Identification of amino acids involved in Cdc42-calmodulin interaction and regulation of Cdc42 activation

by

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Abstract

Cdc42 is a member of Rho family of Ras GTPase superfamily and has been shown to regulate actin cytoskeleton re-organization and filopodia formation. Calmodulin (CaM) is a calcium modulating protein and regulates calcium dependent signal transduction pathways in the cell. According to CaM target database analysis, amino acid region 151-163 of Cdc42 has a potential CaM binding domain that interacts with CaM. In the present work, we have investigated putative CaM binding region in Cdc42. In addition the role of basic amino acids K153 and K163 within this region in Cdc42 interaction with CaM and effect on Cdc42 activity was elucidated.

GST-Cdc42 M (Δ151-163), GST-Cdc42K153A and GST-Cdc42K163A mutants were generated. Binding assay experiments showed that amino acid region 151-163 in Cdc42 is an important regulatory domain for CaM binding. Results also demonstrated that K163A mutant showed significantly reduced binding to CaM, whereas Cdc42 K153A showed reduced but non-significant decrease in its interaction with CaM.

A previous study in our laboratory has shown that CaM plays critical role in maintaining basal activity of Cdc42 suggesting that K153A and K163A mutants may play a role in regulating this basal activity. In CHRF 288-11 cells expressing mutant forms of Cdc42 (K153A & K163A), basal activation was significantly decreased as compared to wild type Cdc42. The decrease in basal activity in Cdc42 mutants was not due to an inability to bind GTP.

In summary, the results demonstrated that K163 in Cdc42 is a critical amino acid for CaM interaction and in the regulation of basal activity of Cdc42.

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I would like to dedicate this thesis to my husband (Harjinder Singh Grewal) and parents. Their continuous support and encouragement are much valued and appreciated.

Table of Contents

List of Tables

List of Figures

List of Copyright Material for which the permission was obtained

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List of Abbreviations

ApoCaM: Calcium free Calmodulin

ARP2/3: Actin related protein 2/3

Ca^{2+}: Calcium

Ca^{2+}/CaM: Calcium bound calmodulin

CRIB: Cdc42- and Rac-interactive binding

EDTA: Ethylenediaminetetraacetic acid

G Protein or GTPases: Guanine nucleotide binding protein

GAPs: GTPase activating protein

GDIs: GDP dissociation inhibitors

GDP: Guanosine-5’ diphosphate

GDPβS: Guanosine 5'-O-(2-thiodiphosphate)

GEFs: Guanine-nucleotide exchange factors

GPCRs: G protein coupled receptors

GST: Glutathione-S-transferase

GTP: Guanosine-5’-triphosphate

GTPγS: Guanosine 5'-O-[gamma-thio]triphosphate

IPTG: Isopropyl β-D-1-thiogalactopyranoside

kDa: kiloDalton

M (Δ151-163): Mutant form of protein (amino acid 151-163 deleted)

Pak: p21-activated kinase

PBS: Phosphate-buffered saline

PVDF: Polyvinylidene Fluoride

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

WASP: Wiskott–Aldrich syndrome protein

WT: wild type version of protein

1. INTRODUCTION

Cells receive process and respond to information from the environment through signal transduction mechanisms. It is the process whereby information from outside of the cell is conveyed into the cell. A membrane associated receptor protein transfers the information across the membrane into the cell. A receptor is an intrinsic membrane protein that has extracellular and intracellular domains (Hendrickson, 2005). A binding site on the extracellular domain recognizes the signal molecule, often referred to as the ligand or first messenger. Second messenger constitutes the next step in the molecular information circuit. It relays information from one location to another (e.g. from plasma membrane to nucleus). Second messengers amplify the signal significantly. Thus, a low concentration of signal in the environment, yields large intracellular signal and response (Murad, 2011). G proteins (Guanine nucleotide binding proteins) play an important role in signal transduction. The two classes of G Proteins are Heterotrimeric G Proteins or large G Proteins and, the small monomeric G Proteins (Konstantinopoulos, Karamouzis, & Papavassiliou, 2007). G Protein signal transduction pathway causes the release of major 2nd messengers such as cyclic AMP or Calcium ions (Ca^{2+}) (Tuteja, 2009). Ca^{2+} binds to a protein known as Calmodulin and Ca^{2+}/CaM -complex acts to activate a number of cellular processes. Cdc42 is a small G Protein that belongs to Rho family of Ras superfamily. Ca^{2+}/CaM complex binds to Cdc42 and plays an important role in its activation (van Nieuw Amerongen & van Hinsbergh, 2001). In this thesis, the region of Cdc42 that binds to Calmodulin is the main focus of study.

1.1 Guanine nucleotide binding proteins

G Proteins play an important role in signal transduction mechanism in the cell (Gilman, 1987; Tuteja, 2009). Most of these proteins act as molecular switches. They cycle between the active GTP bound and the inactive GDP bound form, and transduce upstream signal to downstream effector proteins in the signal transduction pathways (McCudden, Hains, Kimple, Siderovski, & Willard, 2005). There are two classes of G proteins that play a role in the signal transduction mechanism (Table 1) (Konstantinopoulos et al., 2007):

