia (251). The native Bcr and its homologous Abr are multidomain-protein RhoGAP regulators harboring a RhoGEF and a RhoGAP domains flanking a central PH and C2 LBDs, and only in Bcr, an extended N-terminal region with an RA domain. The RhoGAP domain of both proteins is active towards Cdc2 and Rac, whereas the RhoGEF domain stimulates the GTPase activity of Cdc42, RhoA and Rac (162,163,251). The presence of the RhoGEF and RhoGAP domains suggests a simultaneous and/or sequential/spatiotemporal regulation of several Rho GTPase activities, to ensure high precision in specific subcellular-exclusive GTPase activity zones, during processes such as cytokinesis and wound healing (252). Although, due to their domain structure similarity, Abr and Bcr may present similar regulatory mechanisms in some cellular events, in many other processes each protein has its unique mechanism of regulation which could be independent of Rho GTPase modulation. For instance, Abr coordinates the crosstalk between Rho and Cdc42-dependent cytoskeleton remodeling by activating RhoA in the Rho-activity zone by the GEF domain, and inhibiting Cdc42 through the GAP domain during single-cell wound repair (252). On the other hand, Bcr regulates the cell polarity of the migrating astrocytes at the leading edge by inhibiting PKCζ and Rac1

activities. Indeed, Bcr, through its N-terminal region which is lacking in Abr, binds to members of the complex Par (Par3, Par6, and PKCζ)-Tiam1, responsible of PKCζ activation and Rac1 recruitment and activation at the leading edge, leading subsequently to the suppression of both Pac-Tiam1 and Rac1 activities (253).

ArhGAP21 and ARHGAP23 RhoGAPs

ArhGAP 21 (also referred to as ArhGAP10) and it homologous ArhGAP23 are large RhoGAP proteins with in their N-terminus, a PDZ domain, a central PH domain and a C-terminal RhoGAP domain (254,255). ArhGAP21 has been reported to localize to cell-cell junctions bound to α-catenin, wherein it suppresses the Cdc42 GTPase activity and regulates the Arp2/3 complex and F-actin-cytoskeletal remodeling (164,165). Recently, a new role of this RhoGAP in cancer as a tumor-suppressor has been described. Whereas overexpression of this protein in ovarian cancer-cell models inhibited cell adhesion, migration and proliferation through Cdc42 downregulation, low ArhGAP21 expression levels have been associated to ovarian cancer progression (256). The regulation of ArhGAP21 subcellular localization and membrane targeting is crucial for its function, and to date two regulation mechanisms have been described. The first consists of a posttranslational modification by SUMO2/3 at lys-1443 residue, which induces a confinement of the SUMOylated protein and its function to restricted subcellular locations including the PM (257). The second mechanism is well characterized and highlights the cooperation between the LBDs and adjacent motifs. The mechanism consists of the recruitment of ArhGAP21 to the cellular membranes (i.e., Golgi) by binding to active Arf (ADP-ribosylation factor) 1 and 6 small GTPses (165). The binding occurs through a novel Arf binding domain (ArfBD) comprising the PH domain and the adjacent C-terminal region (929–1096) of ArhGAP21. The PH domain alone is not sufficient to drive the interaction with the membrane phospholipids and needs the C-terminal motif arranged in an α helix to bind to GTP-Arf (165,258). The Arf/ArhGAP21 interaction ensures the PM anchorage of the RhoGAP without affecting the GAP activity, since the GAP domain is exposed in a conformation allowing the interaction with membrane Rho GTPases (258).

HMHA-1 (Histocompatibility Minor HA-1) RhoGAP

HMHA-1, which is expressed mostly in hematopoietic cells and solid tumours, is a cancer therapeutic target and a recently characterized RhoGAP (259-262). This protein consists of an N-terminal FCH/BAR domain, a central C1 domain and a C-terminal RhoGAP domain. The full-length protein (FL) colocalizes with Rho GTPases in cellular sites with high actin activity such as peripheral membrane ruffles (245). Intriguingly, the FL HMHA-1 protein displays little or no RhoGAP activity both *in vitro* and *in vivo*. Notably, the RhoGAP activity seems to be blocked by intramolecular interactions involving the N-terminal BAR-domain in an auto-inhibitory action, similar to the one found in other RhoGAPs like GRAF family members (245). However, the expression of HMHA-1 deletion mutants lacking the N-terminal BAR domain, namely the GAP-tail and the C1-GAP mutants, showed strong GAP activities *in vitro* towards Cdc42, RhoA and Rac1, and *in vivo* towards RhoA and Rac1 (262). Furthermore, these mutants via their GAP activity, induced important cell-morphological changes such as inhibition of focal adhesion formation, cell adhesion and spreading, which are all hallmarks of active RhoGAPs (262). Finally, the C1 domain and the C-terminal proline-rich region encoding a PDZ-like binding motif, which likely bind to the negatively-charged membranes, seem to regulate both the subcellular targeting of HMHA-1 and its GAP activity, since expression of the GAP region alone failed to localize properly to the cellular membranes and is unable to inhibit Rho GTPase activities (262).

SYD-1 (Synapse-defective-1)

SYD-1 is a multidomain-RhoGAP protein which is mainly expressed in the brain and localized prominently to the presynaptic terminals, where it is involved in several neuronal regulation processes such as neurite outgrowth and axon guidance (263-265). In addition, SYD-1 is a key regulator of synaptic-vesicle trafficking and synaptic-signal transmission by its association to presynaptic RTKs and to various proteins, notably proteins from the active zone (263). SYD-1 consists of a C-terminal RhoGAP domain, a central C2 domain and several SH3 domains, as well as an N-terminal PDZ domain. The RhoGAP domain of SYD-1 from *C.elegans* has been reported to be catalytically inactive, since it lacks the key catalytic arginine characteristic of RhoGAP activity (266).

However, a RhoGAP domain-truncated SYD-1 decreased both the neurite outgrowth and their guidance, stressing the importance of this domain (266). Meanwhile, recent findings show that the RhoGAP domain could be active. Indeed, it has been shown that SYD-1 binds to active yeast MIG-2 GTPase (homologous of Rac) and inhibits its GTPase activity to promote axon guidance downstream of UNC-40 (DCC) and SAX-3 (Robo) (267).

1.4.7 PBR-containing RhoGAPs

About 18 RhoGAPs of this group present a domain structure without any known LBD, yet similar to the majority of RhoGAP proteins, they need a recruitment to the PM in order to conduct the GAP activity towards their active and PM-bound Rho GTPase targets. Interestingly, all these RhoGAPs contain at least one PBR N-terminal to the GAP domain (Table 1.3 and Figure 1.1C). Consistent with their role in mediating the PM recruitment for some RhoGAPs such as CdGAP and DLC1, the PBRs could be an alternative mechanism directing the PM localization of the RhoGAPs containing them. In this section the mechanisms governing the PM localization and the potential PBR contribution will be explored for these RhoGAPs.

ArhGAP11A and ArhGAP11B

ArhGAP11A and ArhGAP11B are two proteins consisting of an N-terminal RhoGAP domain preceded by a PBR and for ArhGAP11A, a long C-terminal region (268,269). ArhGAP11B, a human-specific RhoGAP, emerged from a partial duplication of its homologous ArhGAP11A, which is expressed in the whole animal kingdom following the separation of human and chimpanzee lineages (268). Interestingly, ArhGAP11B has its highest brain-specific expression in the radial glia cells, through which it seemingly promotes the human neocortex expansion throughout evolution (268). Whereas the RhoGAP domain of ArhGAP11B seems to be catalytically inactive, ArhGAP11A activity preferentially targets RhoA activity both *in vitro* and *in vivo*

Table1.3 – PBRs from RhoGAPs

RhoGAP	Target	Organism	PBR at the N-terminus of the GAP domain	Entry[a]
ArhGAP11B		*H. sapiens*	15-**R**AFYGI**K**V**K**GV**R**GQCD**RRR**[b]	Q3KRB8
ArhGAP11A	RhoA (269)	*H. sapiens* *M. musculus*	5-**R**LV**R**LALLQHL**R**AFYGI**K**V**K**GV**R**GQCD**RRR** 5-**R**LV**R**LALLQQL**R**AVYGI**K**V**K**GG**R**GQCD**RRR**	Q6P4F7 Q80Y19
ArhGAP19	RhoA (270)	*H. sapiens* *M. musculus*	99-**R**SLMSL**KRK**E**K**GVIFGSPLT 99-**R**SLMSL**KRK**E**K**GVVFGSPLT	Q14CB8 Q8BRH3
ArhGAP36	-	*H. sapiens* *M. musculus*	149-**RRR**GNVVR**R**VFG**R**I**RR**FFS**RRR**NEPTLP**R**EFT**RR**G**RR** 137-**RRR**GNVVQ**R**MLG**R**M**RR**FFS**RRR**NEPTLP**R**EFT**RR**G**RR**	Q6ZRI8 B1AUC7
ArhGAP40	-	*H. sapiens* *M. musculus*	103-**RR**LDIYA**R**SV**RR**QH**K**TPV**R**DV**R** 153-**RR**LDIYA**R**SA**RR**RQ**K**APV**R**DV**R**	Q5TG30 E9Q6X9
RalBP1	Rac1 (271)	*H. sapiens* *M. musculus*	126-**K**I**K**E**K**P**K**EE**K**H**K**EE**K**H**K**EE**K**H**K**E**KK**S**K**DLTAADVV**K**QW**K**E**KKKK** 126-**K**I**K**E**K**P**K**EE**K**H**K**EE**K**H**K**EE**K**H**K**E**KK**S**K**DLTAADVV**K**QW**K**E**KKKK**	Q15311 Q62172
p85α	-	*H. sapiens* *M. musculus*	79-**RKK**ISPPTP**K**P**R**PP**R** 79-**RKR**ISPPTP**K**P**R**PP**R**	P27986 P26450
p85β	-	*H. sapiens* *M. musculus*	85-**R**PGP**R**P**R**GP**R**PLPA**R** 85-**R**PGP**R**P**R**GP**R**PLPA**R**	O00459 O08908
ArhGAP6	RhoA and Rac3 (272,273)	*H. sapiens* *M. musculus*	253-**KRKK**SL**RKK**LDSLG**K**E**K**N**K**D**K** 254-**KRKK**SL**RKK**LDSLG**K**E**K**N**K**D**K**	O43182 O54834
ArhGAP18	RhoA and RhoC (274,275)	*H. sapiens* *M. musculus*	307- **K**QQ**K**AV**K**I**K**T**K** 307- **K**QQ**K**AV**K**I**K**T**R**	Q8N392 Q8K0Q5
ArhGAP28	RhoA (276)	*H. sapiens* *M. musculus*	363-**KR**N**K**TE**K**V**K**G**R**DNGIFGVPLTVLLDGD**RKK** 367-**KR**N**K**TE**R**V**R**G**R**DNGIFGVPLTVLLDND**RKK**	Q9P2N2 Q8BN58
Vilse	Rac (277,278)	*H. sapiens* *M. musculus*	849- **K**YCYH**K**LQ**K**AALTGA**KK**GL**KK**PNVEEI**R**HA**K** 873- **K**YCYH**K**LQ**K**AALTGA**KK**GL**KK**PNVEEI**R**HA**K**	Q9C0H5 P59281
TAGAP	RhoA (279)	*H. sapiens* *M. musculus*	52-**KKRKK**VLSWPFLM**RR** 52-**KKRKK**VLSWPSLM**RK**	Q8N103 B2RWW0
p190 RhoGAP-A	Rho, Rac and Cdc42 (280-286)	*H. sapiens* *M. musculus*	1214-**RRR**NIL**R**SL**RR**NT**KK**P**K**P**K**P**R**PSIT**K** 1214-**RRR**NIL**R**SL**RR**NT**KK**P**K**P**K**P**R**PSIT**K**	Q9NRY4 Q91YM2
p190 RhoGAP-B	Rho, Rac and Cdc42 (280-286)	*H. sapiens* *M. musculus*	1225- **KK**I**KKK**TH**K**V**K**ED**KK**Q**KKK**T**K** 1225- **KK**I**KKK**TH**K**V**K**ED**KK**Q**KKK**T**K**	Q13017 P97393
ArhGAP30	Rac1 and RhoA (287)	*H. sapiens* *M. musculus*	2- **K**S**R**Q**K**G**KKK**GSA**K**E**R** 2- **K**S**R**Q**K**G**KKK**GSS**K**E**R**	Q7Z6I6 Q640N3
CdGAP	Rac and Cdc42 (288)	*H. sapiens* *M. musculus*	2-**K**N**K**GA**K**Q**K**L**KRK** 2-**K**N**K**GA**K**Q**K**L**KRK**	Q2M1Z3 A6X8Z5

[a]NCBI protein entry number.

[b]Basic amino acids that make up the PBR are bolted.

(268,269). As such, ArhGAP11A regulates the cell division by controlling RhoA-mediated cortical protrusion formation at M phase that otherwise can lead to cytokinesis failure (269).

Several recent studies report both pro-tumorigenic and tumor-suppressing roles of ArhGAP11A in different cellular contexts. For instance, ArhGAP11A high-expression levels have been described in several human cancer specimens including colon and breast cancers, whereas positive correlations have been established between the expression levels and the aggressiveness of the tumors (126,289,290). Similarly, recent findings from a Rho GTPase-signaling transcriptome analysis by RNA-sequencing revealed that ArhGAP11A, among other RhoGAPs such as RacGAP1, is overexpressed in breast-cancer tissues suggesting a proto-oncogenic function (126,290). Defective ArhGAP11A-dependent cell cycle regulation is pointed out to be at the origin of the tumorigenic transformation noticed when this RhoGAP is overexpressed. Indeed, the tumorigenic role of ArhGAP11A is driven at least partly, through the RhoA activity suppression since ArhGAP11A knockdown resulted in high RhoA activation, cell cycle arrest at G1 phase and random-cell migration inhibition (126). Also, RhoA suppression, which inhibits stress fiber and focal adhesion formation, is coupled to Rac1 activity increase, conferring to the cancer cells enhanced migration and invasion abilities (289). By contrast, ArgGAP11A expression in mouse embryonic oligodendrocytes inhibits cell proliferation by promoting cell cycle arrest in response to DNA damage stress (291). Likewise, ArgGAP11A translocates to the nucleus, where it binds via its RhoGAP domain, to the tetramerization domain (TD) of the tumor suppressor p53 inducing conformation change and gene transcription regulation leading to cell apoptosis (291).

ArhGAP19

This protein consists of an N-terminal RhoGAP domain, that targets RhoA activity both *in vitro* and *in vivo*, flanked by clusters of PBRs. ArhGAP19 is expressed predominantly in cells from hematopoietic origin and in germ cells, and the expression fluctuates during cell cycle progression spiking during mitosis, which suggests a regulatory role at this level (270).

ArhGAP36

ArhGAP36 is a recently characterized RhoGAP and an agonist of the Hedgehog (Hh) pathway. It contains an N-terminal transmembrane motif, a PBR and a RhoGAP domain likely to be catalytically inactive as it lacks the crucial arginine finger motif mediating the GTPase hydrolysis. Interestingly, the five alternative-splicing isoforms of ArhGAP36 have been reported to be overexpressed in medulloblastomas, where they regulate the onset and/or progress of these cancers in a non-canonical Hh pathway (292).

RalBP1 (Ral binding protein 1)

RalBP1 is a protein mediating the Ras and Rho GTPase crosstalk and is therefore, involved in many cellular signaling pathways such as glutathione-conjugated electrophile transport, actin-cytoskeleton dynamics and endocytosis (293-297). It consists of a C-terminal ATP-binding region and several PBRs, a central RhoGAP domain active towards Cdc42 and Rac1 *in vitro*, and an N-terminal Ral binding domain (RBD) which binds Ral GTPases in a GTP-dependent manner (271,294,297). Interestingly, *in vivo* RalBP1 is required for cell adhesion-dependent Rac activation controlled by R-Ras in a Ral-independent manner. Indeed, the RhoGAP domain binds to GTP-R-Ras and activates Arf6 GTPase, which in return stimulates Rac1 resulting in increased cell spreading and migration (298). Additionally, RalBP1 via its interaction with RalA, has been reported to increase cell migration and to promote cancer cell metastasis (i.e., prostate and human bladder cancers)(299).

RalBP1 localization is crucial for its function and the binding to an active Ral is a key factor triggering its PM translocation, but at the same time it blocks its GAP activity towards Cdc42 and Rac1 (293,295,300). At the molecular level, the PBR (154-219aa, Table 1.3) has been identified to be required for the RalBP1 PM translocation and for its function as a membrane transporter of glutathione conjugates, in addition to other domains including, the N-terminal and C-terminal domains, which contribute to the differential cellular distribution of RalBP1 (301).

p85α and p85β

Phosphatidylinositol 3-kinase (PI3K), which catalyzes the generation of phosphoinositides, is a heterodimer complex between a catalytic p110 subunit and regulatory subunits: p85α and p85β. p85α and p85β are very similar and their domain structure comprises, an N-terminal SH3 and a central RhoGAP domains, as well as two SH2 domains, mediating the interaction with phosphorylated RTKs and flanking a p110 binding region (302,303). The RhoGAP domain, although it has a sequence homology similar to functional RhoGAP domains such as that of p50RhoGAP including the arginine finger, and because it lacks some critical residues it is inactive towards most Rho GTPases (304). Nevertheless, *in vitro* the RhoGAP domain of p85 binds to both GTP- and GDP-bound Cdc42 with a preference for the GTP-form, and this binding stimulated the PI3K catalytic activity (305).

ArhGAP6, ArhGAP 18 and ArhGAP28

The members of this subfamily share a high homology domain structure consisting of a central RhoGAP domain with a conserved arginine finger and PBRs mostly in the N-terminal region (274-276). The structure similarity allows, in some cases, functional compensations between these RhoGAPs such as the one in ArhGAP28 null mouse where both ArhGAP6 and ArhGAP28 are overexpressed, possibly to compensate the loss of ArhGAP28 function (276).

ArhGAP6 has been characterized first in the dominant X-linked MLS (Microphthalmia with linear skin defects syndrome) disorder as a protein with a functional RhoGAP domain and a GAP activity towards RhoA and Rac3 (Ras-related C3 botulinum toxin substrate 3)(272,273,306). Likewise, it inhibits stress fiber formation, while mutation of the conserved arginine residue within the RhoGAP domain prevented this effect (272). Also, this RhoGAP seems to play an anti-tumorigenic role since its expression in human cervical-cancer cell models enhanced the expression of tumor-suppressor genes, induced apoptosis and cell cycle arrest, and suppressed cell adhesion, proliferation, migration and invasion (273). Similarly, expression of this gene in athymic nude mouse reduced tumor weight and size, making ArhGAP6 a promising therapeutic target to treat cervical cancers (273). On the other hand, ArhGAP6, which is

overexpressed in mononuclear-blood cells from patients with hypertension, has been shown to bind and stimulate PLCδ1 activity both *in vitro* and *in vivo* leading to increased inositol triphosphate and DAG generation, which might contribute to hypertension (307).

ArhGAP18 has been first characterized in the human epididymis and later found to be ubiquitously expressed. It is active *in vivo* and directs its RhoGAP activity towards RhoA and RhoC (274,275). ArhGAP18 localizes to the leading edge of the migrating cells, wherein it suppresses stress fiber and focal adhesion formation by the suppression of RhoA activity, promoting thereby the cell spreading and migration (275). ArhGAP18 seems to be a major regulator in many signaling pathways such as angiogenesis and its low expression levels are often associated with many cancers including breast and lung cancers, whereas its complete expression loss is associated with severe developmental-vascular defects and enhanced tumor growth and vascularisation (44,121,122,308). Actually, ArhGAP18 has been reported to act as a negative regulator of pro-angiogenic factors to stabilize endothelial cell-to-cell (EC) junctions (308). The underlying mechanism involves its translocation to the sites of destabilized EC junctions where it reduces RhoC activity in a ROCK-dependent way limiting therefore, the tip-cell formation and strengthening the cell-cell junctions (308).

ArhGAP28 has been listed in several DNA-microarray analyses although its cellular functions begin just to be characterized (309). This RhoGAP displays a GAP activity towards RhoA and is involved in several cell events including early osteogenesis, Rho-dependent assembly of the ECM and RhoA-induced stress fiber suppression (276). Similarly, ArhGAP28 is thought to regulate, at the transcriptional level RhoA and many other proteins of the ECM such as Matn3 and Col2a1 (276). It appears therefore, that ArhGAP28 is a crucial regulator of Rho signaling at different levels in various cellular sites and aberrations in its expression translate into enhanced RhoA activity. Of interest, recent findings reported that increased ArhGAP28-promoter region methylation is associated with high RhoA activity, which may contribute to the increased metastatic abilities in cells (310).

Vilse / ArhGAP39 CrGAP (CrossGAP)

Vilse [also known as ArhGAP39 or CrGAP (CrossGAP)] is a conserved RhoGAP that has been first characterized in drosophila for its role in axon guidance downstream of Slit-Robo signaling in the midline (277). Its domain structure consists of an N-terminal WW and a MyTH4 (myosin tail homology 4) domains, and a C-terminal RhoGAP domain. The WW domain, which binds to proline-rich motifs, is required to target Vilse to the PM via a direct interaction between Vilse and the intracellular domain of the receptor Robo. The resulting PM translocation of Vilse permits to the RhoGAP domain to interact with the PM-anchored Rac and hence to stimulate its GTPase activity (277,278). The regulatory mechanism is dose-sensitive, where more or less Vilse activity can lead to axon guidance defects (278). Furthermore, Vilse is involved in spine morphogenesis by binding to the scaffold protein CNK2 through its WW domain to maintain Rac activity at optimal levels for spine formation (311).

TAGAP (T-Cell Activation RhoGTPase Activating Protein)

TAGAP was characterized for its role in male parent-gene transmission ratio to offspring and has been associated with several disorders including rheumatoid arthritis, Crohn's and celiac diseases, and multiple sclerosis (279,312-317). Its domain structure consists of a PBR at the N-terminal region next to a RhoGAP showing a selective RhoGAP activity towards RhoA (279).

p190RhoGAP-A and p190RhoGAP-B proteins

The multidomain p190RhoGAP-A (ArhGAP35) and its closely related p190RhoGAP-B (ArhGAP5) proteins form the p190RhoGAP subfamily, and are among the most prominent actin cytoskeleton and Rho GTPase regulators. They are involved in a variety of cellular processes modulating mainly the actin cytoskeleton and aberrations in their activities are associated often with cell adhesion and migration defects leading to tumorigenesis (123,318). These proteins comprise an N-terminal GTPase binding domain (Ras), several diphenylalanine (FF) motifs in the central region, which mediate protein-protein interactions such as interactions with RNA-binding proteins, and a C-terminal RhoGAP domain. Moreover, they contain in the central region many SH3 motifs which carry out protein-protein interactions. p190RhoGAPs

display an *in vitro* GAP activity towards Rho, Cdc42 and Rac, whereas *in vivo,* studies reported a RhoGAP activity for both Rho and Rac GTPases (280-286). The recruitment to the PM and the regulation the RhoGAP activity of p190RhoGAPs involve in general phosphorylation, lipid-protein and protein-protein interactions leading to protein complex formation with cellular partners including p120RasGAP, integrins, p120 catenin and many other proteins from both intracellular compartments and the extracellular matrix (123,318-322).

One important lipid-protein regulation mechanism implies a PBR N-terminal to the RhoGAP domain of both p190RhoGAP-A and B, a motif that is imperative for the PM recruitment, the GAP activity regulation and substrate selection. This positively-charged domain facilitates the membrane anchoring by interacting directly through electrostatic interactions with the negatively-charged PM phospholipids (35,112). Indeed, p190RhoGAP-A, which regulates both Rho and Rac activities, requires an integral and intact PBR in order to inhibit Rac, whereas its GAP activity towards Rho is PBR-independent. Likewise, a p190RhoGAP-A mutant lacking the PBR fails to inhibit the EGF-induced membrane ruffles (i.e., active Rac) in COS-7 cells, whereas it blocks the lysophosphatidic acid (LPA)-induced stress fibers by suppressing RhoA activity (286). In addition, the PBR of p190RhoGAP-A but not that of p190RhoGAP-B, contains two regulatory phospho-serines, Ser-1221 and Ser-1226, critical for the PM recruitment and substrate selection. As such, following their phosphorylation by PKC, p190RhoGAP-A dissociates from the PM phospholipids and directs its GAP activity towards Rho instead of Rac, which results in opposite changes in both active Rac and Rho levels in different cellular compartments, depending on the PBR phosphorylation status (35).

CdGAP subfamily of RhoGAPs: ArhGAP30, CdGAP, ArhGAP32 and ArhGAP33

The members of this subfamily are well-conserved throughout eukaryotic evolution suggesting evolutionary-conserved cellular functions notably in the regulation of the actin cytoskeleton and cell motility (95,323). They share a domain structure consisting of an N-terminal PBR and a RhoGAP domain, as well as an extended C-terminal region with basic and proline-and-serine residue rich regions in CdGAP, and

two proline-rich regions for ArhGAP30. Additionally, ArhGAP32 and ArhGAP33 harbor in their N-terminal region an SH3 and a PX domain.