Heterotrimeric G Proteins

Monomeric small G Proteins

Heterotrimeric G Proteins

Heterotrimeric G proteins consist of three subunits α, β and γ. Molecular mass of α subunit range between 41 to 45 kDa. β subunit is 35 kDa and the γ subunit is 8 kDa (Milligan & Kostenis, 2006). They are bound to the plasma membrane and are associated with cytoplasmic face of G Protein-Coupled Receptors (GPCRs) (McCudden et al., 2005). In the unstimulated inactive state, the three subunits form a heterotrimeric complex. Gα subunit is bound to GDP (Oldham & Hamm, 2007). Ligand binding to GPCRs induces the α subunit to release its bound GDP and is replaced by GTP, leading to the formation of a stable high affinity complex between G protein and

activated receptor (Oldham & Hamm, 2008). GTP binding causes α subunit and βγ complex to dissociate into two active components that can regulate distinct signal transduction pathways (Ford et al., 1998). α subunit and βγ complex initiate the signal by binding to various downstream effector proteins such as adenylyl cyclase, phospholipase C, inwardly rectifying K^+ channels, phospholipase β1, β2 and β3, Src tyrosine kinase, Ca^{2+} channels, etc. (Milligan & Kostenis, 2006) (Smrcka, 2008). The intrinsic GTPase activity of α subunit causes the hydrolysis of bound GTP to GDP. Thus, RGS (Regulators of G protein signaling) proteins act to stimulate the GTP to GDP conversion. RGS proteins act as GTPase activating proteins and accelerate inactivation of the α subunit (De Vries, Zheng, Fischer, Elenko, & Farquhar, 2000). This results in termination of signal with the inactive GDP bound Gα subunit associating with βY subunit and forming αβγ complex (Figure 1) (Hurowitz et al., 2000; Milligan & Kostenis, 2006).

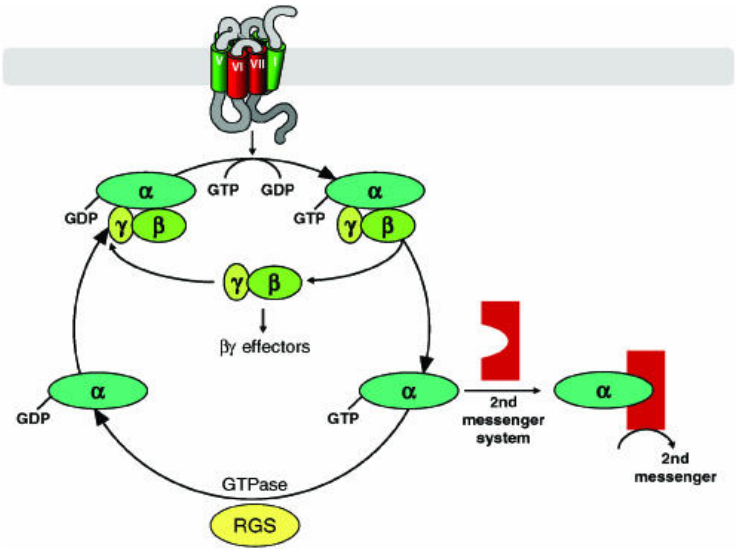

Figure 1: The GTPase cycle for Heterotrimeric G Proteins (Milligan & Kostenis, 2006).

Monomeric small G Proteins

Small G proteins are homologous to the alpha subunit of Heterotrimeric G Proteins (Yang, 2002). They have molecular mass ranging from 20-30 kDa. Small G Proteins play an important role in downstream signaling from various cell surface receptors, thereby controlling various cellular processes such as proliferation, cellular motion, cell division, apoptosis, intracellular transport, etc. (Csepanyi-Komi, Levay, & Ligeti, 2012). There are more than 150 small G Proteins, also known as Ras superfamily (Wennerberg, Rossman, & Der, 2005). The name "Ras" comes from the founding member of the family – Rasp21. Rasp21 is a 21 kDa protein encoded by retroviral oncogene that cause **Ra**t **s**arcome, hence called Rasp21 (Cox & Der, 2010). Ras Superfamily is classified into 5 subfamilies: Ras, Rho, Rab, Ran, Sar1/Arf (Table 1) (Wennerberg, Rossman, & Der, 2005). Rho gene was discovered from Aplysia abdominal ganglia cDNA library as **R**as **ho**molog having 35% sequence homology with HRas (Madaule & Axel, 1985; Takai, Sasaki, & Matozaki, 2001). Rab gene was isolated from a cDNA library from **Ra**t **b**rain using oligonucleotide probes corresponding to conserved amino acid sequence in all Ras proteins and share 30% homology with Ras that is confined to GTP/GDP binding region (Cox & Der, 2010; Touchot, Chardin, & Tavitian, 1987). Ran gene was discovered from human teratocarcinoma cDNA library using mixed oligonucleotide probe and encode for **Ra**s related **n**ucleoproteins (Cox & Der, 2010; Drivas, Shih, Coutavas, Rush, & D'Eustachio, 1990). Arf (**A**DP **r**ibosylation **f**actor) when bound to GTP facilitates cholera toxin catalyzed ADP ribosylation of

adenylyl cyclase and was first purified from rabbit liver membrane using protein purification techniques (Kahn & Gilman, 1984, 1986; Takai et al., 2001).

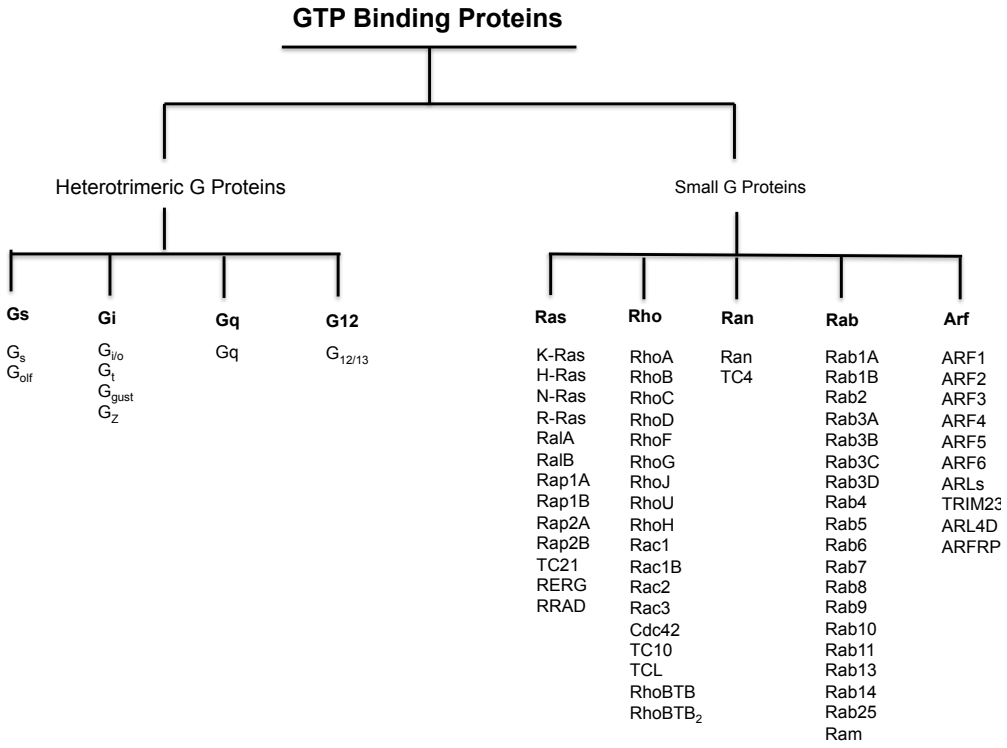

Table 1: Guanine Nucleotide binding proteins: Heterotrimeric G proteins and Monomeric small G proteins

Small G Proteins act as molecular switches in the cell that cycle between the active GTP bound form and inactive GDP bound form (Takai et al., 2001)**.** It is the active form of the protein that interacts with specific effector proteins in the signal transduction pathway**.** Two main classes of proteins, Guanine Nucleotide Exchange Factors (GEFs)

and GTPase activating proteins (GAPs), control the GDP/GTP exchange cycle in the cell. However, the Rab and Rho family members are also regulated by another class of proteins, called GDIs (Guanine nucleotide dissociation inhibitors) (Csepanyi-Komi et al., 2012). The dissociation of GDP from the inactive form of protein is the rate-limiting step in the GDP/GTP exchange cycle (Takai et al., 2001). Guanine Nucleotide Exchange Factors catalyze the dissociation of GDP from GDP bound protein. This facilitates the binding of GTP, as the ratio of GTP is 10 times higher than GDP in the cytoplasm (Bos, Rehmann, & Wittinghofer, 2007). GTPase activating Proteins (GAPs) accelerate the slow endogenous hydrolysis of bound GTP to GDP. These proteins thus promote the formation of inactive GDP bound protein and down regulate the signal transduction pathway (Wennerberg et al., 2005). All of the Guanine Nucleotide Binding Proteins consist of a highly conserved ~20kDa catalytic domain known as G domain (Figure 2 & 3). The G domain binds to GTP and GDP at its switch region I & II and is responsible for activation of GTPases (Paduch, Jelen, & Otlewski, 2001). There is 30-55% homology in the amino acid sequence of different members of Ras superfamily (Takai et al., 2001). Different family members perform different functions: Ras GTPases regulate gene expression, proliferation, and death/survival. Rho/Rac/Cdc42 regulate cytoskeleton reorganization, gene expression, cell shape, migration & contraction. Rab proteins regulate intracellular vesicular trafficking. Ran GTPases regulate nucleocytoplasmic transport during G1, S and G2 phase of the cell cycle and in microtubule organization. Sar1/Arf proteins control vesicle budding (Wennerberg et al., 2005). My focus of discussion will be on Cdc42, a member of the Rho subfamily.

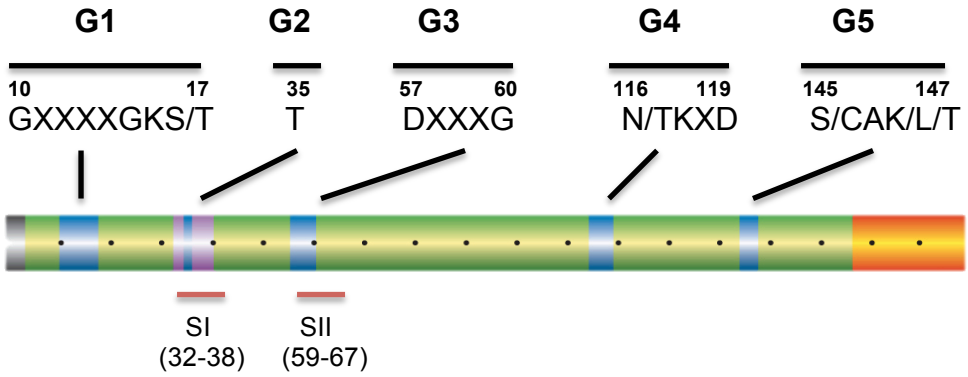

Figure 2: Functional domains for Ras superfamily: : G Domain (5-166), : Membrane targeting sequence (167-188/189), : G Motifs, : Core effector domaiin (32-40), G1-G5: Five G-Motifs that bind GTP/GDP, SI: Switch I, SII: Switch II (Wennerberg, K, Rossman, K.L. & Der, C.J. 2005)

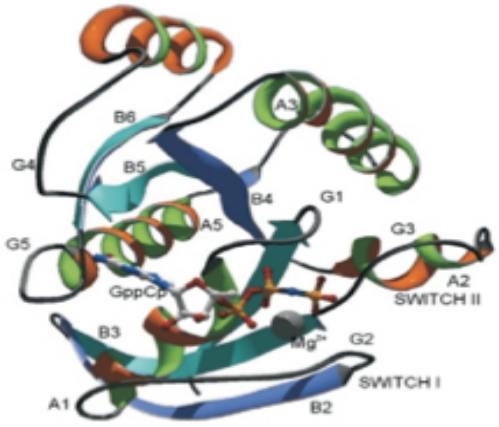

Figure 3: G Domain showing five α helices (A1 – A5), six β sheets (β1 – β6) and five polypeptide loops (G1 – G5) (Paduch, M., Jelen, F., & Otlewski, J. 2001 Structure of small G proteins and their regulators. *Acta Biochim Pol, 48*(4), 829-850.**).**