ArhGAP32 [also designated as p200RhoGAP, p250GAP, RICS (RhoGAP involved in catenin–N-cadherin and NMDA receptor signalling) and Grit (GTPase regulator interacting with TrkA)], which is expressed mainly in the brain, plays key roles in axon growth regulation, and is associated with the autism spectrum disorder in Jacobsen syndrome (324). The membrane localization of ArhGAP32 (i.e., Golgi, ER and endosomes) is achieved through its PX domain which interacts preferentially with the phosphoinositides: PtdIns(3)P, PtdIns(4)P and PtdIns(5)P (325). ArhGAP32 has been first characterized for its regulatory role of neurite outgrowth downstream of activated RTKs, by binding to NGF receptor, TrkA and to N-Shc and CrkL/Crk adapter proteins (326). The control of the neurite growth is achieved chiefly through regulation of key Rho GTPases. As such, ArhGAP32 present a RhoGAP activity both *in vitro* and *in vivo* towards Cdc42, Rac1 and RhoA, while the conserved catalytic arginine (Arg-58) is required for this activity (152,153). ArhGAP32 (RICS) (-/-) neurons develop longer neurites and display increased Cdc42 activity, whereas expression of either the RhoGAP domain alone or the full-length protein prompted the neuronal differentiation phenotype of N1E-115 neuroblastoma cells (152,153). Likewise, both cell migration and axonal defects have been reported in the developing cerebellar cortex of ArhGAP32 (p250GAP) knockdown *in vivo* (327). Finally, ArhGAP32 has been shown to interact through the C-terminal region, with the SH3 domain of RasGAP leading to activation of the Ras-ERK1/2 and PI3K pathways and subsequently to cell transformation and proliferation (328).

ArhGAP33 [also referred to as TCGAP (TC10/Cdc42 GAP)] has been characterized for its interaction with and regulation of TC10 and Cdc42 Rho GTPases, as well as for its regulatory role in insulin signaling pathway. For instance, following insulin stimulation, ArhGAP33 through its PX domain which binds to PtdIns(4,5)P2, translocates to the PM where it blocks the insulin-stimulated glucose uptake and the glucose transporter GLUT4 translocation (154). The RhoGAP activity is directed towards Cdc42 and is negatively regulated by phosphorylation on key residues such as

Tyr-406 in the GAP domain (107). ArhGAP33 is expressed prominently in the developing and mature brain where it regulates positively the neurite outgrowth and branching (107). It is therefore, associated with neuropsychiatric developmental disorders such as the autism spectrum disorder, characterized by severe dendrite-arborisation defects (329). Correspondingly, ArhGAP33 is required for cortical neuron morphogenesis and its depletion by RNA interference results in reduced dendrite branches number and in the overall length of dendrite arbors (330). Likewise, the spine density is rescued by ArhGAP33 re-expression or by expression of a knock-down resistant form of ArhGAP33 (330). In addition, mice lacking ArhGAP33 show aberrant defects during cortical development, characterized by oversimplification of cortical-dendritic arborisation and a decrease of the neocortical volume (331). High Cdc42 activity and actin-cytoskeleton dysregulation may be involved in these defects since ArhGAP33 expression suppresses the GTPase activity and activates cofilin, an actin regulating protein (331). In addition to the above mechanisms, ArhGAP33 function of promoting spine and synapse development involves further regulatory mechanisms either GAP activity-dependent, such as the regulation of PSD-95 in the neocortex, or GAP activity-independent (329).

ArhGAP30 present a high domain homology with its closest homolog CdGAP notably, in the N-terminal PBR and in the RhoGAP domain, suggesting shared conserved functions and regulation mechanisms. The RhoGAP of ArhGAP30 is catalytically active and stimulates the GTPase activity of both Rac1 and RhoA (287). ArhGAP30 is involved in cell adhesion regulation downstream of Wrch-1 and its overexpression induces focal adhesion and stress fiber dissolution in addition to membrane blebbing, reminiscent of the RhoA pathway suppression (287).

Interestingly, ArhGAP30 seems to play an anti-tumorigenic role in cancer, particularly in colorectal cancer (CRC) where its protein expression levels are reduced. Indeed, in both CRC and its precancerous lesions, ArhGAP30 is downregulated and this is associated to poor patient prognosis. The result suggests a potential tumor-suppressing activity of this RhoGAP, whereas its expression level could be used as a potential prognosis biomarker for CRC. The mechanism underlying this role involves at

least a ArhGAP30-driven p53 acetylation and functional activation, achieved through an ArhGAP30-p300 acetyltransferase interaction in a RhoGAP-independent manner (332). In the next section CdGAP regulation and function will be discussed.

1.5 CdGAP regulation and function

CdGAP/ArhGAP31 belongs to a well-conserved CdGAP subfamily comprising in addition to CdGAP orthologues, at least three CdGAP-related genes *ARHGAP30*, *ARHGAP32* and *ARHGAP33* (95,109,287,323,333-336). CdGAP has been discovered in 1998 in a yeast two-hybrid cDNA library screen using Cdc42 mutant Y40C as a bait. A short mouse CdGAP form of 820 amino acids, rich in charged and serine residues was initially isolated and classified among the well-conserved RhoGAP subfamily members (337). Afterwards, a ubiquitously expressed full-length protein has been characterized in both mouse and human with a characteristic enrichment in heart, muscle, liver, brain and lung tissues (334,336-338). CdGAP has been at once characterized for its role in actin reorganization by regulating the GTPase activity of Rac and Cdc42 making it a novel negative Rho GTPase regulator (337). Afterwards, it has been shown to be involved in several cellular pathways and functions such as the regulation of cell migration, embryogenesis, angiogenesis and cancer (Figure 1.2) (127,339-341).

1.5.1 Domain organization

CdGAP is a large RhoGAP protein (mCdGAP, 1425aa; hCdGAP, 1444aa), rich in basic, proline and serine residues, and comprising numerous regulatory domains involved in specific CdGAP functions (Figure 1.1C). CdGAP contains namely: an N-terminal PBR which binds to membrane phospholipids and regulates both CdGAP PM targeting and activity; a conserved RhoGAP domain mediating the GAP activity towards Rac1 and Cdc42; a central highly-phosphorylated basic region (BR) mediating several protein-protein interactions and involved in the regulation of cell migration and adhesion; a highly serine- and threonine-phosphorylated proline-rich domain (PRD) that mediates the transforming growth factor β (TGFβ)-induced effects on cell migration and invasion; and finally, an extended highly-phosphorylated C-terminus (CT), involved in the

regulation of the GAP activity and in the rare developmental disorder, Adams-Oliver Syndrome (AOS) (Figure 1.2) (128,334,340,342-344).

1.5.2 CdGAP regulation

CdGAP is regulated by several mechanisms including protein-protein and lipid-protein interactions, and phosphorylation.

1.5.2.1 Protein-protein interactions

CdGAP contains several structural features such as SH3 and 14-3-3-protein binding motifs which are involved in a variety of protein-protein interactions with different partners including adaptor proteins (e.g. Intersectin), actin cytoskeleton proteins (e.g. Actopaxin), receptors (e.g. vascular endothelial growth factor receptor2, VEGFR2), transcription factors (e.g. Zeb2) and protein kinases (e.g. Erk1/2 MAP Kinases and GSK3) (Figure 1.2)(108,109,127,339,341,345). For instance, the endocytic and Cdc42 GEF adaptor protein, Intersectin interacts with CdGAP and regulates both its subcellular localization and activity in a platelet-derived growth factor (PDGF)-dependent fashion. Likewise, in PDGF-stimulated Swiss 3T3 cells, Intersectin binds and colocalizes with CdGAP at the cell periphery and PM, wherein it suppresses its activity towards Rac1 leading to increased lamellipodia and membrane ruffles. The association involves the SH3D domain of Intersectin and a novel-conserved xKx(K/R)K motif within the BR region of CdGAP (345,346). Similarly, CdGAP has been reported to associate with the actin and paxillin binding protein, Actopaxin, and the interaction targets CdGAP to focal adhesions in an adhesion-dependent manner whereby its activity is suppressed (341). The regulation of CdGAP activity at focal adhesions and the interaction between the two proteins, which is driven through the C-terminal region of Actopaxin and the BR domain of CdGAP, are required for a normal cell spreading, lamellipodia formation and polarization as well as cell migration of human U2OS osteosarcoma cells (341).

1.5.2.2 Phosphorylation

CdGAP is a phosphoprotein displaying high Ser and Thr phosphorylation levels in the BR, PRD and CT regions, and is therefore a potential phosphorylation target of a wide range of Ser/Thr protein kinases (108,109,323). Likewise, many large-scale phosphoproteomic studies report increasing numbers of CdGAP phosphorylation sites

being regulated in many signaling pathways and diseases such as insulin/Akt signaling, angiogenesis and many cancers (listed in www.phosphosite.org) (347-354). CdGAP *in vivo* phosphorylation is increased following growth factor stimulation including serum, PDGF and TGFβ, whereas the Ser/Thr kinases Erk1/2 MAPK, GSK3 and p90 ribosomal protein S6 kinase (RSK) 1 have been shown to both bind and phosphorylate CdGAP(108,109,128,323). As for the phosphorylated sites, Thr-776 residue has been shown to be phosphorylated by both Erk1/2 MAPK and GSK3 in serum-stimulated and serum-starved conditions and this phosphorylation inhibits CdGAP activity (108,109). Yet, little is known about the identity of the many other CdGAP phosphosites or the corresponding protein kinases and their role remains unclear and requires further investigation. Recently, two major phosphosites, Ser-1093 and Ser-1163 in the CT region have been described to be phosphorylated by the AGC kinase RSK1 leading to the recruitment of 14-3-3 adaptor proteins and to the regulation of both CdGAP subcellular localization and activity (to be discussed in Chapter 3).

1.5.2.3 Lipid binding

CdGAP, similar to most active RhoGAPs, requires a membrane translocation in order to suppress the GTPase activity of its membrane-bound effectors, Cdc42 and Rac1. As CdGAP lacks any LBD, an alternative mechanism described recently seems to help its membrane targeting. As such, the N-terminal PBR cluster preceding the RhoGAP domain has been identified to mediate specific binding to the negatively-charged membrane phospholipids, preferentially PtdIns(3,4,5)P3 and thereby, to ensure CdGAP anchoring into the PM (36). Importantly, an intact PBR is required for the *in vitro* full-CdGAP activity toward Rac1 and for the *in vivo* CdGAP effects on cell morphology. Equally, CdGAP protein mutants missing a functional PBR are unable to induce cell rounding in fibroblast cells following PDGF stimulation (36). Collectively, these data describe a PBR-mediated modulation of CdGAP activity and membrane localization through a mechanism common in a number of proteins including some RhoGAPs such as DLC1 and p190RhoGAP-A and B (Table 1.1) (34,35,112,117).

1.5.3 Cellular functions of CdGAP

1.5.3.1 Regulation of actin-cytoskeleton dynamics and cell motility

Consistent with its RhoGAP activity towards the key regulators of the actin cytoskeleton, Rac and Cdc42 GTPases, CdGAP is involved in quite a lot of cytoskeleton -remodeling events including, filopodia and lamellipodia formation, cell spreading, adhesion, migration and invasion. CdGAP overexpression in diverse cell lines has been shown to promote cell rounding, while in a GAP activity-dependent manner, CdGAP inhibits most of the above-cited events as a results of the disruption of cortical actin network. As such, the inhibition of CdGAP activity by CdGAP association to Actopaxin and Intersectin in U2OS and Swiss 3T3 cells respectively, reversed the CdGAP overexpression-induced cell rounding and promoted lamellipodia formation and polarity in these cell lines (345,355). In addition, CdGAP has been shown to localize to focal adhesions formed in three-dimensional (3D) matrix environment and to regulate both random and directed cell migration, as well as adhesion assembly and disassembly dynamics of human U2OS osteosarcoma cells (356). Moreover, CdGAP through localization to cell-extracellular matrix adhesions, modulates both matrix-rigidity sensing and durotaxis, two critical processes in cell migration and invasion (357). The underlying mechanism involves the modulation of Rac1 activity at cell-matrix adhesions, membrane protrusions and focal adhesion dynamics (357).

1.5.3.2 Role in Adams-Oliver Syndrome (AOS)

AOS is a rare developmental disorder, characterized by the presence of aplasia cutis congenita of the scalp vertex and terminal transverse limb-reduction defects, as well as cardiovascular anomalies in some patients (358,359). Recently, mutations in the human *CdGAP/ARHGAP31* leading to autosomal-dominant AOS have been identified in patients suffering from this syndrome. Indeed, using genome-wide linkage analysis and exome sequencing, two premature truncating mutations have been identified in the terminal exon of the *CdGAP* gene from AOS-patients (340). The resulting AOS-protein mutants, hCdGAP(Q683X) and hCdGAP(K1087Sx4) lacking the C-terminal region, exhibit a constitutive GAP activity leading to a loss of available active-Cdc42 disrupting thus, actin cytoskeletal structures. Consequently, the gain-of-function of AOS-causing

proteins, enhances cell rounding and impairs cell migration and proliferation during early development of fibroblasts at the terminal limb buds and craniofacial processes, leading to impaired organogenesis that characterize this syndrome (340). Overall, these data emphasize the requirement for regulated Rac1/Cdc42 pathways during early human development.

1.5.3.3 Role in angiogenesis

One characteristic of AOS patients carrying abnormal CdGAP proteins, is the presence of diverse and frequent vascular and cardiac defects including pulmonary hypertension and cutis marmorata telangiectatica — a rare developmental congenital vascular disorder — suggesting a role of CdGAP in angiogenesis (358,360). In support of this hypothesis, earlier studies have reported that *CdGAP* expression is elevated in endothelial cells notably, in the human umbilical vein endothelial cells (HUVECs), but also in the developing vascular system of zebrafish where CdGAP is among the 17 most highly expressed RhoGAPs (361,362). Accordingly, a very recent study demonstrated a crucial role of CdGAP in embryonic-vascular development and vascular-endothelial-growth factor (VEGF)-induced signaling. The new findings show for the first time, that CdGAP interacts with the VEGF receptor-2 (VEGFR2) and is a key factor for a normal signaling downstream of VEGF stimulation and VEGF-driven angiogenesis. The CdGAP/VEGFR2 interaction seemingly controls the entire signaling pathway leading to Rac1 activation for a typical VEGF-dependent endothelial cell migration and capillary formation (339). Consistent with this role, CdGAP-deficient mouse embryos display impaired vascular development associated to superficial vessel defects and subcutaneous edema. Furthermore, CdGAP depletion both in endothelial cells and in mice is associated with severe VEGF-dependent angiogenesis, highlighting the importance of CdGAP in embryonic-vascular development and VEGF-induced signaling (339).

1.5.3.4 Role in cancer

CdGAP, through the inhibition of Cdc42 and Rac1 activities, controls the actin cytoskeleton-related processes including, cell adhesion, migration and invasion, which are all key aspects associated to cancer progression, suggesting a role of CdGAP in

tumorigenesis and possibly, a negative one. The hypothesis is supported by some recent findings reporting that, in an *in vitro* system, CdGAP-depleted human U2OS osteosarcoma cancer cells displayed both increased migration and invasion through a three-dimensional matrices conferring to CdGAP anti-tumorigenic functions in this context (356). However, early studies have documented steadily elevated CdGAP expression levels following growth factor stimulation (i.e., serum and TGFβ) in immortalized cellular models and cancer cell lines, suggesting a pro-survival role of CdGAP (108,128). The idea of a possible positive CdGAP role in cancer was further supported by the observation that endogenous CdGAP levels were readily upregulated in the ErbB2-induced breast cancer cell model, NMuMG-NT (mouse mammary cancer cell explants expressing the activated Neu/ErbB-2 receptor) comparing to control cells (128). More recently, CdGAP has been found to be highly expressed in basal-type breast cancer cells including, MDA231, MDA468, BT549 and HS578T, and its strong expression correlated with poor prognosis in breast cancer patients (363). By the same token, a study using quantitative-deep RNA sequencing found that CdGAP is the major RhoGAP expressed in HER2/ErbB2-induced mouse breast tumors (364). Collectively the data suggest a positive role of CdGAP in cancer, notably in breast cancer, likely downstream of the HER2/Neu/ErbB-2 and TGFβ signaling pathways. Of note, a cooperation between the two pathways has been observed aiming to amplify the tumorigenic abilities of NMuMG-NT cells, which show high invasive and metastatic activities following TGFβ treatment (365-367). In agreement with this, CdGAP has been reported to mediate TGFβ- and ErbB2-induced cell migration and invasion of breast cancer cells, and does so in a GAP-independent manner. Accordingly, targeting CdGAP by siRNA abolished proliferative abilities of Neu/ErbB2-expressing breast cancer cells and their TGFβ-induced cell motility, and induced the expression of E-cadherin, a tumor suppressor and a cell-cell junction protein (128). Surprisingly, the PRD of CdGAP has been revealed to be sufficient to rescue the TGFβ phenotype which highlights the importance of this region in this process (128). In a follow-up study, a mechanism of E-cadherin-expression regulation by CdGAP downstream of TGFβ/ErbB2 signaling has been shown (363). As such, following TGFβ stimulation, CdGAP via its PRD region,

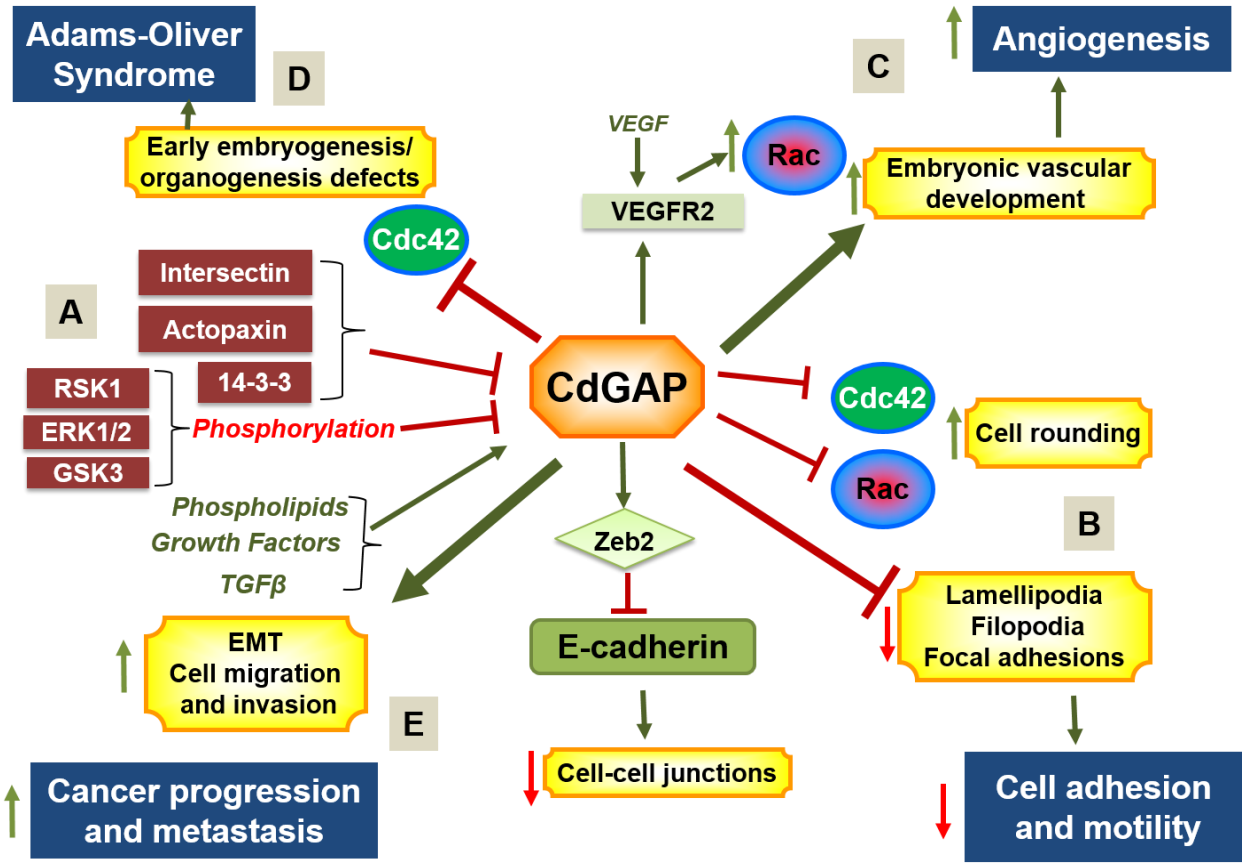
Adams-Oliver Syndrome
D
Early embryogenesis/ organogenesis defects
Cdc42
A
Intersectin
Actopaxin
14-3-3
RSK1
ERK1/2
GSK3
Phosphorylation
Phospholipids
Growth Factors
TGFβ
EMT
Cell migration and invasion
E
Cancer progression and metastasis
VEGF
Rac
VEGFR2
C
Angiogenesis
Embryonic vascular development
CdGAP
Cdc42
Cell rounding
Rac
B
Zeb2
E-cadherin
Cell-cell junctions
Lamellipodia
Filopodia
Focal adhesions
Cell adhesion and motility

Figure 1.2 – Summary of CdGAP functions and cellular partners. CdGAP activity and cellular functions are regulated by different mechanisms including protein-protein and lipid-protein interactions, and phosphorylation (A). CdGAP is involved in various cellular pathways both in a GAP-dependent or – independent manner. Likewise, it suppresses the GTPase activity of Rac and Cdc42 and thereby controls the actin cytoskeleton remodeling and cell motility (B). CdGAP is also involved in early development and is required for embryonic vascular development (C)(339), whereas gain-of-function mutations of CdGAP proteins were associated with the developmental disorder Adams-Oliver Syndrome (D)(340). Finally, a proto-oncogenic role of CdGAP has been recently characterized notably in breast cancer, wherein CdGAP by suppressing E-cadherin expression promotes cancer progression and metastasis (E)(128,363). Abbreviations: E-cadherin, epithelial cadherin; EMT, epithelial to mesenchymal transition; TGFβ, transforming growth factor beta; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; Zeb2, Zinc Finger E-Box Binding Homeobox 2.

interacts and forms a complex at the nucleus with the transcriptional repressor Zeb2 to inhibit E-cadherin expression in a GAP-independent fashion (363,368). Consequently, CdGAP regulates cell junctions and is required for ErbB2 to promote breast tumors and metastasis (363). It appears finally, that CdGAP as a proto-oncogene, is a valuable biomarker and an interesting therapeutic target of human cancers, particularly several breast cancer subtypes.

1.6 14-3-3 adaptor proteins

14-3-3 family members are adaptor/scaffold proteins ubiquitously expressed and highly conserved among eukaryotic organisms. They have been first characterized in a brain protein analysis for their particular elution and migration patterns from which the name 14-3-3 originated, and later the Greek letters were added to describe the early isoforms (369,370). So far seven isoforms have been characterized in mammals: 14-3-3β (also known as α when phosphorylated) γ, ε, σ, ζ (similar to δ when phosphorylated), τ and η (371,372). They interact with their effectors by binding to the phosphorylated Ser/Thr motifs on target proteins thereby regulating their conformation, activity, complex formation and stability, and subcellular localization (371-374). Up to now, more than 200 protein targets have been identified for 14-3-3 proteins including, protein kinases, phosphatases, receptors and transcription factors. Consequently, they exert a widespread influence in various cell processes such as cell cycle regulation, gene transcription regulation, cell adhesion, motility, proliferation and apoptosis (371).

1.6.1 Regulation and substrate binding

The seven 14-3-3 isoforms are found as homo or heterodimer structures, wherein the monomers contain at their N- and C-termini several antiparallel α helices. This conformation allows 14-3-3 dimers to expose a negatively-charged surface that is important in recognising the features of the protein target. 14-3-3 isoforms display also, high variability in their N-terminal regions which contributes to dimer formation and diversity as well as to substrate selectivity and specificity (375,376). Dimeric 14-3-3 isoforms can bind motifs on the same or different protein targets and the binding of two

sites ensures by far, greater stability and more functional interaction than the binding of just one site (106,371-374,377).