1.2 Rho Subfamily

Rho family of GTPases is a subfamily of Ras Superfamily and has molecular weight of ~21 kDa (Tang, Olufemi, Wang, & Nie, 2008). In mammals Rho GTPases contain 20 members, subdivided into 8 subfamilies: Rho, Rnd, Rho D/F, Rho H, Rac, Cdc42, RhoU/V and RhoBTB (Figure 4) (Vega & Ridley, 2008). Rnd, RhoU, RhoV, RhoH and RhoBTB are the atypical Rho GTPases. They are predominantly bound to GTP due to amino acid substitution at residues critical for GTPase activity (Heasman & Ridley, 2008). Rho BTB3 and Miro1 and 2 lack a proper Rho insert domain, hence are not considered part of the Rho family of GTPases (Vega & Ridley, 2008). RhoA, Rac and Cdc42 are the members that are most conserved and extensively studied (Heasman & Ridley, 2008). Rho GTPase family members play an important role in regulating actin dynamics, organelle development, cytoskeleton re-organization and other cellular functions (Csepanyi-Komi et al., 2012). Rho A plays important role in the formation of focal adhesions and actin related stress fibres (Flinn & Ridley, 1996). Rac1 plays important role in the formation of lamellopodia formation, cytokinesis, chemotaxis and cell polarity (Nobes & Hall, 1999). Cdc42 plays significant role in the formation of filopodia (Phillips, Calero, Chan, Ramachandran, & Cerione, 2008).

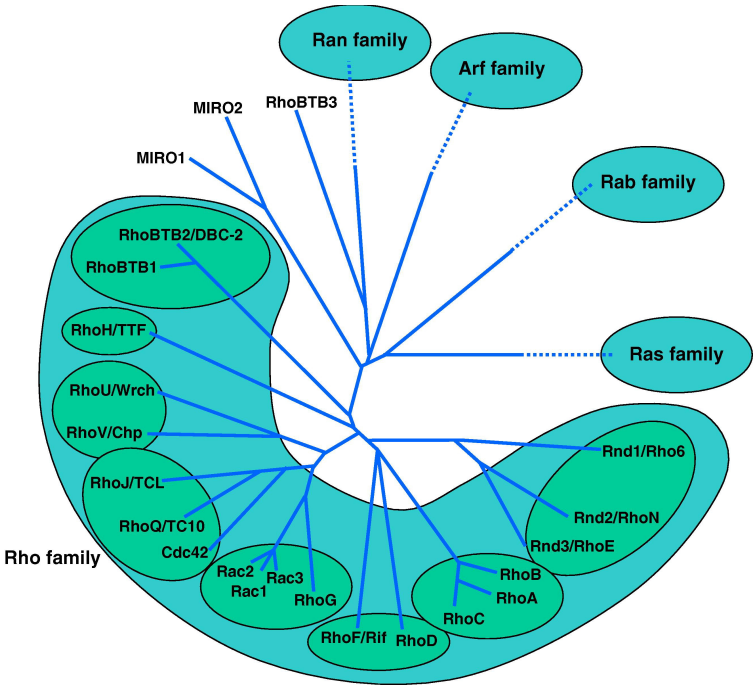

Figure 4: Phylogenetic tree showing the mammalian Ras GTPase superfamily. The 20 Rho GTPase family members are grouped into 8 subfamilies (Vega & Ridley, 2008).

1.3 Cdc42

Cdc42 is a ~21.3 kDa, 191 amino acids long small GTPase that belongs to the Rho family of GTPases (Johnson, 1999). Active form of Cdc42 regulates variety of cellular functions such as actin cytoskeleton organization, cell cycle regulation, intracellular trafficking, cell polarity determination and degradation of epidermal growth factor receptor (Cerione, 2004; Phillips et al., 2008). Cdc42 regulates these functions as it acts as a bimodal switch in the cell. It cycles between an active GTP bound form and the inactive GDP bound form (Heasman & Ridley, 2008; Phillips et al., 2008). It also cycles between the membrane bound form and cytosolic bound form in association with RhoGDIs (Jilkine, Maree, & Edelstein-Keshet, 2007; Takai et al., 2001). Different cellular proteins regulate this cycle that includes: Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs) (Bishop & Hall, 2000). There are over 70 GEFs that are classified into two families: Dbl family GEF and DOCK1 family GEF. Dbl family GEFs contain DH/PH domain & DOCK family GEFs contain DOCK180 homology domain DHR1 & DHR2 (Heasman & Ridley, 2008). GEFs activate the Cdc42 by promoting the exchange from GDP to GTP bound state. In this process, GEF binds to inactive Cdc42, forms Cdc42-GDP-GEF complex and destabilizes GDP. As concentration of GTP is 10 times higher than GDP in the cell, GTP replaces GDP resulting in active Cdc42. Binding affinity of GEF to GTP bound Cdc42 is less and is released from active Cdc42 (Sinha & Yang, 2008). GAPs increase the intrinsic GTP hydrolysis activity, thus convert the protein into the inactive form (Sinha & Yang, 2008). There are ~80 GAPs known for Cdc42 (Heasman & Ridley,

2008). RhoGDI plays significant role in down regulation of Cdc42 signaling and regulate subcellular localization of Cdc42 (Olofsson, 1999). RhoGDI inhibits spontaneous nucleotide exchange by preventing the dissociation of guanine nucleotide. RhoGDI interact with cytosolic face of Cdc42 at switch region I and II limiting the accessibility of Cdc42 to guanine nucleotides (Dovas & Couchman, 2005). It extracts the GDP bound Cdc42 from the cell membrane into cytoplasm (Jilkine et al., 2007; Olofsson, 1999). GDP dissociation stimulators and ERM (ezrin/ radixin/ moesin) proteins interact with RhoGDI at Cdc42 binding pocket and displace Cdc42, resulting in Cdc42 translocation from cytosol to cell membrane (Cerione, 2004; Takahashi et al., 1997). There are three mammalian GDIs: Rho GDIα, Rho GDIβ and RhoGDIγ. Rho GDIα is expressed ubiquitously; Rho GDIβ is expressed in hematopoietic tissues, specifically in B and T lymphocytes and Rho GDIγ is expressed in brain, pancreas, lungs and kidney (Dovas & Couchman, 2005; Olofsson, 1999).

1.4 Mechanism of Action of Cdc42

Various upstream signaling molecules such as growth factors, GPCR ligands, proteoglycans, cytokines and integrins activate Cdc42. It mediates multiple signaling pathways: GPCR pathway, tyrosine kinase receptor pathway, cytokine receptor, integrin and proteoglycan pathway (Cerione, 2004). Activated Cdc42 binds to an array of downstream effector proteins and activates them by disruption of intramolecular auto inhibitory interaction leading to the exposure of functional domain within effector proteins (Bishop & Hall, 2000).

Most of the Cdc42 effector proteins contain conserved GTP-binding motif known as CRIB (Cdc42/Rac interactive binding motif). The effector proteins that contain CRIB motif include MLK2, MLK3, MEKK1, 4, Pak1, Pak2, Pak3, Pak4, Pak5, Pak6, MRCKα, MRCKβ, Ack1, Ack2, WASP, N-WASP, MSE55, BOKGs (Bishop & Hall, 2000). IQGAP1 and IQGAP2 are the effector proteins that bind unique insert region corresponding to amino acids 122-134 of Cdc42 (Li et al., 1999). Other known effector proteins are p70 S6 kinase, PI3K, PLD, PLC-β_2, CIP-4. Cdc42 effector proteins interact specifically with the active form of the Cdc42 (Bishop & Hall, 2000). Cdc42 is unable to discriminate between downstream effector proteins because most of them have the same Cdc42 binding motif with similar binding affinity. Specificity for signaling is derived through upstream signals or by guanine nucleotide exchange factors (Sinha & Yang, 2008).

1.5 Cdc42 Localization

In mammalian cells Cdc42 is predominantly localized at Golgi apparatus but it has also been shown to be present at the plasma membrane and on numerous vesicular structures scattered throughout the cytoplasm (Osmani, Vitale, Borg, & Etienne-Manneville, 2006). Posttranslational modification plays an important role in subcellular localization (Roberts et al., 2008). Posttranslational modification of Cdc42 at CAAX sequence (where C stands for cysteine, A for aliphatic amino acid and X is any amino acid) at the C-terminal promotes its association with cell membranes (Johnson, 1999; Roberts et al., 2008). Geranylgeranylation of cysteine of CAAX motif followed by proteolysis of AAX amino acids allow geranylgeranylated cysteine anchor to lipid bilayer of cell membranes through its geranylgeranyl moiety (Casey & Seabra, 1996; Hoffman, Nassar, & Cerione, 2000).

1.6 Cdc42 Structure

The molecular structure of Cdc42 consists of centrally located 6 stranded β sheets surrounded by α helices, with all the β strands parallel except β_2 (Feltham et al., 1997). Cdc42 consists of 4 functional domains: GTP binding/hydrolysis domain, effector domain, Rho insert domain and membrane localization domain (Figure 5) (Johnson, 1999). Unlike other small G Proteins, GTP and GDP bound forms show little conformational differences that are mostly confined to switch regions I & II (Phillips et al., 2008) and correspond to amino acids 26-45 and 59-74 respectively (Bishop & Hall,

2000). It is the plasticity of switch I that exposes certain residues and allows effector proteins to distinguish between GTP and GDP bound forms of the protein (Phillips et al., 2008). Rho insert domain is only found in the Rho GTPases (Johnson, 1999) and corresponds to 13 amino acids from 122-134 (Feltham et al., 1997). Amino acids 124-128 form short α_1 helix adjacent to β_4 and α_3 loop (Feltham et al., 1997). Effector domain/switch I domain lies between residues 26 and 50 and is important for downstream effector function (Johnson, 1999)

Figure 5: Cdc42 linear schematic diagram showing various functional domains: GTP binding/hydrolysis domain (Blue), effector domain (Red), Rho insert domain (Purple) and membrane localization domain (green).

1.7 Functions of Cdc42

Cdc42 regulates actin cytoskeleton reorganization and filopodia formation (Heasman & Ridley, 2008). Microinjection of serum starved Swiss3T3 cells with Cdc42Hs induced peripheral actin micro spikes formation and promoted filopodia formation (Kozma, Ahmed, Best, & Lim, 1995). Cdc42 interacts with the Wiskott Aldrich Syndrome protein (WASP), which activates ARP2/3 complex and initiates branched actin filament network (Machesky & Insall, 1998). Other target proteins in the Cdc42 signaling pathways such as mDia and IRSp53 also induce filopodia formation by promoting actin polymerization and inducing membrane curvatures (Heasman & Ridley, 2008). Cdc42 mediates formation of myelin sheaths around axons in the central nervous system. Myelin sheaths are thin in the absence of Cdc42 (Heasman & Ridley, 2008; Thurnherr et al., 2006). Cdc42 is also required for Schwann cell proliferation in peripheral nervous system (Benninger et al., 2007). Disruption of Cdc42 leads to polarity defects, resulting in zero or multiple axons (Jaffe & Hall, 2005; Schwamborn & Puschel, 2004). Cdc42 also mediates actin independent signal transduction pathways such as JNK and p38 MAP kinase pathways (Jaffe & Hall, 2005). Cdc42 regulates JNK signaling pathway with the help of Cdc42 regulatory proteins RhoGEFs, RhoGAPs and RhoGDIs**.** RhoGEFs Ost and Onc-dbl promotes JNK activation whereas RhoGAPdp190 and RhoGDI inhibit JNK activation induced by RhoGEFs (Coso et al., 1995)**.** Cdc42 in response to stress related signals activates downstream signaling proteins MEK1/2 and SEK1, which in turn activate JNK in the signal transduction pathway (Auer et al., 1998)**.** Cdc42 induces chemotaxis and migration in various cell types such as macrophages,