14-3-3 proteins and dimer formation are regulated by many posttranslational modifications including Serine and Tyrosine phosphorylation, polyglycylation and acetylation (378-384). Indeed, 14-3-3 proteins are found to be phosphorylated in many signaling pathways and are targeted by various protein kinases, such as cAMP-dependent kinase (PKA), sphingosine-dependent kinase (SDK1), protein kinase B (PKB)/Akt and c-Jun N-terminal kinase (JNK)(385-388). Following their phosphorylation, 14-3-3 dimers are destabilized and the interaction with their effector disturbed releasing thereby, the target protein. For instance, the subcellular localization of apoptosis regulating protein, Bax is controlled by the binding to 14-3-3 proteins which sequestrates it and prevents its mitochondrial translocation. Following their phosphorylation, 14-3-3s dissociate from and release Bax which in return, translocates into the mitochondria where it accumulates and induces cell apoptosis (389). Protein kinases phosphorylate mostly 14-3-3 monomers and rarely 14-3-3 dimers which usually maintain close conformations inaccessible for the catalytic kinase activity (390). However, direct binding to the 14-3-3 dimer of cellular regulators may induce conformational changes rendering them phosphorylable but also, may promote their conversion to monomeric form to promote their phosphorylation (389). As such, the direct binding of the lipid sphingosine to 14-3-3ζ isoform promotes its conversion to a monomer and its subsequent phosphorylation by PKA at Ser-58 (391).

1.6.2 14-3-3 binding motifs

14-3-3 proteins recognize and bind phosphoserine/phosphothreonine motifs on hundreds of substrates with the following sequences: Arg-Ser-Xaa-pSer/Thr-Xaa-Pro (mode I) and Arg-Xaa-Xaa-Xaa-pSer-Xaa-Pro (mode II) (373,374,392). In addition, 14-3-3 proteins recognize and bind some C-terminally phosphorylated proteins bearing a rare motif pSer/pThr (X1-2)-COOH, referred to as the mode III-14-3-3 binding motif (393). While mode II binding motifs are frequent in plant 14-3-3 proteins, mode I motifs seemingly dominate in most 14-3-3 binding effectors (394).The phosphorylated motifs are generated by different Ser/Thr kinases including Ca^{2+}/calmodulin-dependent protein

kinases (CaMK), protein kinase D (PKD), checkpoint kinase 1(Chk1), Chk2 and AMP-activated protein kinase (AMPK) (394). Nonetheless, the majority of mode I binding motifs are generated by AGC kinases such as ribosomal S6 kinases (RSKs), PKB/Akt and PIM kinases. These kinases require usually arginine residues at positions -5 and -3 of the phosphosite, although the phosphorylation of the motif is not always guaranteed and sometimes additional determinant-selection criteria are necessary for each kinase (394,395). Moreover, 14-3-3 proteins can bind unphosphorylated proteins and several studies reported phosphorylation-independent 14-3-3 binding sites. As such, the ExoS protein from the pathogenic bacterium *Pseudomonas aeruginosa* harbors a 14-3-3 binding motif (LLDALDLAS), necessary for the interaction with 14-3-3 proteins and pathogenesis (396). Similarly, an atypical motif $RSX_{(1-3)}$E/D-like motif has been documented to bind 14-3-3 proteins with a comparable efficiency as that of the mode I RSXpSXP motif binding, wherein the negatively charged Asp and Glu are suggested to imitate the phosphoserine/threonine role in the binding (397).

1.6.3 Role in cancer

Given that 14-3-3 proteins are key regulators in many cellular functions (e.g., programmed cell death, cell cycle regulation and DNA-damage repair) and major molecular integrators and crosslinks in different signaling networks, it is logical to find that abnormalities in their abundance and/or regulation are often associated with tumorigenesis. In general, it is the overexpression rather than the depletion of selected 14-3-3 isoforms that is associated to malignancy progression. For instance, elevated expression levels of the 14-3-3 isoforms β, γ, ε, σ and ζ have been associated to hepatocellular carcinoma (HCC), whereas overexpression of the isoforms 14-3-3β and γ is linked to increased metastatic risk and poor patient prognosis (398-400). Similarly, 14-3-3 upregulation in various breast cancer patient tissues has been reported for several isoforms including 14-3-3 ζ, η, δ and γ (401). Additional examples documenting the association of increased 14-3-3 expression levels to specific cancers such as the 14-3-3 ζ in stomach and lung cancers, and 14-3-3γ in melanomas suggest a 14-3-3 isoform-expression signature in numerous tumors (398,402,403). Intriguingly, the isoform 14-3-3σ seems to play opposite roles in different cancers (404). Actually, high

expression levels of this isoform are associated with tumorigenesis and cancer progression in various tumors including prostate, colorectal, pancreatic, ovarian and cervical cancers (404-406). Conversely, for many years 14-3-3σ has been considered as a tumor suppressor and low expression levels are found in many cancers particularly those from epithelial origin such as lung and breast cancers (404,407-409). Overall, the 14-3-3 proteins are involved actively in tumorigenesis and their roles are complex and may vary between tissues and signaling pathways. While overexpression of many isoforms was shown to be proto-oncogenic in a wide range of human cancers, some other isoforms, such as 14-3-3σ seem to be either proto-oncogene or tumor suppressor depending on tumor localization and environment. Therefore, further studies are required for a better understanding and definition of the 14-3-3 proteins' roles in cancer.

1.7 Rationale and objectives

CdGAP is a large-multidomain phosphoprotein required in early development which modulates, in a GAP activity-dependent and -independent manner, many essential cellular events including actin-cytoskeleton remodelling and cell motility. Additionally, both CdGAP protein expression and phosphorylation are modulated downstream of pro-survival signaling pathways including TGFβ, Ras-ERK1/2 MAPK and Akt/GSK3 signaling. Importantly, recent findings assign a proto-oncogenic role for CdGAP, whilst its high expression levels have been associated to tumor progression and metastasis in many human cancers, making it a promising therapeutic target for cancer. However, the molecular mechanisms underlying CdGAP roles remain elusive and need further investigation for a better understanding. Therefore, the goal of the present thesis was to investigate new CdGAP regulatory mechanisms by protein-protein interaction and phosphorylation, in two main objectives:

1) To identify novel CdGAP binding motifs which may be involved in the interaction between CdGAP and the endocytic protein, Intersectin

2) To characterize novel CdGAP phosphorylation sites and investigate their role in CdGAP subcellular localization and activity

Preface to Chapter 2

CdGAP contains an N-terminal GAP domain, a BR central region, and a PRD with an extended C-terminal region. Our previous work identified the endocytic protein Intersectin as a binding partner and a negative regulator of CdGAP. The interaction is mediated by the SH3D domain of Intersectin and the central domain of CdGAP, which does not contain any typical proline-rich domain or known SH3-binding motif. In this study, we investigate the residues in the BR region mediating the interaction with Intersectin-SH3D domain. Using an *in vitro* system, we identify an atypical SH3-binding motif in the BR region that is required for the CdGAP-Intersectin interaction. Moreover, we go on to demonstrate that the identified conserved motif is required for a full CdGAP activity.

Chapter 2: Cdc42 GTPase-Activating Protein (CdGAP) interacts with the SH3D domain of Intersectin through a novel basic-rich motif

Martin Primeau, Ali Ben Djoudi Ouadda, and Nathalie Lamarche-Vane. (2011). **FEBS letters**. 585(6):847-53.

Abstract

The small GTPases Rac1 and Cdc42 are key regulators of the cytoskeleton. We have previously identified the endocytic protein Intersectin as a binding partner and regulator of Cdc42 GTPase-activating protein (CdGAP) with activity towards Rac1 and Cdc42. This interaction is mediated through the SH3D domain of Intersectin and the central domain of CdGAP, which does not contain any typical proline-rich domain or known SH3-binding motif. Here, we have characterized the Intersectin-SH3D/CdGAP interaction. We show that Intersectin-SH3D interacts directly with a small region of CdGAP highly enriched in basic residues and comprising a novel conserved xKx(K/R)K motif.

Introduction

The Rho family of small GTPases, including Cdc42, Rac1, and RhoA, controls a wide variety of cellular processes ranging from cell proliferation, polarization, motility, and adhesion to intracellular membrane trafficking (410). These proteins function as molecular switches that cycle between an active GTP-bound and an inactive GDP-bound form. Three families of regulators tightly regulate this cycle. Guanine nucleotide exchange factors (GEFs) activate GTPases by inducing the exchange of GDP for GTP, whereas guanine nucleotide dissociation inhibitors sequester and maintain inactive Rho GTPases in the cytoplasm (5). GTPase-activating proteins (GAPs) increase the intrinsic GTPase activity, leading to inactivation of Rho GTPases (5,95).

CdGAP (Cdc42 GTPase-activating protein) promotes the inactivation of Rac1 and Cdc42 but not RhoA (288). It comprises a N-terminal GAP domain, a basic-rich (BR) central region, and a proline-rich domain (PRD) with an extended C-terminal domain [20]. We have previously identified CdGAP as a binding partner of the endocytic scaffolding protein Intersectin involved in clathrin-mediated endocytosis and cell signalling (345). Intersectin is composed of two N-terminal Eps-homology (EH) domains, a putative coil-coiled domain and five C-terminal SH3 domains (SH3 A-E) (411). Its neuronal splice-variant, Intersectin-1L, contains an additional GEF domain active towards Cdc42, followed by a C2 domain (412). Intersectin is targeted to clathrin-coated pits through the interaction of its EH domains with epsin (413). Additionally, it can bind to various endocytic and signalling proteins, including dynamin, synaptojanin, Sos1, Numb, Wiskott-Aldrich syndrome protein (WASP), and CdGAP through a subset of its SH3 domains (412-417). Interestingly, we have previously found that the SH3D of Intersectin negatively regulates the GAP activity of CdGAP *in vitro* (345). Although CdGAP-PRD is required for the regulation of its GAP activity by Intersectin, this domain does not mediate CdGAP-Intersectin-SH3D interaction. In fact, Intersectin-SH3D interacts with the central region, lacking the conventional class I ([R/K]xXPxXP) or class II (XPxXPx[R/K]) SH3 binding motifs or other known SH3 binding motifs (418-423).

Therefore, we sought to determine which residues of CdGAP are responsible for the interaction with Intersectin-SH3D.

In this study, we demonstrate that Intersectin-SH3D directly associates with the BR region of CdGAP through a novel conserved SKSKK motif. This BR region is evolutionary conserved within CdGAP closest homologs and we show that the CdGAP-related protein ARHGAP30 is also able to interact with Intersectin-SH3D. Furthermore, the SKSKK motif is essential to CdGAP activity *in vitro*.

Materials and Methods

Plasmids

CdGAP constructs and pGEX4T1-actopaxin were described elsewhere (109,341,345). CdGAP deletion mutants were introduced into BamHI/XbaI of pRK5myc. For S^{35}-methionine *in vitro* translation, CdGAP-(1-515) was subcloned into BamHI/XbaI of pCDNA3.1. The SH3D domain of mouse Intersectin (aa 1070-1131) was subcloned into BamHI/NotI of pGEX-4T3. ARHGAP30 and ARHGAP30 deletion mutants were amplified by polymerase chain reaction (PCR) using mouse ARHGAP30 cDNA (MGC:99989, Mammalian Gene Collection, NIH) and subcloned into BamHI/XbaI of pRK5myc. CdGAP point mutants were generated by a two-step overlap extension PCR strategy using pRK5myc-CdGAP-(1-515) as a template and according to standard protocols. The PCR fragments were subcloned into BamHI/XbaI of pRK5myc or into Cla1 of pEGFPC1-CdGAP (1-820). All plasmids were verified by sequencing.

Cell Culture and transfection

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and antibiotics and maintained in a 5% CO_2 humidified environment at 37°C. Cells were transfected with linear polyethylenimine (PEI) MW 25,000 (Polysciences) using a 1:5 ratio (DNA : PEI) or by calcium phosphate (424).

GST pull-down assay

GST, GST-SH3D, and GST-actopaxin proteins were purified as previously described (341,345). HEK293 cells transfected with the various plasmids were lysed in 20 mM HEPES pH 7.4 and 1% Triton X-100 supplemented with 5 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete™ protease inhibitors. After a 10-minutes centrifugation, supernatants were incubated with 0.6 nmol of GST or GST-SH3D and glutathione-agarose beads for 2 hours at 4°C. Samples were washed three times in lysis buffer and bound proteins were submitted to SDS-PAGE and Western blot analysis using anti-myc antibodies.

In vitro ^{35}S-methionine translation

In vitro transcription and translation were performed using TNT® Quick Coupled Transcription/Translation Systems (Promega). Briefly, TNT Quick Master mix was incubated with 1 µg of pCDNA-CdGAP(1-515) and 63 µCi of [^{35}S]-methionine in a total volume of 50 µL at 30°C for 90 minutes. Fractions of the reaction mix (15 µL) were incubated with 20 µg of GST, GST-SH3D or GST-actopaxin coupled to glutathione-agarose beads, and GST pull-down assays were performed as described above. Bound proteins were submitted to SDS-PAGE, transferred to nitrocellulose membrane, and stained with Coomassie Blue. ^{35}S-methionine labelled proteins were detected by PhosphoImager analysis (PerkinElmer).

Peptide overlay assay

A total of 20 overlapping 15-mer peptides corresponding to CdGAP (249-358) were synthesized directly on a cellulose membrane from their C-terminus (JPT Peptide Technologies GmbH, Berlin, Germany). The membrane was washed once in 100% ethanol and three times in TBS buffer (50 mM TrisHCl pH 8.0, 137 mM NaCl and 2.7 mM KCl) before incubation in blocking buffer (BSA 2% w/v in TBS buffer). The membrane was incubated overnight at 4°C with GST or GST-SH3D (1.4 µmol/mL) in blocking buffer, washed in T-TBS buffer (TBS buffer supplemented with 0.05% Tween-20), and incubated with anti-GST-HRP antibody (GE Healthcare). After several washes, the membrane was subjected to chemiluminescent reaction using Western Lightning Plus-ECL detection kit (Perkin Elmer).

In vitro GAP assays

HEK293 cells were transfected with either pRK5-myc, pRK5-mycCdGAP (1-515), pRK5-mycCdGAP(1-515)(SaSaa), pEGFPC1, pEGFPC1-CdGAP(1-820), or pEGFPC1-CdGAP(1-820)(SaSaa) using Lipofectamine 2000 as per manufacturer. 36h post-transfection, myc-tagged or GFP-tagged proteins were immunoprecipitated using anti-myc or –CdGAP antibodies [20] as described previously [5]. The amount of immunoprecipitated myc-tagged CdGAP was estimated on Coomassie Blue-stained SDS-PAGE by comparison with different amount of purified bovine serum albumin.

Immunoprecipitated GFP-tagged CdGAP was quantified using a Victor X3 2030 Multilabel Reader spectrofluorimeter (PerkinElmer). According to this estimation, 500ng of immunoprecipitated CdGAP was used for the in vitro GAP assays, as described previously [5].

Results

To investigate the interaction between CdGAP and Intersectin-SH3D, we first determined whether the association is direct using an *in vitro* translation assay. A fragment corresponding to the N-terminal 515 amino acids of CdGAP was expressed as a ^{35}S-methionine-labeled protein and incubated in a GST pull-down assay using GST-Intersectin-SH3D or GST-actopaxin as a positive control. The focal adhesion protein actopaxin has previously been shown to interact with a region of CdGAP corresponding to the first 515 amino acids (341). As shown in Fig.2.1A, we found that CdGAP(1-515) binds to both Intersectin-SH3D and actopaxin but not GST, suggesting that the interaction between CdGAP and Intersectin-SH3D is direct. To further define the region of CdGAP binding to Intersectin-SH3D, protein lysates of HEK293 cells expressing various myc–tagged CdGAP deletion mutant proteins were incubated with GST-Intersectin-SH3D immobilized on glutathione-agarose beads (Fig. 2.1B and C). We found that Intersectin-SH3D was able to interact with CdGAP-(1-515), -(1-358), -(254-515), and -(181-358), all encompassing the BR region of CdGAP. On the contrary, CdGAP-(1-312) and CdGAP-(313-515) did not bind to Intersectin-SH3D. Therefore, these results show that an intact BR region is required to mediate the Intersectin-SH3D-CdGAP interaction.

To further define the amino acid motif within the BR region that mediates Intersectin-SH3D/CdGAP interaction, we used a peptide overlay assay comprising 20 overlapping peptides (15-mers), corresponding to the amino acid sequence of the BR region (249-358), immobilized on a cellulose membrane and incubated with purified GST-Intersectin-SH3D or GST proteins as a negative control (Fig.2.2). Interestingly, we found that 8 consecutive peptides corresponding to residues 284 to 333 were able to specifically bind to Intersectin-SH3D with different affinities (Fig.2.2). The interaction

between peptide 10 (residues 294-308) and 14 (residues 314-328) with Intersectin-SH3D showed the strongest affinity. Interestingly, amino acid sequence analysis of the peptides revealed a common SKSK motif, suggesting that these residues are important for the interaction between Intersectin-SH3D and CdGAP.

Furthermore, a close analysis of the amino acid sequences of CdGAP and its protein homologs ARHGAP30, ARHGAP32/GRIT, and ARHGAP33/Noma-GAP revealed that the BR region is highly conserved amongst the CdGAP-related proteins (Fig. 2.3A). In particular, the positively charged residues of the SKSK motif are present in all CdGAP-related proteins (Fig. 2.3A). To determine whether the BR domain of other CdGAP-related proteins is able to interact with Intersectin-SH3D, we examined the interaction between Intersectin-SH3D and ARHGAP30. Indeed, myc-tagged ARHGAP30 expressed in HEK293 cells was able to interact with Intersectin-SH3D (Fig. 2.3B). Moreover, ARHGAP30 (1-334) containing the N-terminus GAP domain followed by the BR region bound to Intersectin-SH3D whereas ARHGAP30 (335-1092) lacking the BR domain was not able to interact with Intersectin-SH3D (Fig.2.3B and C). Thus, these results suggest that the conserved BR domain within CdGAP-related proteins mediates the interaction CdGAP/Intersectin-SH3D.

We then performed alanine-scanning mutagenesis to identify which residues in the BR region of CdGAP mediate the interaction with SH3D. Based on the amino acid sequence similarity between ARHGAP30 and CdGAP, CdGAP point mutants were generated by amino acid substitution of conserved residues (Fig. 2.4A). We found that the interaction between Intersectin-SH3D and CdGAP point mutant 2 was significantly impaired compared with the binding of intersectin-SH3D to other CdGAP point mutants (Fig. 2.4B and C). Interestingly, this CdGAP point mutant 2 has its positively charged lysines replaced by alanines in the first SKSKK motif, previously identified using the peptide overlay assay (Fig. 2.2). However, replacement of the lysines by alanines in the second SKSK motif (CdGAP point mutant 7) did not alter the interaction with Intersectin-SH3D. Altogether, these results show that the SKSKK motif corresponding to residues

296-300 in the BR region of CdGAP is a novel SH3-binding sequence that mediates the interaction of CdGAP with the SH3D of Intersectin.

We then examined if the SKSKK motif is important for the regulation of CdGAP activity. In an *in vitro* GAP assay, [g^{32}P]-GTP-loaded Rac1 was incubated with myc-tagged CdGAP(1-515) or CdGAP(1-515) (SaSaa) immunoprecipitated from HEK293 cell lysates. We observed a reduction in the GAP activity of CdGAP(1-515) (SaSaa) compared with the wild-type protein (Fig.2.5A). However, when point mutations of the SKSKK motif were introduced into the full length CdGAP protein (1-820), the GAP activity of the mutant protein was inhibited (Fig.2.5B). In the presence of GST-SH3D, the GAP activity of CdGAP(1-820) was reduced as previously shown [5] but it has no major effect on the mutant protein (Fig.2.5C). Therefore, these results show that the SKSKK motif is important for the regulation of CdGAP activity *in vitro*.

Discussion

In this study, we have identified key amino acid residues involved in the direct binding of the SH3D domain of Intersectin with CdGAP. These findings suggest that evolutionary conserved lysine residues located within the BR region of CdGAP and its related protein homologs are central to a novel atypical SH3 binding motif xKx(K/R)K. It is well established that most of the SH3 domains characterized to date bind to conventional class I ([R/K]xXPxXP) or class II (XPxXPx[R/K]) peptide motifs (418,419). However, a growing number of atypical motifs containing or not a proline residue have been discovered over the past years. These include the PxxxPR motif recognized by CIN85 in a number of proteins (420), the PxxDY motif that associates with Eps8 SH3 domain (421), the RKxxY motif in SKAP55 that mediates its interaction with the C-SH3 domain of SLAP (422), and the RxxK motif in SLP-76 bound by the C-SH3 domain of Gads (423). Most of these motifs share with the novel identified xKx(K/R)K motif the presence of key basic residues. In contrast to other SH3 domains (A, B, C, and E) of Intersectin that interact with a large number of proteins though the consensus PXXP sequence (412-414), only CdGAP and the adaptor protein Numb have been shown to interact with the SH3D domain of Intersectin. It is noteworthy that the C-terminus of mouse Numb involved in the interaction with Intersectin-SH3D (416) also contains the residues SKSKQ, adding support to

the findings that the novel xKx(K/R)K motif mediates the interaction with Intersectin-SH3D. Although the peptide overlay assay suggests that the second SKSK motif may be involved in the CdGAP/Intersectin-SH3D interaction (Fig.2.2), the alanine scanning mutagenesis experiment shows that only the first SKSKK motif is essential to mediate the interaction with SH3D (Fig.2.4). In this experiment, CdGAP proteins expressed in HEK293 cells may have post-translational modifications, absent in the synthesized peptides, that could alter the interaction with SH3D and account for the discrepancy between the two results. In support of these findings, we demonstrate that the critical SKSKK motif regulates the GAP activity of CdGAP in vitro. Indeed, the replacement of the lysine residues by alanines in the full-length protein CdGAP (1-820) is sufficient to inhibit the GAP activity. Interestingly, point mutations of the SKSKK motif in the shorter protein CdGAP(1-515) lacking the PRD slightly affect the GAP activity, suggesting that the PRD is required for this regulation. These data are in good agreement with our previous study showing that the PRD of CdGAP is required for Intersectin-mediated regulation of CdGAP activity [5].

In addition, it has been reported that the SH3 domains of Intersectin-1L regulate the GEF activity of Intersectin towards Cdc42 through intramolecular interactions with the adjacent DH-PH-C2 domains that do not involve proline peptide binding (416,425). The interaction of Numb with Intersectin-SH3D relieves this autoinhibitory mechanism, resulting in a net activation of Cdc42 activity (416). It will be of interest to determine whether CdGAP binding to the SH3D domain of Intersectin can also act as a positive regulator of the GEF activity of Intersectin-1L towards Cdc42. Combined with the negative regulation of CdGAP activity by Intersectin SH3 domains (345), these SH3 interactions would result in a net activation of Cdc42 activity. Future studies will determine how these molecular interactions are orchestrated in a spatial and temporal manner to affect Cdc42 activity in a physiological context.