fibroblasts and T cells (Heasman & Ridley, 2008). Decrease in Cdc42 activity in macrophages does not inhibit migration but blocks chemotaxis towards source of colony stimulating factor 1 (Allen, Zicha, Ridley, & Jones, 1998). Cdc42 also regulates chemotaxis and motility in neutrophils via MAPK signaling pathways (Szczur, Xu, Atkinson, Zheng, & Filippi, 2006). Cdc42 regulates cell polarity in all eukaryotic cells (Orlando & Guo, 2009). To establish cell polarity Cdc42 associates with Par6, Par3 and atypical protein kinase C and forms a stable Par complex, which regulates the vesicle transport and localization of cytoplasmic proteins to cell membrane (Orlando & Guo, 2009). Cdc42 also regulates various signaling pathways (such as phosphoinositide 3 kinase pathway, heterotrimeric G protein signaling pathway or tyrosine kinase receptor pathway) that participate in regulating cell polarity (Etienne-Manneville, 2004). Cdc42 controls different aspects of cell cycle such as mitosis and G1 progression (Jaffe & Hall, 2005). Cdc42 controls G1 progression by regulating the activity of cyclin dependent kinases such as Cdk4/Cdk6 and Cdk6 (Jaffe & Hall, 2005). Cdc42 plays a central role in mitosis and facilitates microtubule spindles attachment to chromosomes during mitosis (Jaffe & Hall, 2005). Depletion of Cdc42 in the cells leads to mitotic arrest (Jaffe & Hall, 2005; Yasuda et al., 2004).

1.8 Role of Cdc42 in Cancer Cell Biology

Role of Cdc42 in cancer progression is tissue specific. For most cell types Cdc42 is a pro-oncogenic factor. However, in few tissues it inhibits tumor progression (Vega & Ridley, 2008). Unlike Ras oncogenes, Cdc42 oncogenes do not have any activating mutations in human cancers (Rihet et al., 2001; Stengel & Zheng, 2011). Cdc42 protein levels are highly overexpressed; about 3-4 fold higher in breast tumor as compared to normal tissue from the same patient (Fritz, Brachetti, Bahlmann, Schmidt, & Kaina, 2002). Cdc42 protein as well as mRNA levels are enhanced in testicular tumor as compared to non-tumor testis (Kamai et al., 2004). Cdc42 is overexpressed with high incidence of (~60%) in colorectal cancer. The overexpression is associated with silencing of the putative tumor suppression gene ID (Gomez Del Pulgar et al., 2008). Cdc42 knockdown in liver enhances liver cancer development (Vega & Ridley, 2008). LKB1 a tumor suppressor gene maintains the active Cdc42 levels and PAK phosphorylation in a non-small cell lung cancer cell line (Zhang et al., 2008). In mouse lung cancer, Cdc42 plays important role in lung tumor progression. Cdc42 gene is under expressed in adenocarcinoma but showed no changes in adenoma (Yao, Wang, Lubet, & You, 2002). Cdc42 acts as tumor suppressor in Neuroblastoma as 90-95% cases of Neuroblastoma develop due to deletion of short arm of Chromosome 1, region that contains Cdc42 gene, resulting in decreased Cdc42 expression levels in Neuroblastoma (Valentijn et al., 2005).

1.9 Calcium

Calcium impacts various biological processes in the cell. Ca^{2+} ions bind to proteins in the cell and effect their localization, function, shape, charge and association with other proteins (Clapham, 2007). Ca^{2+} ions act as important second messenger in the cell (C. Fischer, Kugler, Hoth, & Dietrich, 2013). Thus, Ca^{2+} ions play significant role in cell signaling and regulate various biological processes such as muscle contraction, cell division, mitochondrial functioning, motility, innate immunity and apoptosis (Clapham, 2007). Intracellular concentration of calcium ions is 10^{-7}M. It is 10^{4} times lower than its extracellular concentration. Extracellular stimuli cause the rapid influx of calcium into the cell (Chin & Means, 2000). Most of the effects of calcium in the cells are mediated by the calcium modulating protein –Calmodulin (CaM). CaM converts calcium signal into physiological functions in the cell (Chin & Means, 2000). As the intracellular calcium concentration increases, CaM binds Ca^{2+} ions forming the Ca^{2+}/CaM complex, which binds to target proteins initiating various signaling cascades (Stevens, 1983). Calcium regulates CaM at cellular, intermolecular and submolecular level. At cellular level, calcium regulates intracellular localization of CaM. At intermolecular level, calcium dependent interactions mediate different ways for CaM to interact with target proteins. At submolecular level, calcium produces change in the conformation of CaM, resulting in change in affinity and functions of specific effector proteins (Chin & Means, 2000).