Acknowledgments

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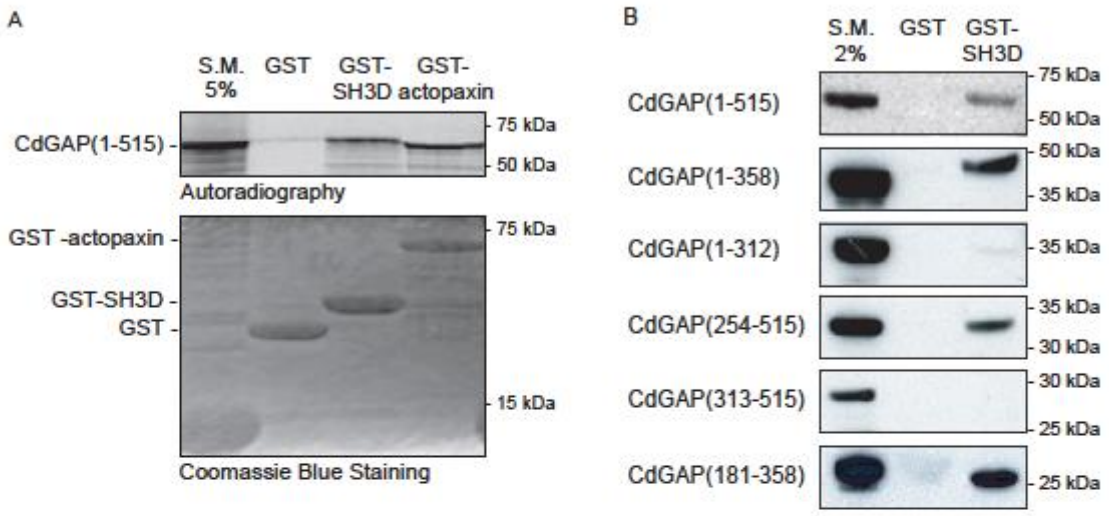
Figure 1


A
S.M. 5%
GST
GST-SH3D
GST-actopaxin
CdGAP(1-515)
75 kDa
50 kDa
Autoradiography
GST -actopaxin
GST-SH3D
GST
75 kDa
15 kDa
Coomassie Blue Staining
B
S.M. 2%
GST
GST-SH3D
CdGAP(1-515)
CdGAP(1-358)
CdGAP(1-312)
CdGAP(254-515)
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25 kDa
25 kDa

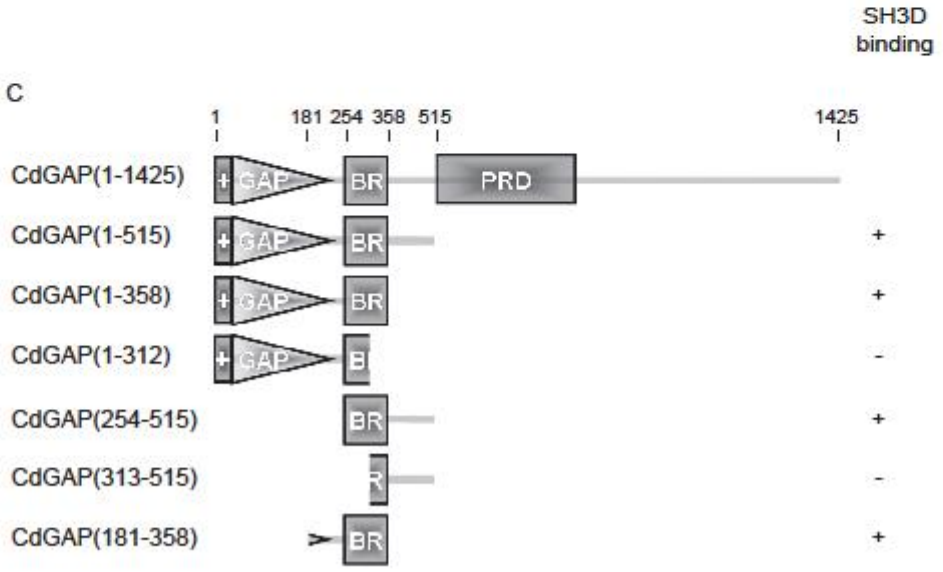
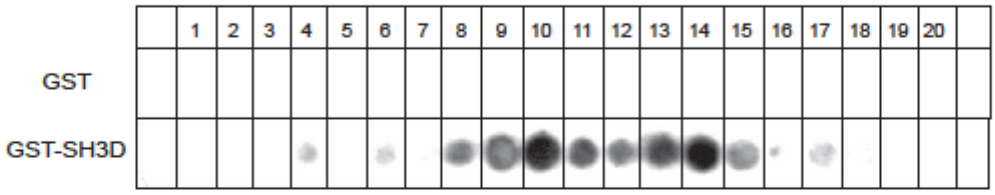

SH3D binding
C
1
181
254
358
515
1425
CdGAP(1-1425)
CdGAP(1-515)
CdGAP(1-358)
CdGAP(1-312)
CdGAP(254-515)
CdGAP(313-515)
CdGAP(181-358)
GAP
BR
PRD
+
+
+
-
+
-
+

Figure 2.1 – An intact BR domain of CdGAP is required to bind to Intersectin-SH3D.

(A) *In vitro* [^{35}S]-methionine translated CdGAP (1-515) was incubated with GST, GST-SH3D or GST-actopaxin coupled to glutathione-agarose beads in a GST pull down assay. 5% of the starting material (S.M.) was loaded in the first lane. (B) Myc-tagged deletion mutants of CdGAP expressed in HEK293 cells were incubated with GST or GST-SH3D proteins coupled to glutathione-agarose beads in a GST pull down assay. CdGAP was revealed by western blotting using anti-myc antibodies. 2% of starting material (S.M.) was loaded in the first lane. (B) Schematic representation of mouse full-length CdGAP (a.a. 1-1425) and CdGAP deletion mutants. +: polybasic residue cluster, GAP: GTPase-activating Protein, BR: basic-rich, PRD: proline-rich domain.

Figure 2




```
247-LVSLEEAQARSLATNHPARKERRENSLPEIVPPPFHTVLELPDNKRKLSSKSKKWKSIFNLGRSGSDSKSKLSRNGSVFVRGQRLSVEKATIR-339
 8  ------------------------------------VLELPDNKRKLSSKS
 9  -----------------------------------------DNKRKLSSKSKKWKS
 10 ----------------------------------------------LSSKSKKWKSIFNLG
 11 --------------------------------------------------KKWKSIFNLGRSGSD
 12 ------------------------------------------------------IFNLGRSGSDSKSKL
 13 ---------------------------------------------------------RSGSDSKSKLSRNGS
 14 ------------------------------------------------------------SKSKLSRNGSVFVRG
 15 ---------------------------------------------------------------SRNGSVFVRGQRLSV
```

Figure 2.2 – Peptide overlay assay with GST-SH3D.

The C-terminus of twenty 15-mer overlapping peptides corresponding to amino acids 249-358 of CdGAP were covalently bound to a cellulose membrane as per manufacturer. The membrane was incubated with GST or GST-SH3D proteins and bound proteins were revealed using an anti-GST-HRP antibody. Protein sequence alignment of peptides 8 to 15 is shown. Two distinct SKSK motifs are highlighted in bold.

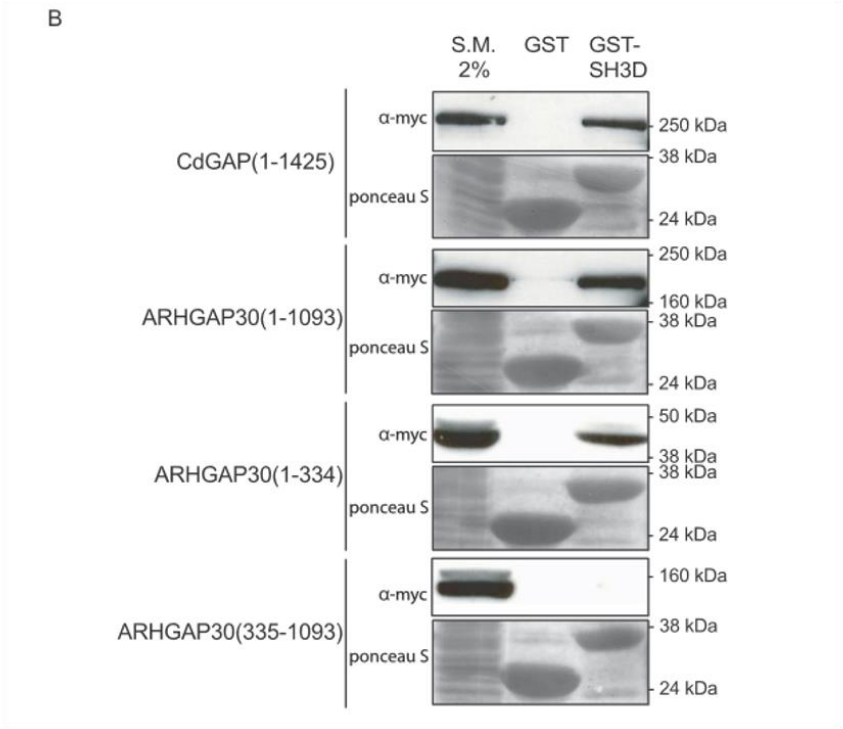
Figure 3

A

```
Mm CdGAP      254- QARSLATNHPARKERRENS----LPEIV----PPPFHTVLELPDNKRK--LSSKSKK-----WKSIFNLG-308
Hs CdGAP      254- QARSLATNHPARKERRENS----LPEIV-PPMGTLFHTVLELPDNKRK--LSSKSKK-----WKSIFNLG-311
Hs ARHGAP30   245- MPRPLPYHLPSILQAGDG-----PPQMR------PYHTIIEIAEHKRK--GSLKVRK-----WRSIFNLG-296
Hs ARHGAP32   604- QARTQAQVNSPIVTENKYIEVGEGPAALQG----KFHTIIEFPLERKR--PQNKMKKSPVGSWRSFFNLG-667
Hs ARHGAP33   549- QARTQGRLGTPTEPTTPK-----APASP--------------AERRKGERGEKQRKPGGSSWKTFFALG-598

Mm CdGAP      309- RSGSDSKSK-LSRNGSVF------VRGQRLSVE---KATIRPAKSMDSLCS-VPVEGKENK -358
Hs CdGAP      312- RSGSDSKSK-LSRNGSVF------VRGQRLSVE---KATIRPAKSMDSLCS-VPVEGKETK -361
Hs ARHGAP30   297- RSGHETKRK-LPR----------GAEDREDKSN---KGTLRPAKSMDSLSA-AAGASDEPE -343
Hs ARHGAP32   668- KSSSVSKRK-LQRN----ESEPSEMKAMALKGGRAE-GTLRSAKSEESLTSLHAVDGDSKL -722
Hs ARHGAP33   599- RGPSVPRKKPLPWLG-------GTRAPPQPSGSRPDTVTLRSAKSEESLSS---QASGAGL -649
```

B



C

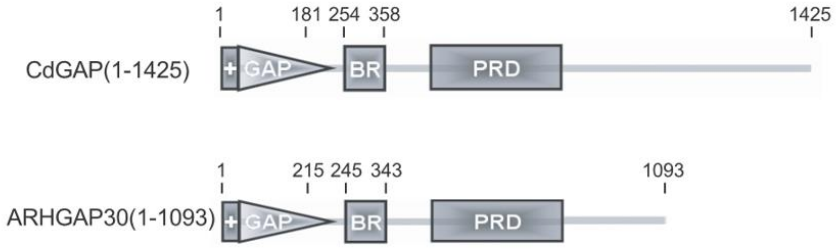


Figure 2.3 – Intersectin-SH3D interacts with the CdGAP-related protein ARHGAP30.

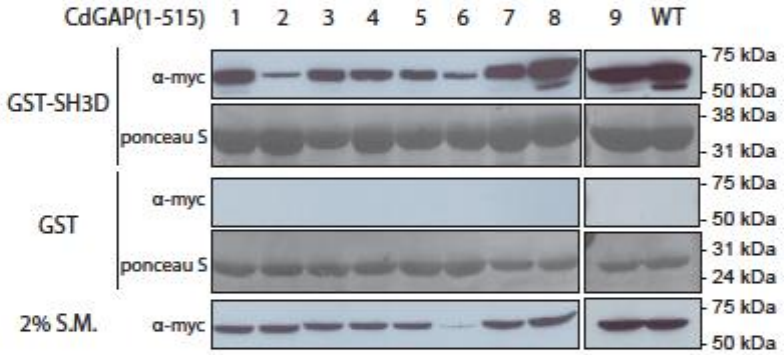
(A) Amino acid alignment of the BR region of mouse and human CdGAP with protein homologs. Conserved amino acids are highlighted in bold. (B) Myc-tagged CdGAP, ARHGAP30, and ARHGAP30 deletion mutants were transiently expressed in HEK293 cells and protein lysates were incubated with GST or GST-SH3D proteins coupled with glutathione-agarose beads. GST pull down proteins were revealed by western blotting using anti-myc antibodies. 2% of starting material (s.m.) was loaded as a control. (C) Schematic representation of mouse full-length CdGAP (a.a. 1-1425) and ARHGAP30. +: polybasic residue cluster, GAP: GTPase-activating Protein, BR: basic-rich, PRD: proline-rich domain.

Figure 4

A


CdGAP
ARHGAP30
1. CdGAP (K291A, R292A, K293A)
2. CdGAP (K297A, K299A, K300A)
3. CdGAP (W301A)
4. CdGAP (K302A, S303A)
5. CdGAP (I304A, F305A, L307A)
6. CdGAP (R309A)
7. CdGAP (D313A, S314A, K315A, S316A, K317A, L318A)
8. CdGAP (K335A, T337A, I338A, R339A)
9. CdGAP (K342A, S343A, D345A, S346A, L347A)

B


CdGAP(1-515) 1 2 3 4 5 6 7 8 9 WT
GST-SH3D
α-myc
ponceau S
GST
α-myc
ponceau S
2% S.M.
α-myc
75 kDa
50 kDa
38 kDa
31 kDa
75 kDa
50 kDa
31 kDa
24 kDa
75 kDa
50 kDa

C

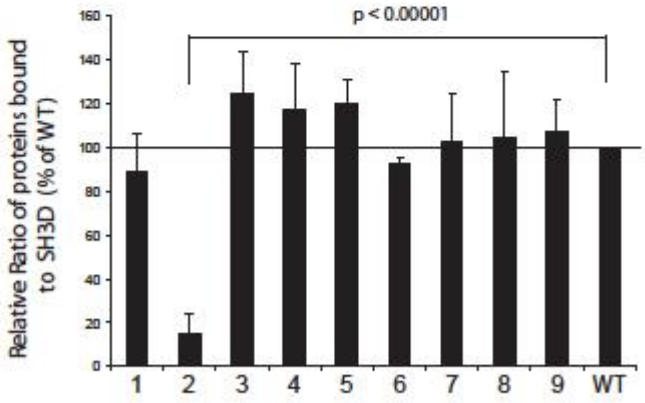

p < 0.00001
Relative Ratio of proteins bound to SH3D (% of WT)
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 WT

Figure 2.4 – The basic residues in the first SKSK motif are required for the interaction between CdGAP and Intersectin-SH3D.

(A) Amino acid sequence alignment of the BR domains of CdGAP and ARHGAP30. CdGAP point mutants have been generated by alanine scanning mutagenesis based on the conserved residues between CdGAP and ARHGAP30. (B) Myc-tagged wild-type (WT) CdGAP (1-515) and CdGAP point mutants 1-9 expressed in HEK293 cells were incubated with GST or GST-SH3D proteins coupled to glutathione-agarose beads. Proteins specifically bound to the beads were revealed by western blotting using an anti-myc antibody. 2% of starting material (S.M.) was loaded as a control. (C) Quantitative analysis of blots as in (B), showing the relative ratio of CdGAP point mutants bound to GST-SH3D. A ratio of bound to total proteins was calculated for each myc-tagged protein and is represented relative to wild-type (WT) CdGAP (1-515). Error bars represent standard errors of the mean for at least five independent experiments. P value was determined by unpaired student's *t* test.

Figure 5

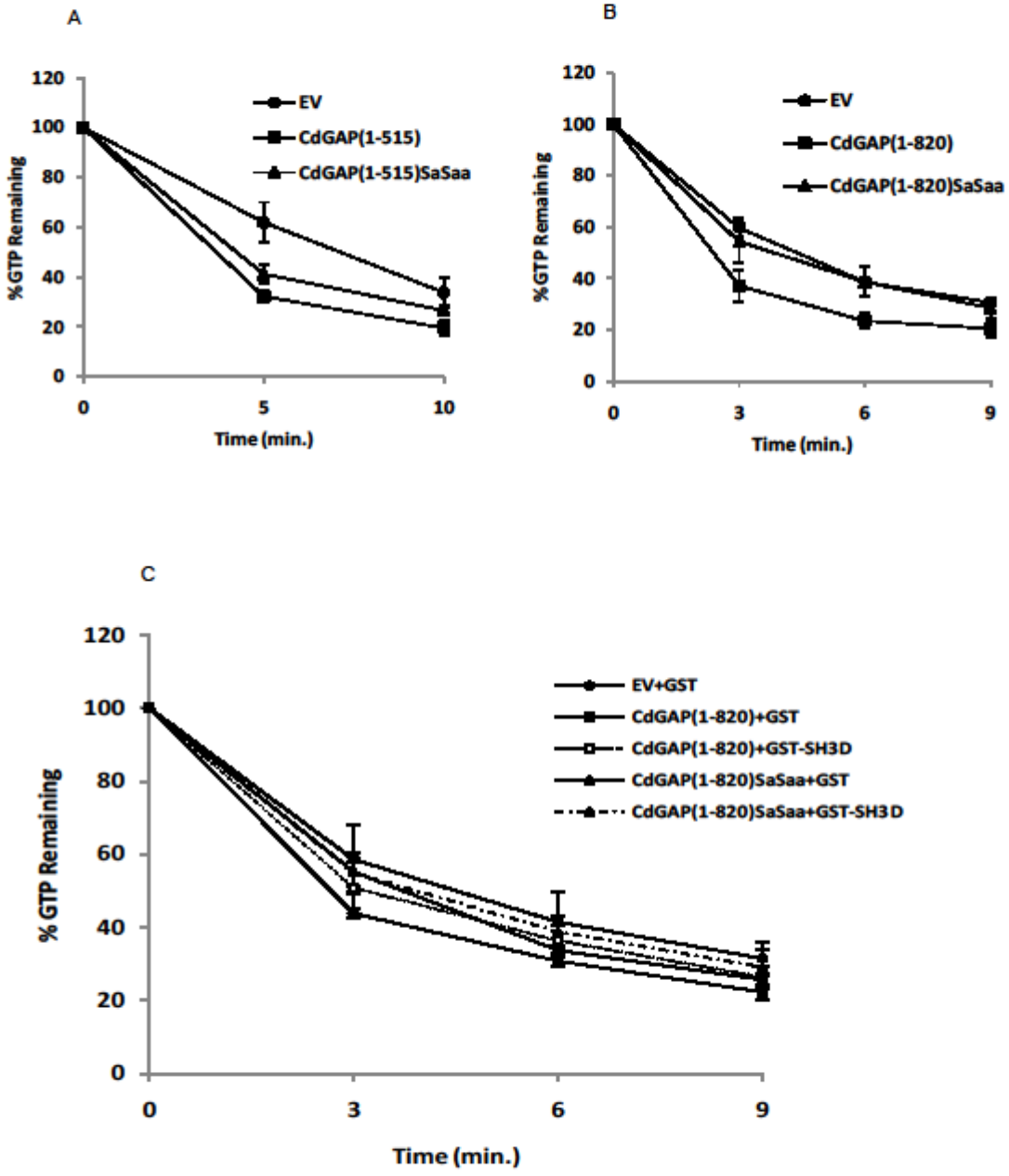

A
120
100
80
60
40
20
0
% GTP Remaining
EV
CdGAP(1-515)
CdGAP(1-515)SaSaa
0
5
10
Time (min.)
B
120
100
80
60
40
20
0
% GTP Remaining
EV
CdGAP(1-820)
CdGAP(1-820)SaSaa
0
3
6
9
Time (min.)
C
120
100
80
60
40
20
0
% GTP Remaining
EV+GST
CdGAP(1-820)+GST
CdGAP(1-820)+GST-SH3D
CdGAP(1-820)SaSaa+GST
CdGAP(1-820)SaSaa+GST-SH3D
0
3
6
9
Time (min.)

Figure 2.5 – The SKSKK motif is critical for the GAP activity of CdGAP in vitro.

[γ-^{32}P]-GTP loaded Rac1 was incubated with myc-tagged immunoprecipitated CdGAP proteins (A) or with GFP-tagged immunoprecipitated CdGAP proteins (B and C) and a GAP assay was performed. (C) The *in vitro* GAP assay was performed in the presence of 10um GST or GST-SH3D. Error bars represent standard errors of the mean for at least three independent experiments.

Preface to Chapter 3

CdGAP is highly phosphorylated in its BR region, PBR and CT regions in response to growth factor stimulation, and is predicted to be a phosphorylation target of many protein kinases. In addition, numerous phosphoproteomic studies identified several potential phosphorylation sites on CdGAP in different cellular environments, suggesting regulatory roles of the phosphorylation. Despite these data, the function of CdGAP phosphorylation remains unknown and this led us to investigate CdGAP phosphorylation in the context of growth factor stimulation. To do so, we used cellular models and different biochemical and imaging techniques to map, identify and characterize the role of potential CdGAP phospho-residues. Using site-directed mutagenesis we were able to identify two conserved phospho-serines in the CT tail, Ser-1093 and Ser-1163, that are RSK1 (AGC kinase)-phosphorylated residues. Furthermore, we go on to identify a novel function of CdGAP phosphorylation involving the recruitment of adaptor 14-3-3 family members leading to the regulation of CdGAP activity and subcellular localization.

Chapter 3: CdGAP/ARHGAP31 is regulated by RSK phosphorylation and binding to 14-3-3β adaptor protein

Ali Ben Djoudi Ouadda, Viviane Calabrese, Yi He, Hidetaka Ishii, Rony Chidiac, Jean-Philippe Gratton, Philippe P. Roux and Nathalie Lamarche-Vane. (2016). Submitted to **Molecular Biology of the Cell**

ABSTRACT

Cdc42 GTPase-activating protein (CdGAP, also named ARHGAP31) is a negative regulator of the small GTPases Rac1 and Cdc42. Associated with the rare developmental disorder Adams-Oliver Syndrome (AOS), CdGAP is critical for embryonic vascular development and for VEGF-mediated angiogenesis. Moreover, CdGAP is an essential component in the synergistic interaction between the TGFβ and Neu/ErbB-2 signaling pathways during breast cancer cell migration and invasion. CdGAP is highly phosphorylated on serine and threonine residues in response to growth factors and is a substrate of ERK and GSK-3. Here, we identified two residues, Ser1093 and Ser1163, in the C-terminal region of CdGAP, which are phosphorylated by RSK (p90 ribosomal protein S6 kinase) in response to phorbol ester (PMA). These phospho-residues create docking sites for binding to 14-3-3 adaptor proteins. The interaction between CdGAP and 14-3-3 proteins impairs the GAP activity of CdGAP and sequesters CdGAP into the cytoplasm. Consequently, the nucleocytoplasmic shuttling of CdGAP is inhibited and CdGAP-induced cell rounding is abolished. Furthermore, we show that 14-3-3□ is unable to regulate the activity and subcellular localization of the AOS-related mutant proteins lacking these phospho-residues. Altogether, we provide a novel mechanism of regulation of CdGAP activity and subcellular localization, which impacts directly on a better understanding of the role of CdGAP in the molecular causes of AOS.

INTRODUCTION

The Rho family of small GTPases holds central functions in cell proliferation, migration, and adhesion (426,427). Alterations in *Rho* genes are linked to many human cancers and indicate a role in tumor invasion and metastasis (46,428,429). Rho proteins act as molecular switches by cycling between an active GTP- and an inactive GDP-bound state. This GDP/GTP exchange is regulated by guanine nucleotide exchange factors (GEFs), which induce the replacement of GDP by GTP, guanine nucleotide dissociation inhibitors (GDIs) binding and sequestering the GDP-bound form of the GTPase in the cytoplasm, and finally GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity, leading to deactivation of the GTPase (6,48,95).

Cdc42 GTPase-activating protein (CdGAP, also known as ARHGAP31), a member of the large family of RhoGAPs, negatively regulates the activity of Rac1 and Cdc42, but not RhoA (323,337). Recently, the loss of CdGAP in mice unveiled the importance of CdGAP in embryonic vascular development (339). CdGAP has also been shown to control directional membrane protrusions of migrating osteosarcoma cells (341,356,357). In addition, CdGAP mediates transforming growth factor (TGFβ)- and ErbB2-induced cell motility and invasion of breast cancer cells in a GAP-independent manner (128). Of interest, a quantitative RNA profile analysis of Rho GTPases and their regulators in ErbB2-induced mouse breast tumors revealed Rac1 and CdGAP as the major GTPase and RhoGAP expressed in these tumors, respectively (364). CdGAP is a large protein, comprising several regulatory domains, each of them being associated with a specific function. Notably, CdGAP consists of an N-terminal GAP domain preceded by a stretch of polybasic residues (PBR) binding to phosphatidylinositol 3,4,5-trisphosphate (PI (3,4,5) P3) that regulates its GAP activity by targeting the protein at the plasma membrane (36). The N-terminal GAP domain is followed by a basic-rich (BR) central region, a proline-rich domain (PRD) with an extended C-terminal region. The BR region interacts through an atypical basic-rich motif with the SH3D domain of

the endocytic scaffolding protein intersectin leading to inhibition of CdGAP activity (342,343) while the PRD is responsible for the ability of CdGAP to facilitate TGFβ-mediated cell motility and invasion of breast cancer cells (128). Furthermore, truncating mutations in the terminal exon of the *CdGAP* gene have been identified in patients with a rare developmental disorder, the Adams-Oliver Syndrome (AOS), characterized by the combination of aplasia cutis congenita (ACC) and limb defects (340,360,430). These mutations result in the removal of the C-terminal region and part of the PRD of CdGAP, which increase the GAP activity of the truncated proteins through a gain-of-function mechanism (340). CdGAP is highly phosphorylated on serine and threonine residues in response to growth factors and is a substrate of extracellular signal-regulated kinase (ERK)/GSK-3. Indeed, phosphorylation of T776 in the PRD by ERK1/2 and GSK-3 negatively regulates the GAP activity of CdGAP (108,109). CdGAP was also found to interact with members of the mitogen-activated protein kinase (MAPK) signaling pathway, ERK1/2 and p90 ribosomal protein S6 kinase (RSK) (109). Mutations of key residues in the ERK docking site impair ERK binding and phosphorylation of CdGAP (109). Here we report the identification of two important serine residues S1093 and S1163 phosphorylated by RSK, which creates 14-3-3 docking sites in the C-terminal region of CdGAP. We show that 14-3-3β interacts with CdGAP through these phosphoserines and sequesters the protein into the cytoplasm, which inhibits the nucleocytoplasmic shuttling of CdGAP, cell rounding, and its GAP activity towards Rac1. In this way, we highlight a novel mechanism of regulation of CdGAP and we provide mechanistic insights into understanding the function of the C-terminal region of CdGAP, lacking in the truncated mutants expressed in AOS patients.

RESULTS

CdGAP is a substrate of the AGC family kinases in response to growth factors and mitogens

To determine if AGC family kinases, such as Akt and RSK (431,432), phosphorylate CdGAP in response to agonists of the Ras/MAPK pathway, we used the phosphorylation site-specific antibody recognizing the consensus motif Arg/Lys-X-Arg/Lys-X-X-pSer/Thr (RXRXXpS/T) found in substrates of the AGC family kinases (431,432). COS-7 fibroblast cells transfected with GFP-tagged CdGAP were serum-starved overnight before stimulation with TGFβ, serum, or phorbol ester (phorbol-12-myristate-13-acetate, PMA) for 5 to 30 minutes before harvesting. Immunoprecipitated CdGAP was then examined for phosphorylation by immunoblotting using the anti-RXRXXpS/T antibodies. CdGAP phosphorylation at the basic consensus motif was increased in response to the agonists and peaked at 30 minutes after stimulation (Fig. 3.1 A-F).

We next investigated whether endogenous CdGAP is phosphorylated by AGC kinases in response to TGFβ stimulation of mammary epithelial cells. We found that CdGAP phosphorylation at the basic consensus motif was significantly increased after 30 minutes of TGFβ stimulation (Fig. 3.1G and H). CdGAP phosphorylation was concomitant with the phosphorylation of RSK (T359/S363) and Smad2/3 (Fig. 3.1I and J), suggesting that activation of RSK by the canonical TGFβ pathway may be responsible of CdGAP phosphorylation on basic consensus motifs. Altogether, these data demonstrate that CdGAP is a substrate of the AGC family kinases, which likely implicate RSK activation in response to growth factor and mitogen stimulation.

Identification of S1093 and S1163 as RSK-dependent phosphorylation sites

Because we have previously shown that RSK interacts and directly phosphorylates CdGAP *in vitro* (109), we next sought to determine whether RSK phosphorylates CdGAP at the basic consensus sites. COS-7 cells were co-transfected with GFP-CdGAP and wild-type (WT) RSK1, kinase-deficient (KD) RSK1, or

constitutively active (CA) RSK1. RSK1-CA induced a robust CdGAP phosphorylation at the basic consensus motif compared to RSK1-WT (Fig. 3.2A and B). RSK1-KD, which retains some ability to autophosphorylate (433), did not significantly increase CdGAP phosphorylation, relative to the amount of total immunoprecipitated CdGAP (Fig. 3.2A and B). Additionally, we used an RSK inhibitor (BI-D1870) that selectively blocks RSK activity (434). Treatment of cells with BI-D1870 prior to PMA stimulation significantly reduced CdGAP phosphorylation at the basic consensus site (Fig. 3.2C and D). Taken together, these results demonstrate that RSK induces CdGAP phosphorylation at basic consensus sites in response to PMA.

To identify CdGAP residues phosphorylated by RSK, we analyzed the basic consensus residues surrounding all Ser/Thr amino acids in the CdGAP protein sequence, with at least one Arg residue in the -3 position. According to the RSK consensus phosphorylation sequence determined using an oriented peptide library (435), we identified 8 potential phosphorylation sites (Table 1). Based on the number of phosphoproteomic studies reporting these potential CdGAP phosphorylation sites (http://www.phosphosite.org/), we chose to replace each of these 4 residues Ser272, Ser765, Ser1093, and Ser1163 to an alanine residue. We found that the mutation of Ser272 and Ser765 located in the BR and PRD, respectively, did not significantly affect CdGAP phosphorylation compared to wild-type CdGAP in response to PMA treatment of COS-7 cells using anti-RXRXXpSer/Thr antibodies (Fig. 3.2E). However, the phosphorylation of CdGAP-S1093A and –S1163A was significantly reduced compared to wild-type CdGAP in response to PMA treatment whereas the phosphorylation of the double mutant CdGAP-S1093A/S1163A was almost completely absent (Fig. 3.2C and D). Furthermore, phosphorylation of the double mutant CdGAP-S1093A/S1163A on the basic consensus motif was completely inhibited in the presence of wild-type or constitutively active RSK1 compared to wild-type CdGAP (Fig. 3.2A and B). Treatment of the cells with the RSK inhibitor BI-D1870 prior to PMA completely inhibited the phosphorylation of the single mutants and the double mutant CdGAP-S1093A/S1163A (Fig. 3.2C and D). Collectively, these data demonstrate that Ser1093 and Ser1163

located in the C-terminal region of CdGAP are major phosphorylation sites targeted by RSK in response to PMA stimulation.

14-3-3 adaptor proteins isoforms β and σ interact with CdGAP

Because the minimum RSK consensus motif RXXpSer/Thr overlaps with the 14-3-3 mode 1 binding site RXXpSer/ThrXP (373,374,392), we next investigated whether CdGAP interacts with 14-3-3 proteins. Myc-tagged CdGAP was expressed in HEK293 cells and subjected to a GST-14-3-3 pull-down. Consistent with a phospho-dependent interaction, CdGAP interacted with wild-type 14-3-3**ε** but not with the mutant 14-3-3**ε**K49E, which shows reduced binding to phosphorylated substrates (374) (Fig. 3.3A). Then, we examined which 14-3-3 isoforms interact with CdGAP in co-immunoprecipitation assays. Myc-tagged CdGAP was immunoprecipitated from HEK293 cells expressing Myc-CdGAP together with the HA-tagged 14-3-3 isoforms. CdGAP interacted specifically with 14-3-3β and σ isoforms (Fig. 3.3B). Similarly, immunoprecipitated HA-tagged 14-3-3 isoforms showed an interaction between 14-3-3β or σ with CdGAP (Fig. 3.3C). To delineate the regions of CdGAP permitting the association between CdGAP and 14-3-3, GFP-CdGAP deletion mutants (Fig. 3.4A) were expressed in COS-7 cells and the interaction with 14-3-3β was assessed by immunoprecipitation in unstimulated or PMA-treated cells (Fig. 3.4B). In this assay, the N-terminal PBR-GAP fragment (1-221), the BR (181-515) and C-terminal domains (1083-1425) but not the PRD (516-820) interacted with 14-3-3β (Fig. 3.4B). However, we did not observe an increased interaction in PMA-stimulated cells (Fig. 3.4B). We next examined the interaction between 14-3-3β and CdGAP phospho-mutants co-expressed in COS-7 cells unstimulated or treated with PMA. CdGAP-S272A was still able to interact with 14-3-3β (Fig. 3.4C), suggesting that this basic consensus motif was not responsible for the binding of CdGAP-BR to 14-3-3β. Conversely, the interaction between CdGAP double mutant S1093A/S1163A and 14-3-3β was greatly reduced compared to wild-type CdGAP or to each single CdGAP mutant in unstimulated and PMA-treated cells (Fig. 3.4D and E). Therefore, these results show that the RSK-

dependent phosphorylation residues S1093 and S1163 in the C-terminal region of CdGAP create 14-3-3 binding sites.

14-3-3β regulates CdGAP subcellular localization and inhibits CdGAP-mediated cell rounding

We have previously demonstrated that the expression of CdGAP in various cell types induces cell rounding in a GAP-dependent manner (36,288,340,341). Thus, we assessed whether 14-3-3β regulates the ability of CdGAP to induce cell rounding and its subcellular localization by microscopy. Wild-type CdGAP was expressed alone or with 14-3-3β into fibroblast cells and the percentage of cells showing a rounded phenotype was determined (Fig. 3.5). As previously reported, CdGAP-WT induced cell rounding in 45% of transfected cells compared to 20% of control GFP-transfected cells (Fig. 3.5A and B). Co-expression of 14-3-3β and CdGAP-WT abolished the ability of CdGAP to induce cell rounding, showing a flat and elongated phenotype (Fig. 3.5A and B). In addition, we determined that CdGAP showed a nuclear localization in 35% of transfected cells (Fig. 3.5A and C) whereas the expression of 14-3-3β with CdGAP significantly reduced the nuclear localization of CdGAP to 15% of transfected cells (Fig. 3.5A and C). We assessed the degree of co-association of 14-3-3β and CdGAP in the cytoplasm by calculation of the mean Pearson's correlation coefficient between 14-3-3β and CdGAP. By this mean, we observed a significant colocalization of CdGAP with 14-3-3β in the cytoplasm (r=0.55+/-0.06) (Fig. 3.5D). We next examine the effect of 14-3-3β on the localization of the double mutant CdGAP-S1093A/S1163A and its ability to induce cell rounding. Similar to wild-type CdGAP, the mutant CdGAP-S1093A/S1163A induced cell rounding in 45% of transfected cells (Fig. 3.5A and B). However, the expression of 14-3-3β with the double mutant did not affect its ability to induce cell rounding (Fig. 3.5A and B). Furthermore, 14-3-3β did not affect the percentage of transfected cells with CdGAP-S1093A/S1163A nuclear localization (Fig. 3.5A and C) and consequently, 14-3-3β did not colocalize with CdGAP-S1093A/S1163A (r=0.10+/-0.02) (Fig. 3.5D). Together, these data show that 14-3-3β sequesters CdGAP into the

cytoplasm and inhibits its ability to induce cell rounding, which is dependent on the RSK-dependent phosphorylation sites Ser1093 and Ser1163.

14-3-3β docking sites negatively regulate the GAP activity of CdGAP towards Rac1

We next sought to determine whether 14-3-3β directly modulates CdGAP activity towards Rac1. To this end, we performed pull-down assays with the Cdc42/Rac1 interactive binding (CRIB) domain of PAK fused to GST to assess the levels of active GTP-Rac1 in HEK293 cell extracts. As expected, overexpression of CdGAP led to a significant reduction in GTP-Rac1 levels ($p< 0.05$), whereas 14-3-3β had no significant effect on GTP-Rac1 levels alone ($P= 0.45$) (Fig. 3.6A and B). However, the expression of 14-3-3β with CdGAP resulted in the inhibition of CdGAP activity with higher levels of GTP-Rac1 ($P< 0.05$) (Fig. 3.6A and B). To examine whether the 14-3-3β docking sites in the C-terminal region of CdGAP regulate CdGAP activity, we determined the GAP activity of the double mutant CdGAP-S1093A/S1163A alone or with 14-3-3β. The expression of CdGAP-S1093A/S1163A resulted in a significant higher GAP activity with lower levels of GTP-Rac1 ($P <0.01$), whereas co-expression with 14-3-3β did not significantly modulate its GAP activity (Fig. 3.6A and B). Together with the previous findings that 14-3-3β abolished CdGAP-mediated cell rounding (Fig. 3.5A and B), these results support the hypothesis that 14-3-3β inhibits the GAP activity of CdGAP towards Rac1 through interaction with the RSK-dependent phosphorylation sites Ser1093 and Ser1163.

The AOS-related CdGAP mutant proteins show a reduced interaction with 14-3-3β

Because the AOS-related CdGAP-Q683X and –K1087Sx4 mutants are lacking the C-terminal region and show an increased GAP activity (340), we next determined the interaction between 14-3-3β and the AOS-related CdGAP mutant proteins in co-immunoprecipitation assays (Fig. 3.7A). Myc-tagged human CdGAP (hCdGAP) wild-type, CdGAP-Q683X or –K1087Sx4 were immunoprecipitated from COS-7 cells expressing HA-tagged 14-3-3β. Both AOS-related CdGAP mutants showed a reduced, but not completely abolished, interaction with 14-3-3β compared to the wild-type

hCdGAP (Fig. 3.7A). Consistent with the previous findings that 14-3-3β can also interact with the N-terminal GAP and BR of mouse CdGAP (Fig. 3.4B), these data suggest that 14-3-3β association with human CdGAP may be in part mediated by the N-terminus and BR, though the C-terminal region of human CdGAP is important to mediate an efficient interaction with 14-3-3β, which is lacking in the AOS-related mutants.

14-3-3β is unable to modulate AOS-related CdGAP mutant localization and activity

We next explored the role of 14-3-3β on the subcellular localization and the ability of the AOS-related mutants to induce cell rounding. As previously reported (340), human CdGAP induced cell rounding in 40% of transfected cells, whereas the AOS-related mutants showed a significant increased rounded phenotype with 55% and 60% of transfected cells for CdGAP-Q683X and –K1087Sx4 expression, respectively (Fig. 3.7B and C). In addition, human CdGAP-WT and AOS-related mutants showed a similar nuclear localization in 38% to 42% of transfected cells (Fig. 3.7B and D). Co-expression of 14-3-3β inhibited the ability of hCdGAP-WT to induce cell rounding, whereas it has a small but significant capability of reducing the ability of AOS-related mutant proteins to induce cell rounding (Fig. 3.7B and C). Furthermore, 14-3-3β decreased the percentage of cells with nuclear hCdGAP-WT localization to 15%, whereas it has no effect on the localization of the AOS-related mutants (Fig. 3.7B and D). Consequently, we did not find a significant co-localization between the AOS-related mutants and 14-3-3β by assessing the degree of co-association with the Pearson's correlation coefficient, whereas hCdGAP-WT co-localized with 14-3-3β with an r=0.43+/-0.03 (Fig. 3.7E). Collectively, these results demonstrate that the negative regulation of CdGAP activity by 14-3-3β is impaired for the AOS-related protein mutants, which correlates with its increased GAP activity (340) and ability to induce cell rounding.

DISCUSSION

In this report, we provide evidence for a novel mechanism of regulation of CdGAP activity and subcellular localization by RSK-dependent phosphorylation and 14-3-3 interaction. We demonstrate that CdGAP is phosphorylated at the C-terminal region on Ser1093 and Ser1163 residues by RSK, which creates docking sites for 14-3-3 binding. We show that 14-3-3 binding to these phosphosites sequesters CdGAP in the cytoplasm to inhibit the GAP activity of CdGAP towards Rac1 (Fig. 3.8). Previous studies have identified CdGAP as a molecular target of the Ras/MAPK pathway in response to serum and PDGF (108,109,345). Indeed, we identified Thr776 in the proline-rich domain of CdGAP as a major phosphorylation site of ERK1/2, which negatively regulates the GAP activity of CdGAP towards Rac1 (109). In addition, this consensus ERK1 regulatory site is phosphorylated by GSK-3β in serum-starved cellular conditions (108). Here, we report that CdGAP is also connected to the Ras/MAPK pathway via its phosphorylation by RSK, which is a downstream ERK effector involved in the control of cell proliferation, survival, and motility (431,435,436). The Ser1093 and Ser1163 residues located in the C-terminal region of CdGAP appear to be the major phosphorylation sites of RSK, although we cannot rule out the possibility that other residues may be RSK targets as well. According to phosphosite.org, phospho-Ser1163 has been reported in 23 large-scale proteomic studies, including the phosphoproteomic analysis of breast cancer, lung cancer, and the liver (350-353). Moreover, phospho-Ser1093 was reported in 3 large-scale proteomic studies, including the phosphoproteome of the liver (347,348), and more recently, this phosphosite was identified in a comparative phosphoproteomic analysis of VEGF and angiopoietin-1 signaling in endothelial cells (349). Of interest, we have recently shown that CdGAP is a critical regulator of VEGF-mediated signaling in angiogenesis (339). Therefore, phospho-Ser1093 and Ser1163 at the C-terminal tail of CdGAP appear to be regulated by various agonists in different cellular contexts.

We show that the RSK-dependent phospho-Ser1093 and –Ser1163 residues create docking sites for binding to 14-3-3β and σ adaptor proteins. The 14-3-3 family consists of 7 isoforms (β, γ, ε, σ, ζ, τ, η) sharing a high degree of homology amongst vertebrates (398,401,437). They are crucial regulators of several intracellular signaling pathways. They form homo- and heterodimers that interact with a variety of target proteins containing the consensus motif, RSXpS/pTXP, thereby affecting their activity, subcellular localization, and protein stability (371-374,438). Consequently, dysregulation of 14-3-3 proteins is often associated with tumorigenesis with a 14-3-3 isoform expression signature emerging in many types of cancer (398,402). Because of the requirement of an Arg residue at the -3 position, 14-3-3 binding sites are often regulated by Ser/Thr basophilic protein kinases of the AGC family (435). A recent phosphoproteomic analysis of the 14-3-3 interactome in melanoma cells has indeed identified a large number of potential RSK substrates, including CdGAP (435). Here we show that the RSK-dependent phospho-Ser1093 and –Ser1163 are the major binding sites of 14-3-3β, which is supported by a phosphoproteomic study reporting these phospho-residues as 14-3-3 binding sites with excellent scores (439). However, the residual interaction between the CdGAP-S1093A/S1163A mutant and 14-3-3β suggests that other residues may be involved in the interaction. In fact, we found that the N-terminus PBR-GAP domain and the basic region (BR) of CdGAP are also able to bind to 14-3-3β. Although Ser272 in CdGAP-BR does not mediate the interaction with 14-3-3β, we cannot exclude the possibility that additional AGC family kinase-dependent consensus sites within CdGAP-BR as indicated in Table 1 could be involved in the interaction between 14-3-3β and CdGAP. However, no phospho-dependent basic motif can be identified in the PBR-GAP domain, suggesting an interaction through an unphosphorylated residue. Interestingly, a basic motif RSKKIE similar to the unphosphorylated basic $RSx_{1-3}E$-like motif previously reported to interact with 14-3-3 (397) could mediate the interaction between the PBR-GAP domain and 14-3-3 proteins. Altogether, the association between 14-3-3 dimers and CdGAP may be mediated in part by the N- and C-terminus of CdGAP, though both regions may differently cooperate in the context of the full-length protein.

Furthermore, our study demonstrates that the recruitment of 14-3-3 proteins to CdGAP through the phospho-Ser1093 and S1163 residues sequesters CdGAP in the cytoplasm and inhibits CdGAP–induced cell rounding and consequently, its GAP activity towards Rac1. This regulatory mechanism of CdGAP is similar to the regulation of a number of GEFs and GAPs previously identified in global 14-3-3 interaction screens (440-442), including Deleted in liver cancer 1 (DLC1), ARHGAP22, and the RhoGEF AKAP-Lbc. Notably, the RhoGAP DLC1, a tumor suppressor protein inactivating RhoA in many types of cancer (443), is phosphorylated by PKC/PKD protein kinases on Ser residues, which create binding sites for 14-3-3 proteins. This phosphorylation results in the inhibition of the RhoGAP activity and nucleocytoplasmic shuttling of DLC1 (440). Therefore, these studies support a general role for 14-3-3 adaptor proteins in the control of small GTPase regulators, and consequently, cytoskeletal regulation and organization. We have previously shown that PI-3 kinase activation causes the recruitment of CdGAP to the plasma membrane, likely via the binding of PI-3,4,5 to the PBR preceding the GAP domain (36). This lipid interaction is essential for CdGAP activity to induce cell rounding. Therefore, the interaction of 14-3-3 with CdGAP may impede the recruitment of CdGAP to the plasma membrane by inhibiting the interaction between PI-3,4,5 and the N-terminus PBR-GAP domain. We also report here that CdGAP localizes to the nucleus in a proportion (35%) of transfected cells and that co-expression with 14-3-3β significantly reduces CdGAP nuclear localization. In a separate study, we have uncovered a previously unknown nuclear function for CdGAP where it functions in a GAP-independent manner as a critical E-cadherin transcriptional co-repressor with Zeb2 to promote breast tumorigenesis and metastasis to the lungs (127). Thus, 14-3-3 binding to CdGAP may behave as an important negative regulator of CdGAP transcriptional activity by cytosolic sequestration, leading to the inhibition of epithelial-to-mesenchymal transition (EMT), cell motility and invasion of breast cancer cells.

The AOS-related CdGAP-Q683X and –K1087Sx4 mutants have been first identified in autosomal-dominant AOS patients and displayed an increased GAP activity towards Cdc42, highlighting the importance of Rac1/Cdc42 regulation in the developmental processes of scalp and limb formation (340). Moreover, we have showed

that the C-terminus of CdGAP was able to interact with the N-terminal PBR-GAP domain, suggesting a mechanism of regulation of CdGAP activity by the C-terminus (340). The results presented in this study show that the AOS-related CdGAP-Q683X and –K1087Sx4 mutants lacking the C-terminal tail displayed a higher proportion of cell rounding and lost the nucleocytoplasmic regulation by 14-3-3 proteins, which correlate with the reduction of interaction between the mutants and 14-3-3β. Therefore, these novel findings strongly support a mechanism, whereby the binding of 14-3-3 proteins to the C-terminus of CdGAP is necessary to regulate the subcellular localization and GAP activity of CdGAP. We propose that impaired 14-3-3 regulation of CdGAP in AOS patients may cause profound effects during early human development. In addition, we have recently reported that vascular development is impaired in CdGAP-deficient mouse embryos, associated with superficial vessel defects and subcutaneous edema, resulting in 44% perinatal lethality (339). Altogether, these findings unveil the importance of a tight regulation of CdGAP activity in cells and tissues, which otherwise may lead to developmental disorders and cancer.

EXPERIMENTAL PROCEDURES

DNA constructs

pEGFPC1-mCdGAP and deletion mutants, pRK5myc-hCdGAP and AOS-related mutant, pKH3-avRSK1 wild-type (WT), pKH3-avRSK1-K112/464R kinase-deficient (KD), pRK7-Myr-avRSK1 constitutively active (CA), pcDNA-HA-14-3-3 isoforms (β, γ, σ and ζ), GST-14-3-3ε wild-type (WT) and GST-14-3-3ε K49E, pRK5myc- Rac1 constructs have been previously described (36,108,109,323,340,374,433,444,445). CdGAP point mutants were derived from the wild-type form of CdGAP in pEGFPC1 using the QuikChange® Site-Directed Mutagenesis Kit (*Stratagene*), according to the manufacturer's instructions. The following primers were used:
S272A (forward-5' GAAAGACGAGAGAACGCCCTGCCCGAGATCGTC 3'
and reverse-5' GACGATCTCGGGCAGGGCGTTCTCTCGTCTTTC3'),
S765A (forward-5' GGCCCAAGGAATCTCGCTCCCCCTCTTACTCC 3'and reverse-5' GGAGTAAGAGGGGGAGCGAGATTCCTTGGGCC 3'),
S1093A (forward-5' GAAACACAGGCCGTCTGCCCTCAACCTGGACTCTG 3' and reverse-5' CAGAGTCCAGGTTGAGGGCAGACGGCCTGTGTTC 3')
S1163A (forward-5' GACAGGCCGCAGGAATGCGGCTCCTGTAAGTGTG 3' and reverse-5'
CACACTTACAGGAGCCGCATTCCTGCGGCCTGTC 3'). All plasmids were verified by sequencing.

Antibodies

The following antibodies were used: Anti-rabbit IgG (whole molecule), anti-CdGAP (Sigma); anti-myc (clone 9E10), anti-phospho-RSK1 (Thr359/Ser363) (Millipore); rabbit and mouse anti-GFP, anti-rabbit-conjugated Alexa-488, anti-mouse-Cy3 (Molecular Probes); anti-rabbit and anti-mouse-HRP (GE Healthcare); anti-HA, anti-RSK1(Santa Cruz Biotechnology**)**; anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (Erk1/2), anti-phospho-Smad2/3 (Ser465/467), anti-Smad2/3 and anti-RXRXXpS/T [Phospho-(Ser/Thr) Akt Substrate] (Cell Signaling).

Cell culture, transfection, and treatment

NMuMG mammary epithelial cells were grown in DMEM supplemented with 10% Fetal Bovine serum (FBS) (Wisent), 10 mM HEPES, 1 mM sodium pyruvate, 1 mM L-glutamine, 10 µg/ml insulin and antibiotics as previously described (128). HEK293 and COS-7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics in a humidified incubator at 37 °C with 5% CO_2. HEK293 and COS-7 cells were transfected with the indicated constructs using linear polyethylenimine (PEI) (Polysciences) at a 1:8 ratio (cDNA:PEI) following the manufacturer's instructions. Thirty hours post-transfection, cells were serum-starved for 18h and stimulated for 30 min with either 20% FBS, 200 nM PMA (phorbol-12-myristate-13-acetate, Cell Signaling) or 5 ng/ml recombinant Human TGF-β1 (Invitrogen). For PMA and BI-D1870 (Stemgent) treatments, cells were treated for 30min with PMA (200nM) and BI-D1870 (20nM) following a pre-treatment with BI-D1870 (20nM) for 1h.