1.10 Calmodulin

Calmodulin is a highly conserved calcium binding protein (Kovalevskaya et al., 2013). Calmodulin is ~16.7kDa in weight and is found ubiquitously in all the eukaryotic cells (Kumar, Chichili, Tang, & Sivaraman, 2013). It constitutes 0.1% of total protein in the cell and is distributed throughout the cytosol and nucleus (Chin & Means, 2000). CaM acts as a calcium sensor and regulates calcium dependent signal transduction pathways by binding to different target proteins in the cells in response to changes in intracellular calcium concentration (Chin & Means, 2000). Posttranslational modifications such as methylation, acetylation, phosphorylation and proteolytic cleavage regulate CaM functions (Murtaugh, Rowe, Vincent, Wright, & Siegel, 1983). CaM regulates various cellular processes such as cell proliferation, migration, autophagy, apoptosis, cytokinesis, cell division, etc. (Geiser, van Tuinen, Brockerhoff, Neff, & Davis, 1991; Kumar et al., 2013).

1.11 Structure of Ca^{2+}/CaM Complex

Calmodulin is a dumbbell shaped protein with two globular domains at the N and C terminal separated by a central α helix (Figure 6b) (Babu, Bugg, & Cook, 1988; Chou, Li, Klee, & Bax, 2001). These domains are homologous to each other and share 46% sequence identity (Kumar et al., 2013; Yap, Ames, Swindells, & Ikura, 1999). Each globular domain consists of a pair of calcium binding EF hand motif: EF-1 & EF-2 in the N terminal domain and EF-3 & EF-4 in the C terminal domain (Babu et al., 1988;

Lakowski, Lee, Okon, Reid, & McIntosh, 2007). Each EF hand motif consists of 2α helix placed perpendicular to each other and joined by 12 amino acid loop (Kumar et al., 2013; Wilson & Brunger, 2000). The EF hand motifs provide electromagnetic environment that attracts calcium ions (Kumar et al., 2013; Wilson & Brunger, 2000). Hence each molecule of CaM can bind to four Ca^{2+} ions (Vogel, 1994; Yamniuk & Vogel, 2004). Calcium binding causes a change in the configuration of CaM (Vogel, 1994; Yamniuk & Vogel, 2004) that results in exposure of hydrophobic amino acids such as methionine that interact with target enzymes (Stevens, 1983). Calcium binding releases large amount of energy that alter CaM affinity for effector proteins (Chin & Means, 2000).

Calcium free CaM or ApoCaM, also regulates various target proteins and signaling pathways in the cell. It is globular ellipsoidal in shape (Yamniuk & Vogel, 2004). In ApoCaM, domain at N terminal is present in closed conformation such that hydrophobic amino acids are located in the inner side of the domain. Whereas the C terminal domain is present in semi open orientation that allows its interaction with target proteins in the absence of Ca^{2+} ions (Figure 6a) (Chin & Means, 2000).

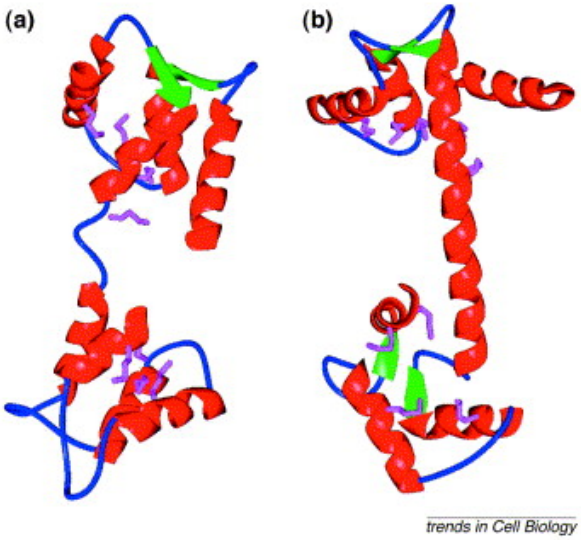

Fig: 6 (a) Structure of Calcium free Calmodulin – ApoCAM (Globular Ellipsoid) (b) Structue of Calcium bound Calmodulin (dumbbell shaped) (Chin & Means, 2000).

When Ca^{2+} binds ApoCaM, the N and C terminal globular domains move outward resulting in change in the shape of CaM. Globular ellipsoidal shape of ApoCaM changes to dumbbell shaped in Ca^{2+} bound CaM (Yamniuk & Vogel, 2004). Unlike Ca^{2+} saturated CaM where α helices in the EF hand motifs are perpendicular to each other, in ApoCaM they are arranged in antiparallel orientation (Babu et al., 1988; Kumar et al., 2013). Globular domains are in closed confirmation in the ApoCaM, where the hydrophobic amino acids are located within the interior of domain. Ca^{2+} binding changes the closed confirmation to an open confirmation (Kumar et al., 2013).

1.12 Mechanism of action of Ca^{2+}/CaM complex

Calmodulin has no catalytic activity of its own. Its role is to transduce the function of calcium to other target proteins in the cell (Swulius & Waxham, 2008). The exact mechanism by which CaM transduces calcium signals to downstream effector proteins is not very clear (Dagher et al., 2011). CaM specifically interacts with different CaM binding proteins in the cell. Numerous orientations of the N and C terminal lobes of CaM, due to flexible central α helix linker, provide this specificity. CaM adopts different conformations depending upon the number of calcium molecules bound (Dagher et al., 2011). CaM in the cell is divided into two different types (dedicated and promiscuous); depending upon the sequence in which it interacts with calcium and CaM binding proteins. In the dedicated type, CaM is bound to target proteins and regulates them upon calcium binding (Berchtold & Villalobo, 2014). Binding of CaM to target proteins increases its affinity for calcium (Olwin & Storm, 1985). However, the promiscuous type is the free CaM that first interacts with calcium followed by target protein regulation (Berchtold & Villalobo, 2014).