Immunoprecipitation, western blotting, and quantitative densitometry

NMuMG cells were lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate (Na_3VO_4) , 1 µg/ml aprotinin and leupeptin, and 50 mM sodium fluoride (NaF) (BioShop). COS-7 and HEK293 cells were lysed in lysis buffer containing 25 mM Hepes pH 7.5, 1% Nonidet P-40 (NP-40), 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF and 1 µg/ml aprotinin and leupeptin. Protein lysates were centrifuged at 10,000 × g for 10 min at 4°C to remove insoluble materials. For immunoprecipitation, 2-3mg of protein lysates were incubated with the indicated antibodies for 1h followed by Protein-G-Sepharose beads (GE Healthcare). Beads were washed twice with lysis buffer and heated to 95°C in SDS sample buffer. Protein samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes for Western blotting with the indicated antibodies, and visualized by enhanced chemiluminescence (ECL) (Millipore). Quantitative densitometry was assessed using Image J software (446).

GST-protein purification and pull down assays

GST-fusion proteins (GST, GST-14-3-3ε wild-type and K49E) were produced in BL21 *E. coli* as described previously (435). For pull-down experiments, HEK293 cells transfected or not with Myc-tagged wild-type CdGAP were lysed as described above. Cellular debris were removed by centrifugation for 10 min at 13,000 × g, and the supernatant was divided equally and incubated with 10 µg of GST, GST-14-3-3ε wild-type, or GST-14-3-3ε K49E bound to glutathione beads for 2 hours. The beads were then washed 4 times with lysis buffer prior to elution with reducing sample buffer, SDS-PAGE and immunoblotting.

Rac1 activation assay

Transfected HEK293 cells were lysed in buffer containing 25mM Hepes pH 7.5, 1% NP-40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 1mM Na_3VO_4, 50 mM NaF, 1mM PMSF, 1 µg/ml aprotinin and leupeptin. Protein lysates were centrifuged at 10,000 × g for 10 minutes to remove insoluble materials. Myc-tagged Rac1-GTP was pulled down by incubating 2-3mg of protein lysates for 60 min at 4 °C with 30 µg of purified GST-CRIB (amino acids 73-146 of mouse PAK3 fused to GST) (447) coupled to glutathione-sepharose beads (Sigma). The beads were washed three times with the lysis buffer and then boiled in SDS sample buffer. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting with anti-myc antibodies. The levels of GTP-bound Rac1 were assessed by densitometry using Image J software and normalized to total Rac1 detected in total cell lysates.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (36,128). Briefly, transfected COS-7 cells grown on glass coverslips were fixed in 3.7% formaldehyde (BioShop**)** for 10 min before permeabilization in 0.25% Triton X-100 for 5

min. Cells were then blocked for 30 min with 0.1% BSA and incubated in blocking buffer containing the indicated primary antibodies at 4 °C overnight. After washing twice with PBS, cells were incubated with Alexa Fluor 488 or Cy3-conjugated secondary antibodies (Molecular Probes) with 4', 6'-diamidino-2-phenylindole (DAPI) for nucleus staining. Coverslips were mounted on glass slides using Prolong Gold antifade reagent (Invitrogen). Cells were examined using a laser-scanning Zeiss LSM780 confocal microscope with a Plan-Neofluar 40x/0.60 oil or a Plan-Apochromat. 63x/1.40 oil immersion objective lenses and analyzed with Zen2010 software (Carl Zeiss). Colocalization analysis and Pearson's correlation coefficient (r) were performed using Zen2010 software, analyzing > 30 cells per condition in at least three independent experiments. For quantification of cell rounding and nuclear localization, cells were imaged with a motorized inverted Olympus microscope IX81 using a 60X U PLAN S-APO oil objective lens. Images were recorded with a CoolSnap 4K camera (Photometrics) and analyzed with MetaMorph software (Molecular Devices). At least 100 cells per condition were analyzed in at least three independent experiments.

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.

ACKNOWLEDGMENT

We thank Dr Min Fu and the imaging core facility of the Research Institute of the McGill University Health Centre for assistance with confocal microscopy. This research was supported by the Canadian Institute of Health Research grant MOP-119544 (to N. L.V.) and MOP-123408 (to P.P.R.). ABDO was a recipient of a Fonds de la Recherche en Santé du Québec doctoral studentship.

Figure 1

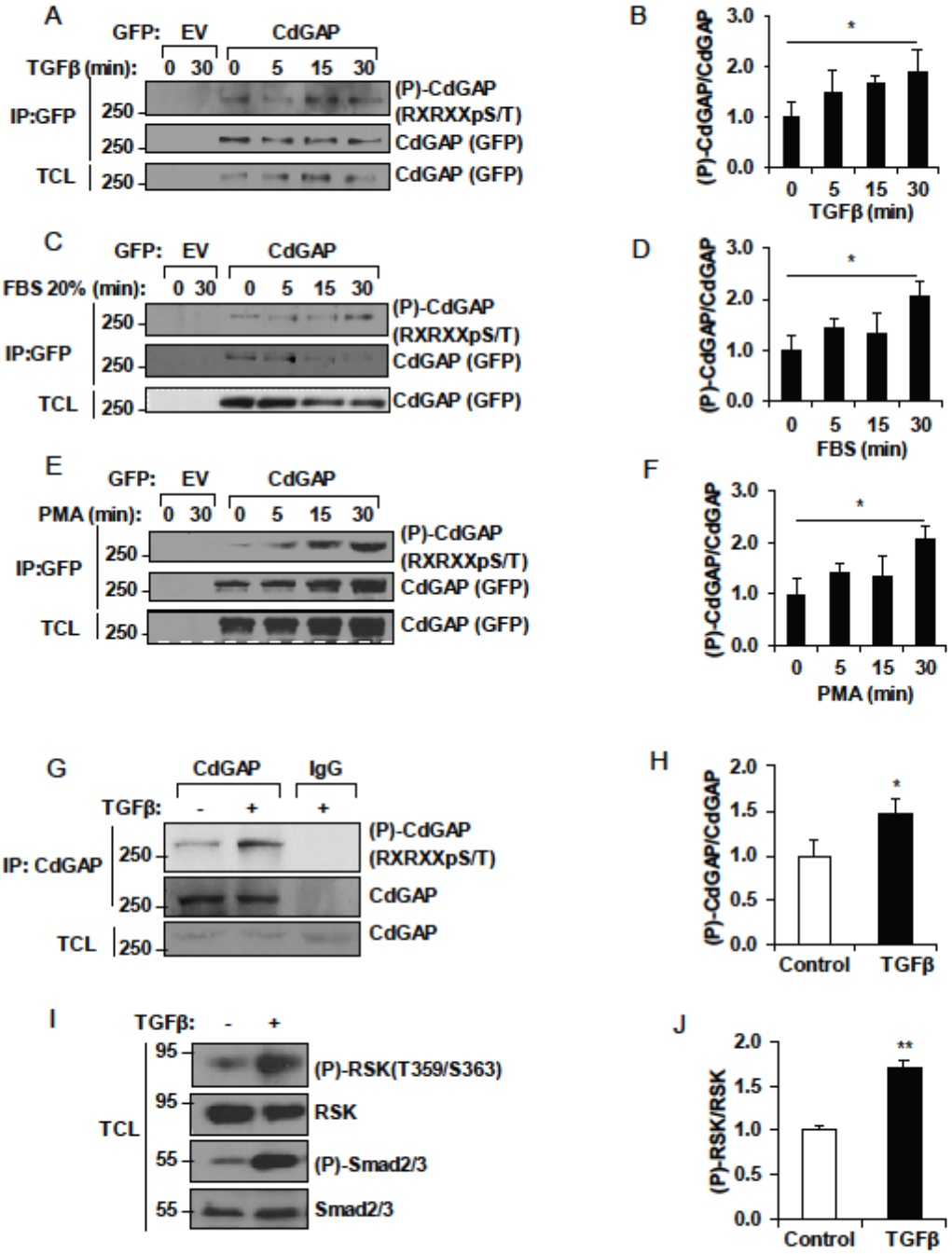

A
GFP: EV CdGAP
TGFβ (min): 0 30 0 5 15 30
IP:GFP
250
(P)-CdGAP (RXRXXpS/T)
250
CdGAP (GFP)
TCL
250
CdGAP (GFP)
B
(P)-CdGAP/CdGAP
3.0
2.0
1.0
0.0
0 5 15 30
TGFβ (min)
C
GFP: EV CdGAP
FBS 20% (min): 0 30 0 5 15 30
IP:GFP
250
(P)-CdGAP (RXRXXpS/T)
250
CdGAP (GFP)
TCL
250
CdGAP (GFP)
D
(P)-CdGAP/CdGAP
3.0
2.0
1.0
0.0
0 5 15 30
FBS (min)
E
GFP: EV CdGAP
PMA (min): 0 30 0 5 15 30
IP:GFP
250
(P)-CdGAP (RXRXXpS/T)
250
CdGAP (GFP)
TCL
250
CdGAP (GFP)
F
(P)-CdGAP/CdGAP
3.0
2.0
1.0
0.0
0 5 15 30
PMA (min)
G
CdGAP IgG
TGFβ: - + +
IP: CdGAP
250
(P)-CdGAP (RXRXXpS/T)
250
CdGAP
TCL
250
CdGAP
H
(P)-CdGAP/CdGAP
2.0
1.5
1.0
0.5
0.0
Control TGFβ
I
TGFβ: - +
TCL
95
(P)-RSK(T359/S363)
95
RSK
55
(P)-Smad2/3
55
Smad2/3
J
(P)-RSK/RSK
2.0
1.5
1.0
0.5
0.0
Control TGFβ

Figure 3.1 – CdGAP is phosphorylated by the AGC family kinases in response to growth factors and mitogens.

COS-7 cells transfected with empty vector (EV) or GFP-CdGAP were stimulated with TGFβ (5 ng/ml) (A, B), FBS 20% (C, D), or PMA (200 nM) (E, F). GFP-CdGAP proteins were immunoprecipitated (IP) from cell lysates and the phosphorylation of CdGAP (P-CdGAP) on the consensus motif RXRXXpS/T was detected by immunoblotting with the indicated antibodies. TCL, total cell lysates. (B, D, F) Densitometric analysis of P-CdGAP/CdGAP ratio is represented as the fold change relative to 0 min of stimulation from A, C, and E. (**p*<0.05, unpaired Student's *t* test). (G) Endogenous CdGAP was IP from lysates of mouse mammary epithelial (NMuMG) cells, stimulated with TGFβ (5 ng/ml) for 30 min with anti-CdGAP antibodies or rabbit IgG as control. IP proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (H) Densitometric analysis of P-CdGAP/CdGAP ratio from G. (**p*<0.05, unpaired Student's *t* test). (I) Total cell lysates (TCL) from NMuMG cells stimulated with TGFβ (5ng/ml) for 30min were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (J) Densitometric analysis of (P)-RSK/RSK from I. (***p*<0.01, unpaired student's *t* test).

Figure 2

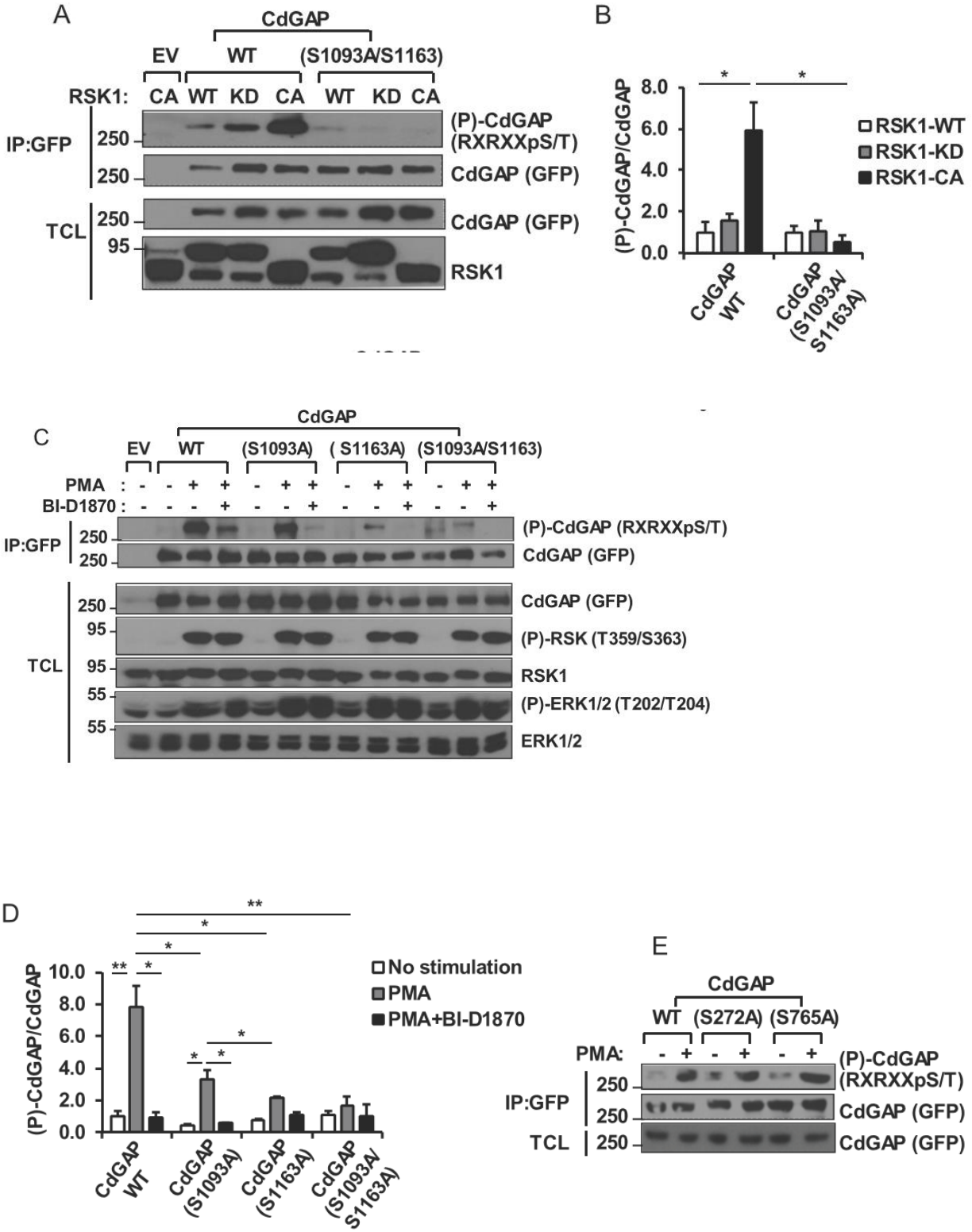

A
CdGAP
EV
WT
(S1093A/S1163)
RSK1: CA WT KD CA WT KD CA
IP:GFP
250
250
(P)-CdGAP (RXRXXpS/T)
CdGAP (GFP)
TCL
250
95
CdGAP (GFP)
RSK1
B
(P)-CdGAP/CdGAP
8.0
6.0
4.0
2.0
0.0
RSK1-WT
RSK1-KD
RSK1-CA
CdGAP WT
CdGAP (S1093A/ S1163A)
C
CdGAP
EV
WT
(S1093A)
(S1163A)
(S1093A/S1163)
PMA : - - + + - + + - + + - + +
BI-D1870 : - - - + - - + - - + - - +
IP:GFP
250
250
(P)-CdGAP (RXRXXpS/T)
CdGAP (GFP)
TCL
250
95
95
55
55
CdGAP (GFP)
(P)-RSK (T359/S363)
RSK1
(P)-ERK1/2 (T202/T204)
ERK1/2
D
(P)-CdGAP/CdGAP
10.0
8.0
6.0
4.0
2.0
0.0
No stimulation
PMA
PMA+BI-D1870
CdGAP WT
CdGAP (S1093A)
CdGAP (S1163A)
CdGAP (S1093A/ S1163A)
E
CdGAP
WT (S272A) (S765A)
PMA: - + - + - +
IP:GFP
250
250
TCL
250
(P)-CdGAP (RXRXXpS/T)
CdGAP (GFP)
CdGAP (GFP)

Figure 3.2 – S1093 and S1163 are RSK-dependent phosphorylation sites.

(A) COS-7 cells were transfected with empty vector (EV), CdGAP, and RSK1 constructs as indicated. GFP-CdGAP proteins were IP from total cell lysates (TCL) and the phosphorylation of CdGAP (P-CdGAP) on the consensus motif RXRXXpS/T was detected by immunoblotting with the indicated antibodies. (B) Densitometric analysis of (P)-CdGAP/CdGAP from A. (*p<0.05, unpaired student's t test). (C) COS-7 cells transfected with empty vector (EV), CdGAP and RSK1-WT constructs were treated with PMA in the presence or absence of the RSK inhibitor BI-D1870 as indicated. GFP-CdGAP proteins were IP from TCL. IP proteins and TCL were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (D) Densitometric analysis of (P)-CdGAP/CdGAP from C. (*p<0.05, **p<0.01, unpaired student's t test). (E) COS-7 cells were transfected with the indicated CdGAP constructs and analyzed as in C.

Figure 3

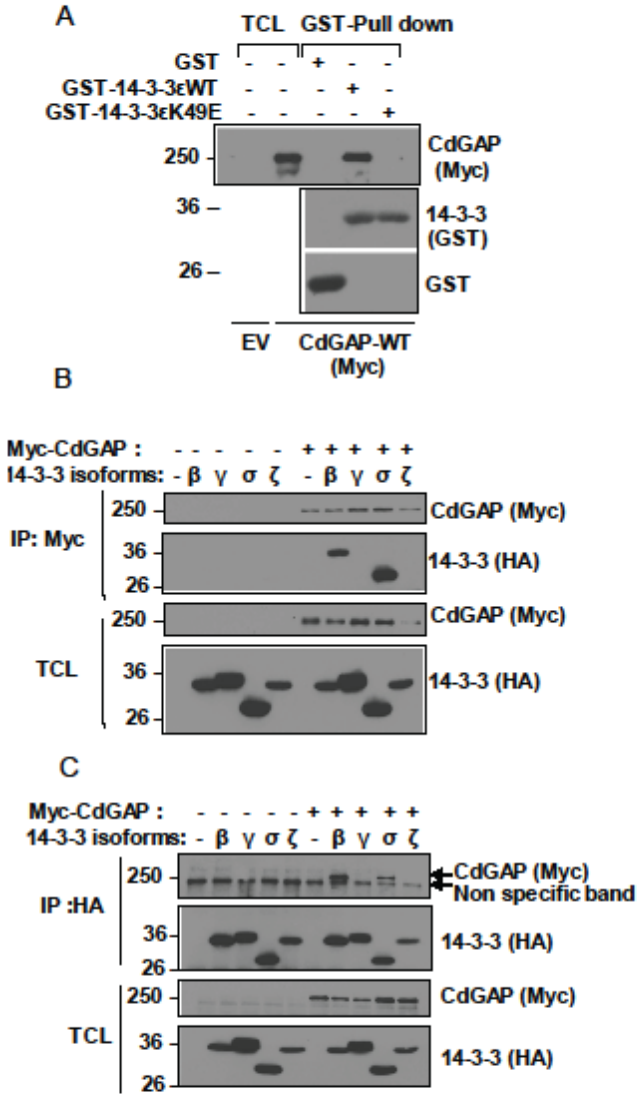

A
TCL
GST-Pull down
GST - - + - -
GST-14-3-3εWT - - - + -
GST-14-3-3εK49E - - - - +
250
36
26
CdGAP (Myc)
14-3-3 (GST)
GST
EV
CdGAP-WT (Myc)
B
Myc-CdGAP : - - - - - + + + + +
14-3-3 isoforms: - β γ σ ζ - β γ σ ζ
IP: Myc
TCL
250
36
26
CdGAP (Myc)
14-3-3 (HA)
C
Myc-CdGAP : - - - - - + + + + +
14-3-3 isoforms: - β γ σ ζ - β γ σ ζ
IP :HA
TCL
CdGAP (Myc)
Non specific band
14-3-3 (HA)

Figure 3.3 – 14-3-3 adaptor proteins isoforms β and σ interact with CdGAP

(A) HEK293 cells were transfected with empty vector (EV) or myc-CdGAP constructs. Proteins from total cell lysates (TCL) were pulled down with GST, GST-14-3-3e wild type (WT) or mutant 14-3-3eK49E proteins. Associated proteins and TCL were resolved by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies. (B and C) HEK293 cells were transfected with myc-CdGAP and HA-14-3-3 isoform constructs as indicated. Myc-CdGAP proteins (B) or HA-14-3-3 (C) proteins were IP from total cell lysates (TCL), resolved by SDS-PAGE and immunoblotted using anti-Myc and anti-HA antibodies.

Figure 4

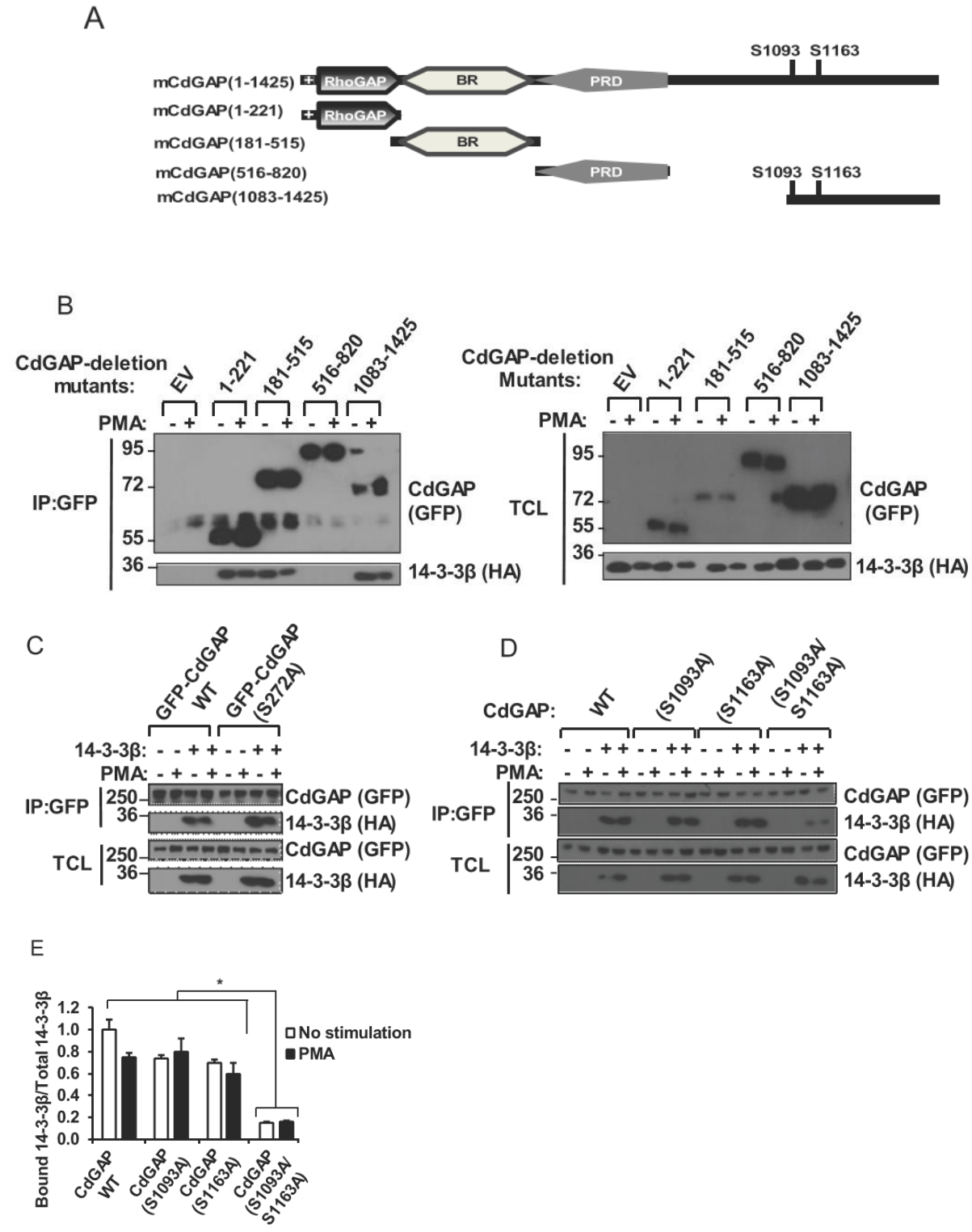

A
S1093 S1163
mCdGAP(1-1425)
RhoGAP
BR
PRD
mCdGAP(1-221)
RhoGAP
mCdGAP(181-515)
BR
mCdGAP(516-820)
PRD
S1093 S1163
mCdGAP(1083-1425)
B
CdGAP-deletion mutants:
EV
1-221
181-515
516-820
1083-1425
PMA:
IP:GFP
95
72
55
36
CdGAP (GFP)
14-3-3β (HA)
CdGAP-deletion Mutants:
TCL
C
GFP-CdGAP WT
GFP-CdGAP (S272A)
14-3-3β:
PMA:
IP:GFP
250
36
CdGAP (GFP)
14-3-3β (HA)
TCL
D
CdGAP:
WT
(S1093A)
(S1163A)
(S1093A/ S1163A)
E
Bound 14-3-3β/Total 14-3-3β
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
No stimulation
PMA
CdGAP WT
CdGAP (S1093A)
CdGAP (S1163A)
CdGAP (S1093A/ S1163A)

Figure 3.4 – The RSK-dependent phosphorylation residues S1093 and S1163 are required for the interaction between CdGAP and 14-3-3β.

(A) Schematic representation of mouse CdGAP (mCdGAP) deletion mutants, including the major RSK target residues. +, stretch of polybasic residues; rhoGAP, GTPase-activating protein; BR, basic region; PRD, proline-rich domain. (B) COS-7 cells were transfected with empty vector (EV) or GFP-CdGAP deletion mutant constructs with HA-14-3-3β and treated with PMA as indicated. GFP-CdGAP proteins were immunoprecipitated (IP) from total cell lysates (TCL). IP proteins and TCL were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (C and D) COS-7 cells were transfected with the indicated CdGAP constructs with HA-14-3-3β and analyzed as in B. (E) Densitometric analysis of bound 14-3-3β to CdGAP/total 14-3-3β from D. (*p<0.05, unpaired student's t test).

Figure 5

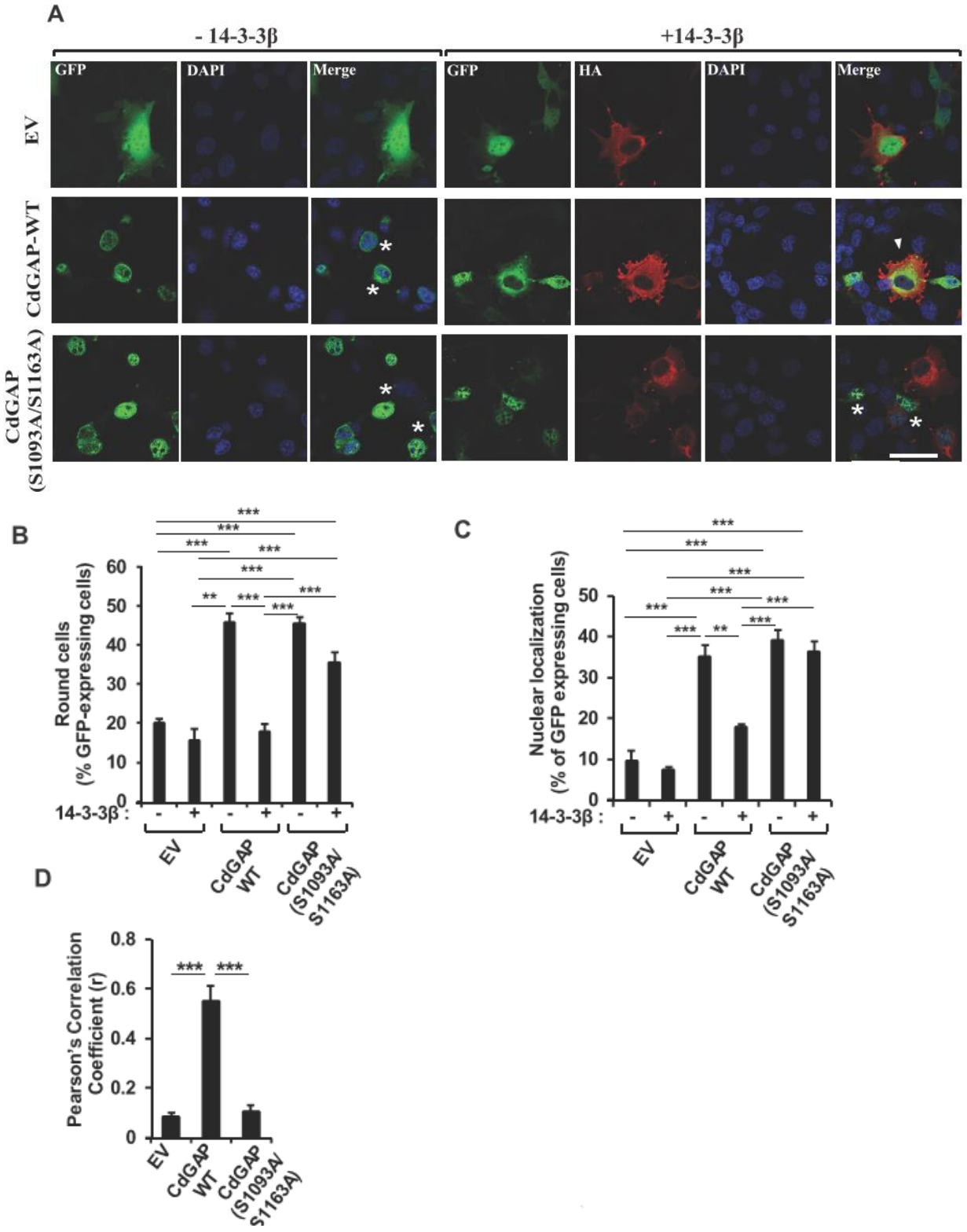

A
- 14-3-3β
+14-3-3β
GFP
DAPI
Merge
GFP
HA
DAPI
Merge
EV
CdGAP-WT
CdGAP (S1093A/S1163A)
B
Round cells (% GFP-expressing cells)
14-3-3β :
EV
CdGAP WT
CdGAP (S1093A/ S1163A)
C
Nuclear localization (% of GFP expressing cells)
14-3-3β :
EV
CdGAP WT
CdGAP (S1093A/ S1163A)
D
Pearson's Correlation Coefficient (r)
EV
CdGAP WT
CdGAP (S1093A/ S1163A)

Figure 3.5 – 14-3-3β regulates CdGAP subcellular localization and inhibits CdGAP-mediated cell rounding

(A) GFP-CdGAP constructs with or without 14-3-3β were transfected into COS-7 cells before fixation. CdGAP and 14-3-3β localization was assessed by indirect immunofluorescence and confocal microscopy. CdGAP (green), 14-3-3β (red), nucleus (blue) were visualized using anti-GFP, anti-HA antibodies, and DAPI staining, respectively. Bar, 50µm. star, round cells, arrowheads, cytoplasmic localization of CdGAP. (B and C) The percentage of GFP-expressing cells showing cell rounding (B) or CdGAP nuclear localization (C) was calculated manually using Metamorph software. Cells with nuclei occupying ≥ 50% of the total cell area were counted as being round and cells with ≥70% of their GFP staining in the nucleus were counted as nuclear localization. More than 100 GFP-positive cells were counted in at least three independent experiments. (**p<0.01, ***p<0.001, unpaired student's t test). (D) The correlation between CdGAP and 14-3-3β fluorescence intensities in A was measured with ZEN2010 software using Pearson's correlation coefficient (r). At least 30 cells from three independent experiments were analyzed (***p<0.001, unpaired student's t test).

Figure 6

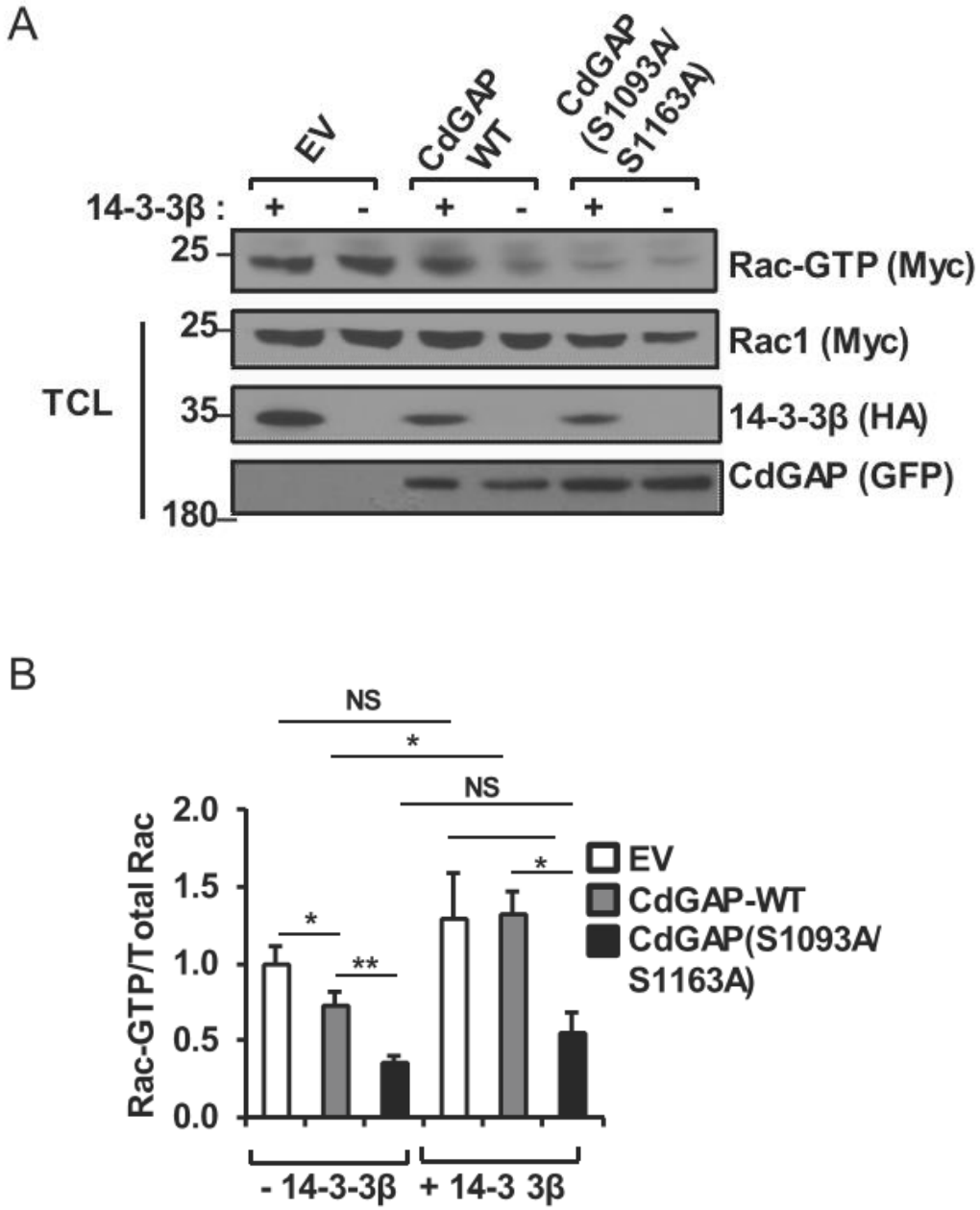

A
EV
CdGAP WT
CdGAP (S1093A/ S1163A)
14-3-3β :
+
-
+
-
+
-
25
Rac-GTP (Myc)
25
Rac1 (Myc)
TCL
35
14-3-3β (HA)
CdGAP (GFP)
180
B
NS
*
NS
*
*
**
2.0
1.5
1.0
0.5
0.0
Rac-GTP/Total Rac
EV
CdGAP-WT
CdGAP(S1093A/ S1163A)
- 14-3-3β
+ 14-3 3β

Figure 3.6 – 14-3-3β negatively regulate the GAP activity of CdGAP towards Rac1

(A) HEK293 cells were transfected with pRK5-mycRac1 with empty vector (EV), the indicated GFP-CdGAP constructs, and HA-14-3-3□ as indicated. GTP-loaded Rac1 was pulled down from total protein lysates (TCL) by GST-CRIB. GTP-bound Rac1, total Rac1, and the indicated proteins were detected by immunoblotting . (B) Densitometric ratio of GTP-bound Rac1/total Rac1 from A. (*p<0.05, NS, non significant, unpaired student's t test).

Figure 7

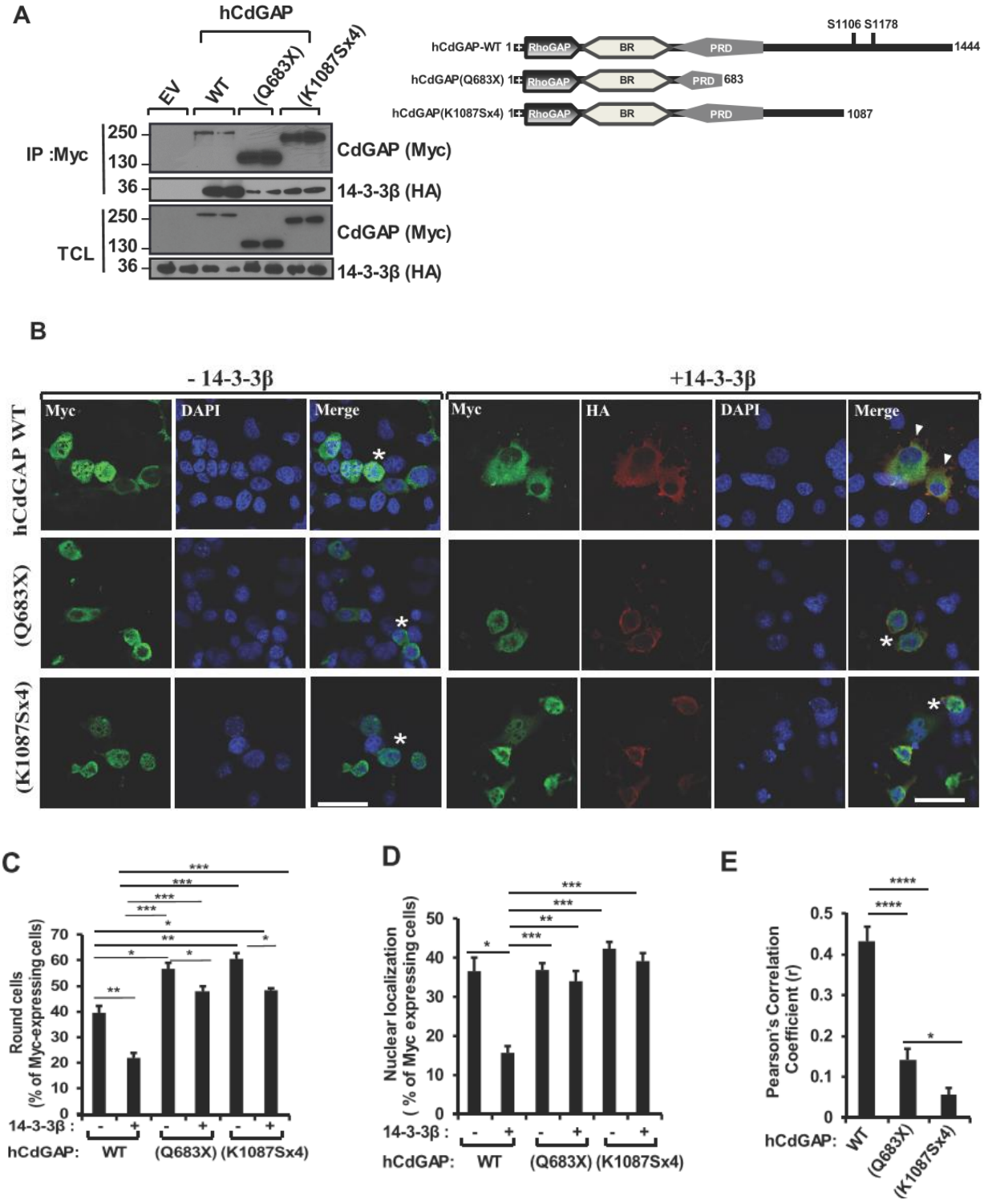

A
hCdGAP
EV
WT
(Q683X)
(K1087Sx4)
IP :Myc
250
130
36
CdGAP (Myc)
14-3-3β (HA)
TCL
250
130
36
CdGAP (Myc)
14-3-3β (HA)
S1106 S1178
hCdGAP-WT 1
RhoGAP
BR
PRD
1444
hCdGAP(Q683X) 1
RhoGAP
BR
PRD
683
hCdGAP(K1087Sx4) 1
RhoGAP
BR
PRD
1087
B
- 14-3-3β
+14-3-3β
hCdGAP WT
(Q683X)
(K1087Sx4)
Myc
DAPI
Merge
Myc
HA
DAPI
Merge
C
Round cells (% of Myc-expressing cells)
14-3-3β :
hCdGAP:
WT
(Q683X)
(K1087Sx4)
D
Nuclear localization (% of Myc expressing cells)
14-3-3β :
hCdGAP:
WT
(Q683X)
(K1087Sx4)
E
Pearson's Correlation Coefficient (r)
hCdGAP:
WT
(Q683X)
(K1087Sx4)

Figure 3.7 – Reduced interaction and modulation of AOS-related CdGAP mutant proteins by 14-3-3β.

(A) COS-7 cells were transfected with HA-14-3-3β together with empty vector (EV) or myc-human CdGAP (hCdGAP) constructs as indicated. Myc-hCdGAP proteins were IP from total cell lysates (TCL), resolved by SDS-PAGE and immunoblotted using anti-Myc and anti-HA antibodies. Schematic representation of hCdGAP-WT and AOS-associated mutants. (B) myc-hCdGAP constructs with or without 14-3-3β were transfected into COS-7 cells before fixation. CdGAP and 14-3-3β localization was assessed by indirect immunofluorescence and confocal microscopy. hCdGAP (green),14-3-3β (red), nucleus (blue) were visualized using anti-GFP, anti-HA antibodies, and DAPI staining, respectively. Bar, 50µm. star, round cells, arrowheads, cytoplasmic localization of CdGAP. (C and D) The percentage of GFP-expressing cells showing cell rounding (C) or hCdGAP nuclear localization (D) was calculated manually using Metamorph software as in Fig. 3.5 (*p<0.05, **p<0.01, ***p<0.001, unpaired student's t test). (E) The correlation between hCdGAP and 14-3-3β fluorescence intensities in B was measured with ZEN2010 software using Pearson's correlation coefficient (r). At least 30 cells from three independent experiments were analyzed (*p<0.05, ****p<0.0001, unpaired student's t test).

Figure 8

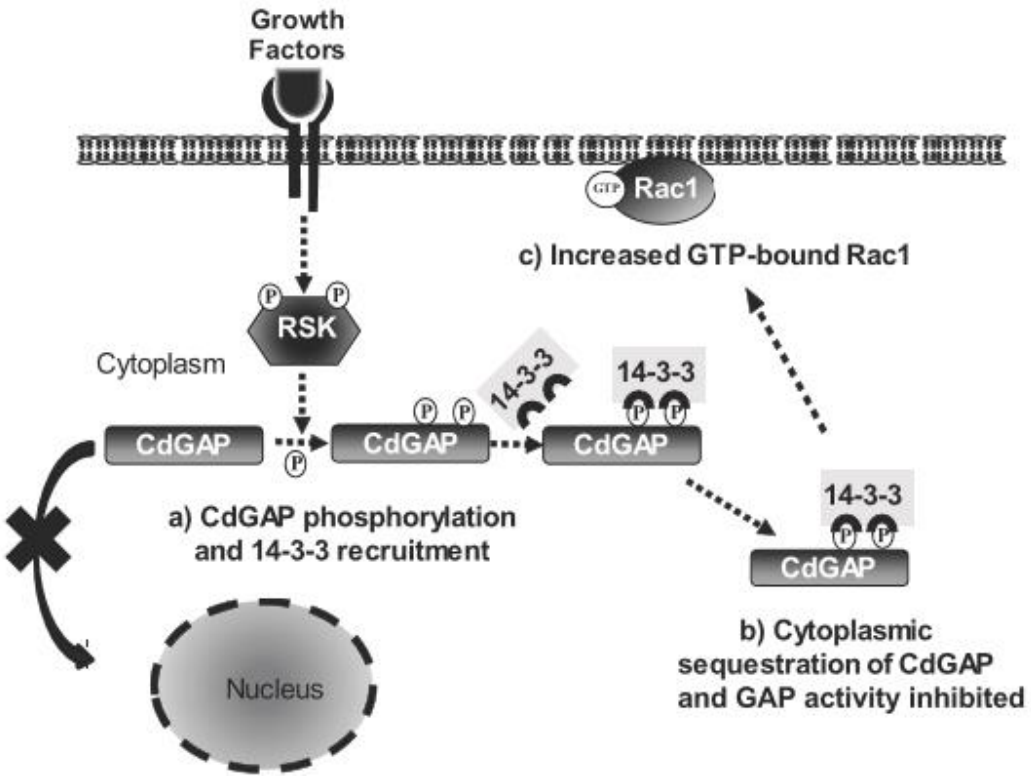

Growth Factors
Rac1
c) Increased GTP-bound Rac1
RSK
Cytoplasm
14-3-3
14-3-3
CdGAP
CdGAP
CdGAP
a) CdGAP phosphorylation and 14-3-3 recruitment
14-3-3
CdGAP
Nucleus
b) Cytoplasmic sequestration of CdGAP and GAP activity inhibited

Figure 3.8 – Model of CdGAP regulation by 14-3-3 adaptor proteins.

(a) In response to agonist stimulation, CdGAP is phosphorylated by RSK on Ser1093 and Ser1163, which permits the recruitment and binding of 14-3-3 proteins. (b) 14-3-3 interaction with CdGAP inhibits the nucleocytoplasmic shuttling of CdGAP and leads to CdGAP sequestration in the cytoplasm and inhibition of the GAP activity. (c) Consequently, Rac1-GTP levels are increased, inducing cell spreading.

Table 3.1 - Potential RSK phosphorylation sites identified in the CdGAP protein sequence.
[a] 8 potential phosphorylation sites were identified according to the RSK consensus phosphorylation sequence determined using an oriented peptide library (29) (ranked from the highest to the lowest). [b] Number of phosphoproteomic studies reporting the phosphorylated residue according to phosphosite.org. [c]Phosphorylated in response to VEGF and /or Angiopoietin-1 (42).
BR, basic region; PRD, proline- rich region. CT, C-Terminal region.

Ranking No. [a]	Residue *Mouse (Human)*	CdGAP sequence *Mouse (Human)*	CdGAP-domain	Records[b]
1	Ser1163[c] (Ser1178)	TLTGRRN**pS**APVSVSA (ALTGRRN**pS**APVSVSA)	CT	23
2	Ser312 (Ser315)	FNLGRSG**pS**DSKSKLS (FNLGRSG**pS**DSKSKLS)	BR	0
3	Ser1093[c] (Ser1106)	KGKHRPS**pS**LNLDSAT (KGKNRPS**pS**LNLDPAI)	CT	3
4	Ser323 (Ser327)	SKLSRNG**pS**VFVRGQR (SKLSRNG**pS**VFVRGQR)	BR	0
5	Ser765 (Ser778)	IGGPRNL**pS**PPLTPAP (VGGPGNL**pS**PPLPPAP)	PRD	13
6	Ser1331 (Ser1346)	SRPGRPQ**pS**LILFPIM (SRPGRPQ**pS**LILFSPP)	CT	2
7	Ser272 (Ser272)	RKERREN**pS**LPEIVPP (RKERREN**pS**LPEIVPP)	BR	18
8	Ser998 (Ser1011)	LKAFREF**pS**GLKGLEV (LRSFREF**pS**GLKGAEA)	CT	0

Chapter 4: General Discussion and Conclusions

4.1 Major Findings

4.1.1 Identification of a novel conserved SH3-binding motif

The central region of CdGAP, which is highly phosphorylated and enriched in basic residues, drives the interactions with Intersectin and actopaxin but the motifs mediating the interactions were not known. In the study presented in chapter 2 we identified and characterized for the first time a novel atypical-SH3 binding motif, xKx(K/R)K (SKSKK) in the CdGAP central region, which is highly conserved within CdGAP subfamily members. We then characterized the Intersectin-SH3D/CdGAP interaction and provided evidence that Intersectin-SH3D interacts directly with the xKx(K/R)K motif. Furthermore, we demonstrated that the identified motif is required for CdGAP activity *in vitro* and substitution of the lysine residues by alanine (SaSaa) impaired CdGAP activity, mimicking the Intersectin-binding effect. Together, our data support the previous results investigating the Intersectin-SH3D/CdGAP interaction and the regulation of CdGAP activity by Intersectin-SH3D binding.

4.1.2 Phosphorylation of CdGAP

CdGAP is a phosphoprotein that is highly phosphorylated particularly in the BR, PRD and C-terminal region and this study is the first to characterize the CdGAP-phosphorylated residues targeted by AGC kinase family members. We first demonstrated that the endogenous CdGAP is a phosphorylation target of AGC kinases in different cell models and that its phosphorylation levels augmented following stimulation by growth factors. We then mutated to phospho-null alanine residue the potential phosphorylation sites and identified two conserved phospho-serines in the C-terminal tail, Ser-1093 and Ser-1163 as AGC-kinase phosphorylated residues. Furthermore, we provided evidence that AGC-kinase RSK1 is the major protein kinase phosphorylating the identified residues.

4.1.3 Regulation of CdGAP function and subcellular localization by 14-3-3 adaptor proteins

Next, we demonstrated for the first time that 14-3-3 adaptor proteins interact with CdGAP and regulate both its GAP activity and subcellular localization. We first showed that the 14-3-3 isoforms β and σ interact with CdGAP. We then identified three 14-3-3-binding regions on CdGAP namely: the GAP domain, the BR and the C-terminal regions. We next provided evidence that the binding to the C-terminus is mediated by RSK1-phosphorylated residues, the Ser-1093 and Ser-1163, and further the binding therein is required for a stable and strong 14-3-3/CdGAP interaction. Additionally, we showed that 14-3-3β binding to CdGAP impairs its GAP activity and induces its sequestration in the cytoplasm. Furthermore, 14-3-3β induced cell spreading and abolished CdGAP-induced-cell rounding.

Altogether, our results illustrate a novel regulatory mechanism of CdGAP involving phosphorylation by RSK1 and 14-3-3 binding. As such, following CdGAP phosphorylation, 14-3-3 proteins bind and sequestrate it in the cytoplasm preventing both its GAP activity and its nuclear function. Consequently, the GTPase activities of Rac1 and Cdc42 are augmented resulting in increased cell spreading.

4.2 Regulation of CdGAP by Intersectin

In this work we identified a key SH3-binding motif in CdGAP BR region mediating the interaction with Intersectin-SH3D domain and involved in the GAP activity regulation. Importantly, the BR region bearing the novel xKx(K/R)K motif is well conserved and retained the binding specificity to Intersectin-SH3D domain across CdGAP subfamily members. As such, we demonstrated that the BR region of CdGAP-related protein, ArhGAP30 containing an xKx(K/R)K (LKVRK) motif is required for the interaction Intersectin-SH3D/ArhGAP30 (Figure 2.3). Interestingly, this motif is present in adaptor protein Numb, also known to interact with Intersectin-SH3D, adding support to the findings that the novel xKx(K/R)K motif mediates the interaction with Intersectin-SH3D.

In this study and in the previous findings (345), we used a short form of CdGAP (1-820aa) lacking the C-terminal tail (820-1425) to investigate the interaction with Intersectin. However, recent results from our laboratory attribute interesting regulatory functions to the extended C-terminal region, particularly an autoinhibitory role of the GAP activity (340). In addition, we noticed that the PRD, although it does not bind to Intersectin-SH3D domain (345), is still required for inhibition of the GAP activity stressing the importance of the protein structure in the SH3D domain-mediated activity regulation. Therefore, it may be of interest to investigate the Intersectin-SH3D/CdGAP interaction in CdGAP and Intersectin full-length protein contexts.

Intersectin is a large-multidomain adaptor protein known as a general endocytic scaffold but also as an integrator in many signalling pathways, such as actin cytoskeleton wherein for instance, it regulates and coordinates the function of Cdc42, Nck and N-WASP as well as Arp2/3 complex to promote actin polymerisation (448). It is therefore not surprising to find interactions between Intersectin and CdGAP which is a well-characterized regulator of Cdc42 and Rac1 activities and actin cytoskeleton.

Although the biological function of this interaction is unclear, it appears that a priori, Intersectin antagonizes the action of CdGAP by abolishing its GAP activity in specific-subcellular sites (i.e., exclusive zones) which may as a result, enhance the GTPase activities of CdGAP effectors, Rac1 and Cdc42 in these exclusive zones. Likewise, Intersectin by binding and sequestrating CdGAP in the PM, will prevent CdGAP from exerting its effects in other cellular locations such as the nucleus. Also, since both Intersectin and CdGAP are multidomain proteins interacting with many other protein partners, the interaction between them in the physiological context, may involve the formation of regulatory complexes including exclusive zone proteins for a selective and precise spatiotemporal regulation of designated Rho GTPase activities.

In support for this model, we find that the same CdGAP BR region is involved in the binding to Actopaxin and in the subsequent regulation of CdGAP activity at focal adhesions suggesting a subcellular site-dependent competitive or synergic protein binding to this region (341).

Interestingly, both Intersectin and CdGAP are involved in cancer notably in TGFβ-mediated tumorigenesis where they play opposite roles. Indeed, CdGAP which mediates TGFβ- induced effects on cell migration and invasion, exerts its role at the nucleus level wherein it represses transcription of E-cadherin, a key protein in cell-cell junctions inducing therefore EMT (127,128). Conversely, Intersectin seems to inhibit tumorigenesis and its deficiency has been found to activate the non-canonical TGFβ-dependent Ras/Erk1/2 MAPK pathway, particularly in endothelial cells leading to increased cell proliferation and survival (449). Therefore, it would be of interest to investigate whether the Intersectin-mediated PM sequestration of CdGAP would prevent its nuclear translocation and the subsequent E-cadherin transcription repression and EMT onset.

Finally, the mechanism identified in this work involving the CdGAP-SKSKK motif could be used to design therapeutic tools to inhibit CdGAP function. As such, the strategy may imply a development of CdGAP-binding small molecules to inhibit CdGAP function in diseases such as breast cancer, in an Intersectin-like fashion similar to a compound developed to inhibit Cdc42 activity (450).

4.3 Regulation of CdGAP by phosphorylation

In this study we shed light on a novel mechanism of CdGAP phosphorylation-dependent regulation involving the Ras-Erk1/2 MAPK-cascade effector RSK1 and 14-3-3 adaptor proteins. The mechanism involves RSK1-dependent phosphorylation of CdGAP and 14-3-3-protein recruitment on the phosphorylated residues regulating thereby CdGAP subcellular localization and function as well as the whole cell morphology (Figure 3.8).

We have previously shown using *in vivo* [^{32}P] orthophosphate labeling that CdGAP is highly phosphorylated in different cellular models in response to growth factor stimulation including FBS, PDGF and TGFβ (95,109,128), with high phosphorylation levels detected in the BR, PRD and CT regions (Figure 4.1) (108,109,323). We also identified the Thr-776 residue as being targeted by both ERK1/2 and GSK3, and its phosphorylation inhibited CdGAP activity *in vitro* (108,109). With the exception of this

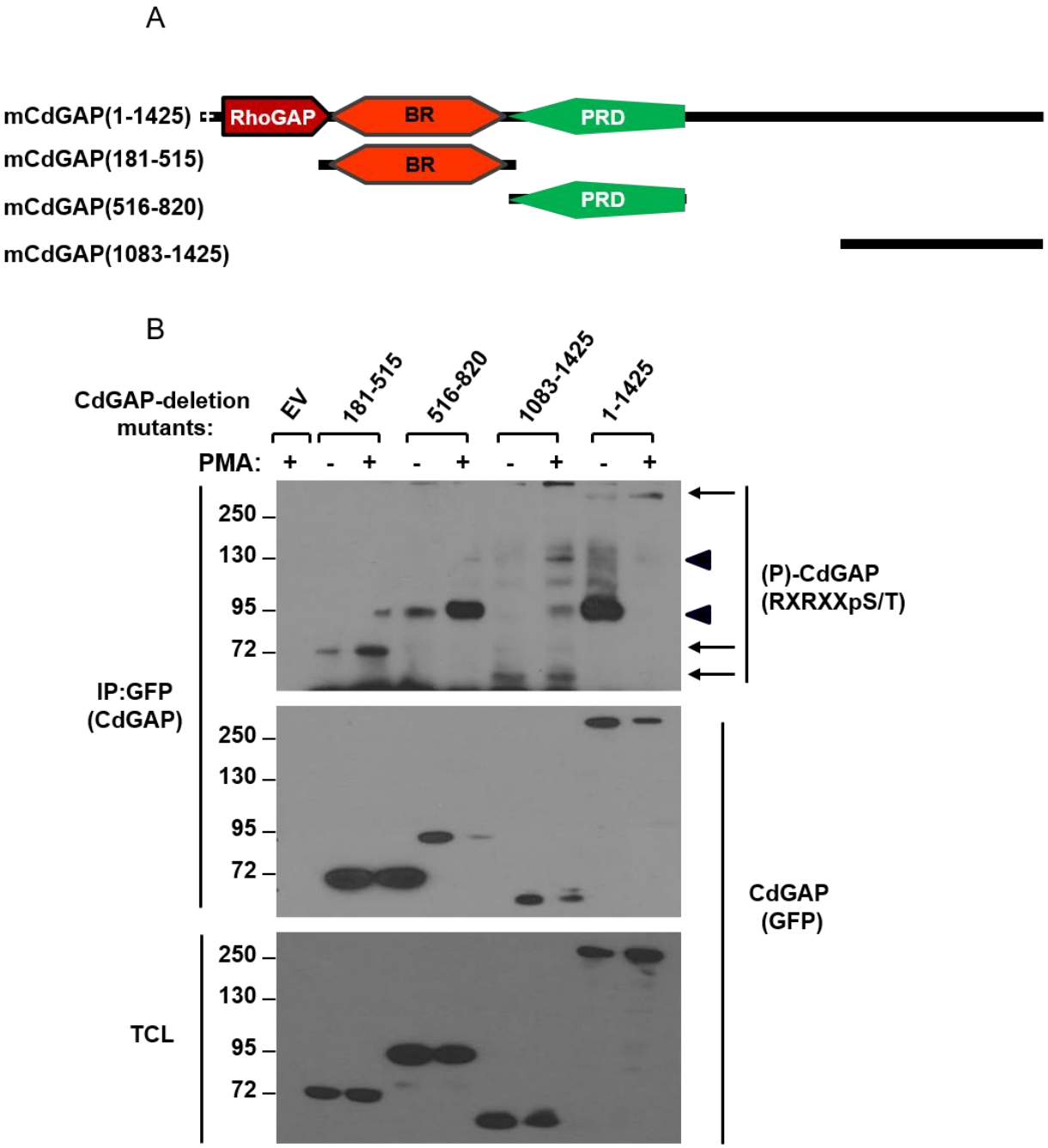

A
mCdGAP(1-1425)
RhoGAP
BR
PRD
mCdGAP(181-515)
BR
mCdGAP(516-820)
PRD
mCdGAP(1083-1425)
B
CdGAP-deletion mutants:
EV
181-515
516-820
1083-1425
1-1425
PMA: + - + - + - + - +
IP:GFP (CdGAP)
250
130
95
72
(P)-CdGAP (RXRXXpS/T)
CdGAP (GFP)
TCL

Figure 4.1 – CdGAP deletion mutant phosphorylation

(A) Schematic representation of mouse CdGAP (mCdGAP) deletion mutants. +, stretch of polybasic residues; RhoGAP domain, Rho GTPase-activating protein domain; BR, basic region; PRD, proline-rich domain. (B) COS-7 cells were transfected with empty vector (EV) or GFP-CdGAP deletion mutant constructs and treated with PMA for 30 min. GFP-CdGAP proteins were immunoprecipitated (IP) from total cell lysates (TCL). IP proteins and TCL were resolved by SDS-PAGE and immunoblotted with the AGC kinase phosphorylation site-specific, RXRXXpS/T and GFP antibodies.

Arrows, (P)-CdGAP; arrowheads, nonspecific bands.

Table 4.1 – Predicted CdGAP phosphorylation targets of RSK family members

Mouse CdGAP (NCBI Entry number: A6X8Z5) amino acid sequence was analyzed by GPS 3.0 software for phosphorylation by selected RSK kinases. Predicted phosphosites with highest scores are shown.

Site	Phosphopeptide	Region	kinase	Score	Cutoff
S272	RKERREN**pS**LPEIVPP	BR	RSK3 RSK RSK1	13.206 5.702 3.104	10.513 5.671 2.462
S295	PDNKRKL**pS**SKSKKWK	BR	RSK3 RSK RSK1	12.441 7.957 2.938	10.513 5.671 2.462
S296	DNKRKLS**pS**KSKKWKS	BR	RSK2 RSK1	8.333 2.688	6.617 2.462
S312	FNLGRSG**pS**DSKSKLS	BR	RSK3 RSK	10.794 6.479	10.513 5.671
S323	SKLSRNG**pS**VFVRGQR	BR	RSK3 RSK	11.441 6.723	10.513 5.671
S765	IGGPRNL**pS**PPLTPAP	PRD	RSK	6.33	5.671
S941	PSLRQSH**pS**LDSKTTG	CT	RSK1	2.688	2.462
S1066	SPRAQD**pS**TLPGEHPL	CT	RSK1	2.688	2.462
S1093	KGKHRPS**pS**LNLDSAT	CT	RSK1	3.049	2.876
S1163	TLTGRRN**pS**APVSVSA	CT	RSK3 RSK RSK1	12.941 7.489 2.812	10.513 5.671 2.462
S1223	RSQEEPG**pS**TPEIPQK	CT	RSK1	2.938	2.462
S1255	PKQETGA**pS**ASRRQAS	CT	RSK1	2.542	2.462
S1262	SASRRQA**pS**ITSCMYE	CT	RSK3 RSK	12.971 6.521	10.513 5.671
S1284	PSASTLA**pS**TQDAVVQ	CT	RSK1	3.292	2.462

GAP domain, GTPase-activating protein domain; BR, basic region; PRD, proline-rich domain; CT, C-terminus.

phosphosite, little is known about neither the identity of the phosphorylated residues nor the role of the CdGAP abundant phosphorylation.

In this study we used a phosphorylation site-specific antibody recognizing a consensus motif (RXRXXpS/T) found in substrates of AGC family kinases (431,432), and reported that CdGAP is a phosphorylation target of this class of kinases. We next mapped the potential phosphosites and identified two major regulatory phospho-residues namely: Ser-1093 and Ser-1163 in the CT region. We further provided evidence that the Ras-Erk1/2 MAPK pathway effector, RSK1 is the major kinase phosphorylating the identified residues.

Despite the increase of the phosphorylation signal in CdGAP segments expressed separately (Figure 4.1), the predicted phosphosites (Table 4.1) turned out to be not major phosphorylation targets of AGC kinases since the phosphorylation signal remains unchanged when mutated to alanine residue (Figure 3.2). This suggests that in the context of three-dimensional structure of the full-length protein these sites are inaccessible to the kinase action.

Interestingly, the Ser-1093 and Ser-1163 residues have been listed in many phosphoproteomic studies according to phosphosites.org. For instance, Ser-1163 has been reported in large-scale phosphoproteomic analyses of liver, lung and breast cancers, whereas Ser-1093 surfaced in the phosphoproteome of the liver (347,348,350-353). Moreover, Ser-1093 has been recently identified as being regulated by VEGF and angiopoietin-1 signaling in endothelial cells (349). Intriguingly, in each process displaying a regulation of these phosphosites, such as breast cancer or angiogenesis, CdGAP has been shown to play a role, emphasising a possible involvement of its phosphorylation in the above cellular events (127,339).

4.4 CdGAP/14-3-3 protein interaction

14-3-3 family members of adaptor/scaffold proteins are found as homo or heterodimers and seven isoforms have been so far characterized in mammals (i.e., β, γ,

Table 4.2 – 14-3-3-predicted binding sites of CdGAP

Mouse CdGAP (NCBI Entry number: A6X8Z5) amino acid sequence was analyzed by 14-3-3-Pred software. Predicted 14-3-3 binding sites with highest scores by two and three methods are indicated (439).

			Method			
			ANN	**PSSM**	**SVM**	**Consensus**
Site	14-3-3-binding motif	Region	*Score*	*Score*	*Score*	*Score*
pS272	KERREN**pS**LPEI	BR	0.974	1.723	1.831	1.51
pS343	TIRPAK**pS**MDSL	BR	0.52	0.487	0.071	0.359
pT666	PPAAQK**pT**SPIP	PRD	0.458	0.354	-0.139	0.224
pS685	FPEAPG**pS**LPSS	PRD	0.366	0.313	-0.236	0.148
pS690	GSLPSS**pS**APRE	PRD	0.621	0.464	-0.049	0.346
pS765	GGPRNL**pS**PPLT	PRD	0.622	0.934	0.443	0.666
pS1092	KGKHRP**pS**SLNL	CT	0.21	0.028	-0.8	-0.187
pS1093	GKHRPS**pS**LNLD	CT	0.817	0.943	0.921	0.894
pS1163	LTGRRN**pS**APVS	CT	0.905	1.537	1.042	1.161

ANN - Artificial Neural Network (cut-off = 0.55)

PSSM - Position-Specific Scoring Matrix (cut-off = 0.80)

SVM - Support Vector Machine (cut-off = 0.25)

Consensus - Average of the scores provided by the three methods (cut-off = 0.50)

BR, basic region; PRD, proline-rich domain; CT, C-terminus

ε, σ, ζ, τ and η)(106,371,372). They can bind motifs on the same or different protein targets which they regulate in diverse ways including conformational change, sequestration/retention and inducing/preventing protein-protein interaction (106,371-374). Phosphorylation of substrates by AGC kinases generates binding sites for 14-3-3 proteins including a crucial phosphoserine comprised in their mode I consensus binding motif (Arg-Ser-Xaa-pSer-Xaa-Pro) (373,374,392). In addition, 14-3-3 proteins can also bind directly unphosphorylated motifs with similar affinity as that of phosphoserine sites (106).

Here we report for the first time that 14-3-3 proteins (i.e., isoforms β and σ) bind and regulate CdGAP in a phosphorylation-dependent manner, and we identified three binding sites of 14-3-3β namely: the GAP domain, the BR and CT regions. In addition, we demonstrated that the CT identified residues, Ser-1093 and Ser-1063 are required for the 14-3-3β/CdGAP interaction. We further validated this requirement using the AOS-associated hCdGAP(Q683X) and hCdGAP(K1087Sx4) mutants lacking the phosphosites (Figure 3.7 A and E).

14-3-3 proteins function exclusively as dimers and the presence of two binding sites on the same protein target instead of one increases the binding affinity for their ligand by up to 30-fold (374,451). In this work we show that the expression of CdGAP (Ser-1093A/Ser-1163A) double mutant altered severely the interaction with 14-3-3β. We propose therefore, a binding model in which 14-3-3 dimers interact simultaneously with at least Ser-1093 or Ser-1063 residues at the CT, and with another binding site at the GAP domain or the BR region. The BR region which contains several predicted AGC-phosphorylated and 14-3-3 binding sites (Table 4.1 and Table 4.2), could mediate the binding in a phosphorylation-dependent manner but it is also the interacting site for other partners such as Actopaxin and Intersectin suggesting a competitive binding to this region (341,346). Alternatively, the binding to 14-3-3 proteins could be mediated in a phosphorylation-independent manner, through an unphosphorylated-14-3-3 binding site, 179-RSKKIE (RSx_{1-3}E-like motif), an atypical 14-3-3-docking site in the GAP

domain (397). As such, it would be of interest to replace the residues of this binding site and investigate whether the interaction CdGAP/14-3-3 is affected.

4.5 Regulation of CdGAP activity and subcellular localization

We next characterized the role and cellular function of both CdGAP phosphorylation and the subsequent 14-3-3 binding. As such,14-3-3β binding to CdGAP resulted in inhibition of the RhoGAP activity in a (Ser-1093 and Ser-1163) phosphorylation-dependent manner, concomitant with increased cell spreading and reduced cell rounding, both hallmarks of suppressed GAP activity (36,340,341). 14-3-3 proteins could neutralize CdGAP activity by at least two mechanisms: the first by directly interacting with the GAP domain residues and disturbing its catalytic activity; the second by binding CdGAP and sequestering it in the cytoplasm preventing it therefore, from reaching its effectors at the membrane.

We have previously reported a CdGAP-autoinhibitory mechanism by intramolecular interactions involving the CT and the GAP domains leading, seemingly to reduced GAP activity (340). Accordingly, AOS-associated mutants, hCdGAP(Q683X) and hCdGAP(K1087Sx4) lacking the CT portion, displayed constitutive CdGAP activity resulting in a loss of active Cdc42 in AOS patients (340). Given that 14-3-3β binds to both the GAP domain (179-RSKKIE) and the CT region (Ser-1093/Ser-S1163), we can hypothesize that 14-3-3 proteins bridge the autoinhibitory interaction between the two domains, which results in the loss of GAP activity.

On the other hand, and consistent with CdGAP cellular functions, the subcellular localization data show CdGAP-WT protein in several cellular compartments including the PM and the nucleus. CdGAP localizes to cellular membranes to regulate mainly Rac and Cdc42 activities, while it translocates to the nucleus to drive its transcriptional regulation function (36,127,341). As such, we identified several nuclear localization sequences (NLSs), particularly in 14-3-3β binding regions, the GAP and BR domains as well as in the CT (Table 4.3). Collectively, the actual data suggest a model wherein, 14-3-3β dimers upon binding CdGAP in the cytoplasm, will disguise the NLSs and will consequently prevent its nucleocytoplasmic shuttling.

Table 4.3 – Identification of NLS motifs of CdGAP

Mouse CdGAP (NCBI Entry number: A6X8Z5) amino acid sequence was analyzed by cNLS Mapper software (452). Putative NLSs with highest scores are indicated**.**

Monopartite NLS

Position	Region	Sequence	Score
288	BR	PDNKRKLSSK	7

Bipartite NLS

7	PBR	KQKLKRKGAASAFGCDLTEYLESSGQDVPYV	4.2
265	BR	RKERRENSLPEIVPPPFHTVLELPDNKRKLSS	4.3
1053	CT	DSSKESSPRAQDSTLPGEHPLQLQLKNTEC	4.3
1293	CT	RKRTSETEPSGDNLLSSKLERAS	4.2

PBR, polybasic region ; BR, basic region; CT, C-terminus

This retention mechanism is very common among 14-3-3 proteins which bind and sequestrate their effectors in a subcellular compartment frequently the cytoplasm (371,372). For instance, 14-3-3 proteins regulate the cellular localization of Notch4 by retention in the cytoplasm through binding to phosphosites generated by the AGC kinase, Akt on the Notch4-intracellular domain (453).

The findings and the model presented here are supported further by a similar regulatory mechanism reported for DLC1, which involves both 14-3-3 proteins and AGC kinases, PKC and PKD (440). The mechanism documents a PKC and PKD-dependent phosphorylation of two 14-3-3 binding sites: Ser-327 and Ser-431. Later on, the recruitment of 14-3-3 proteins to these sites and the ensuing masking of key NLSs will induce a retention of DLC1 in the cytoplasm and a suppression of its GAP activity (440).

4.6 Potential applications of the 14-3-3/CdGAP regulation model in cancer research therapy

High expression levels of CdGAP in clinical patients have been recently associated to tumorigenesis in a wide range of cancers and many breast cancer subtypes such as basal-like subtype, making it a newly discovered biomarker and cancer therapeutic target (127). This proto-oncogenic role is driven chiefly through a novel nuclear function, wherein, in association with the transcription factor Zeb2, CdGAP co-represses E-Cadherin, a key protein in cell-cell junctions, promoting thereby EMT and tumorigenesis (127).

On the other hand, many 14-3-3 isoforms have been documented to play crucial roles in many cancers. For instance, the 14-3-3σ isoform, which has been considered as a tumor suppressor affects negatively several key survival pathways such as the Akt signaling, cell cycle regulation and apoptosis (454-456). As such, 14-3-3σ low protein expression has been recorded in many cancers notably breast cancer where it is associated to poor prognosis, making it a potential biomarker and treatment target for breast cancer (408,457,458).

Given the opposite effects of CdGAP and 14-3-3σ in cancer, specifically breast cancer, we assume that similar to 14-3-3β (Figure 3.3),14-3-3σ binds and sequestrates CdGAP in the cytoplasm preventing thus its nuclear translocation, E-Cadherin repression, EMT and tumorigenesis.

This model will allow at least two applications in the breast cancer research field: first, the CdGAP/14-3-3σ ratio can be used as a signature and a biomarker; second, the CdGAP/14-3-3σ interaction can be targeted for breast cancer treatment. For the first application, and since both CdGAP and 14-3-3σ expression profiles are associated with poor patient prognosis, it would be of interest to investigate whether the CdGAP to 14-3-3σ expression ratio is associated to a specific breast cancer-subtype aggressiveness and malignancy. For the second application, CdGAP/14-3-3σ interaction could be investigated in order to generate molecules that mimic the binding to prevent CdGAP nuclear translocalization. The treatment could be used for instance, in basal-like breast cancer patients to prevent progression/recurrence and metastasis.

4.7 Conclusion and perspectives

Recent findings provided useful insights into the role of CdGAP in cancer as well as the mechanism involved in this function. This thesis has provided insights into two important mechanisms of CdGAP regulation involving protein-protein interaction and phosphorylation. Combining various techniques, we identified an atypical SH3-binding motif that is important for the regulation of CdGAP activity by Intersectin, a mechanism that could be further used to elaborate CdGAP inhibitors. We next deciphered a key role of CdGAP phosphorylation and discovered a critical CdGAP nucleocytoplasmic-shuttling regulatory mechanism involving both RSK-induced phosphorylation and 14-3-3-protein binding. Further investigation is needed to better characterize the two mechanisms in different *in vitro* and *in vivo* systems. Finally, this thesis has provided valuable tools to pursue the research on CdGAP role in cancer, tools that we hope ultimately, will contribute to breast cancer treatment.

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