1.13 Calmodulin binding domain

CaM binding domain is the amino acid sequence within the target proteins that bind to CaM. CaM binding domain consists of 16-30 amino acids long amphipathic α helices with bulky hydrophobic and basic groups present at the end of the domain. CaM binding domains of different target proteins do not have conserved amino acid sequence. Based on the location of bulky hydrophobic amino acids region, CaM binding domain is classified into CaM binding motifs (C. Fischer et al., 2013; Yamniuk & Vogel, 2004). 1- 14, 1-10 and 1-16 are the most common recognition motifs, where number indicates the terminal hydrophobic amino acids (O'Day & Myre, 2004). Depending upon the position of intervening conserved hydrophobic amino acids, CaM binding motifs are divided into subgroups: 1-8-14, 1-5-8-14, 1-5-10 (Table 1) (O'Day & Myre, 2004; O'Neil & DeGrado, 1990). CaM binding proteins mediate calcium independent binding through IQ motif (IQXXXRGXXXR) where X is any amino acid. Similar to Ca^{2+}/CaM binding motif, IQ motif is highly hydrophobic and basic in nature (Yap et al., 2000).

Calcium-Dependent Motifs	
1-10 Motif	
1-10	(FILVW)xxxxxxxx(FILVW)
1-5-10	(FILVW)xxx(FALVWY)xxxx(FLVW)
1-5-10 basic	(RK)(RK)(RK)(FAILVW)xxx(FILV)xxxx(FILVW)
1-14 Motif	
1-14	(FILVW)xxxxxxxxxxxx(FILVW)
1-8-14	(FILVW)xxxxxx(FAILVW)xxxxx(FILVW)
1-8-14 basic	(RK)(RK)(RK)(FILVW)xxxxxx(FAILVW)xxxxx(FILVW)
1-5-18-14	(FILVW)xxx(FAILVW)xx(FAILVW)xxxxx(FILVW)
1-16 Motif	
1-16	(FILVW)xxxxxxxxxxxxxx(FILVW)
Calcium-Independent Motifs	
IQ Motif	(FILV)Qxxx(RK)Gxxx(RK)xx(FILVWY)
IQ-Like	(FILV)Qxxx(RK)xxxxxxxx

Table 2: Calcium dependent and Calcium independent CaM binding motifs (O'Day & Myre, 2004)

1.14 Biological Role of Calmodulin

CaM controls different cellular functions by its interaction with various target proteins in the cell. CaM regulates cyclic nucleotide metabolism by activating cyclic nucleotide phosphodiesterase and adenylyl cyclase (Walsh, 1983). CaM mediates platelet aggregation through CaM dependent enzyme phosphorylase A_2 (Walsh, 1983). CaM dependent protein kinases such as CaM Kinase I, II and IV transduce Ca^{2+}/CaM signal through phosphorylation of target proteins (Hudmon & Schulman, 2002). CaM kinase cascade modulates cell survival by activating protein kinase B (PKB) and MAP kinase (Soderling, 1999) . CaM plays a significant role in cell cycle regulation at G_1/S phase transition and at G_2 & mitotic phase (Yu, Dai, Pan, Chen, & Li, 2005). CaM mediates cytoskeleton reorganization by its association with CaM binding protein Kir/Gem (Berchtold & Villalobo, 2014). CaM also regulates intercellular communication between cells through another CaM binding protein: Connexins (Berchtold & Villalobo, 2014). CaM plays important role in energy metabolism by regulating NAD^+ kinase in plants. It regulates neurotransmitter synthesis by controlling tryptophan and tyrosine 3-monooxygenase kinase (Stevens, 1983).

1.15 Regulation of small G Proteins by Calmodulin

CaM has been shown to interact with a number of small G-proteins. It interacts with RalA and RalB small GTPase to their N-terminal CaM binding regions in Ca^{2+} independent manner and to their C-terminal CaM binding regions in Ca^{2+} dependent manner (Clough, Sidhu, & Bhullar, 2002). CaM plays a central role in regulating the thrombin-induced activation of Ral GTPases in platelets, as incubation of platelets with W7 (CaM inhibitor) decreased the thrombin-induced activation of Ral in platelets (Clough et al., 2002). Ca^{2+}/CaM causes 3 times increase in the activation of RalA as well as enhance the GTPase activity of RalA, hence regulating the GTP/GDP state of RalA (Park, 2001; Wang & Roufogalis, 1999). CaM binds to KRasB, but not to KRasA, HRas or NRas and regulates the activation of KRas but not HRas or NRas (Villalonga et al., 2001). This difference in interaction with CaM between different Ras isoforms is due to variable C-terminal regions, as C- terminal truncated HRas showed binding with CaM similar to full length KRas (Villalonga et al., 2001). CaM interacts with the active form of Rab3A and inhibits the calcium dependent exocytosis (Wang, Khan, & Roufogalis, 1997). CaM interacts with Rac1 and Cdc42 in platelets. It also regulates the activation of Rac1 and Cdc42, thus playing a significant role in the cytoskeleton rearrangement (Elsaraj & Bhullar, 2008). CaM interacts directly with Rin small GTPase in a Ca^{2+} dependent manner and regulates Rin mediated neuronal signaling pathways leading to neurite overgrowth, as incubation of PC12 cells with W13 (CaM inhibitor) resulted in decreased neurite overgrowth (Hoshino & Nakamura, 2003). CaM binds to members of Rad family small GTPases (Rad, Kir, Gem) in a calcium dependent

manner and regulates their activation by inhibiting GTP binding to Kir/Gem (R. Fischer, Wei, Anagli, & Berchtold, 1996). CaM exhibits 5-fold higher binding to GDP-Rad as compared to GTP-Rad. Moreover, S105N (dominant negative Rad mutant) when expressed In C2C12 cells showed increased binding to CaM as compared to wild type Rad (Moyers, Bilan, Zhu, & Kahn, 1997). CaM interacts with Rad, Kir and Gem at their CaM binding region located at the C-terminal region corresponding to amino acids 278-297 of Rad, 264-288 of Kir and 265-289 of Gem (R. Fischer et al., 1996; Moyers et al., 1997).

1.16 CHRF 288-11 cells

CHRF 288–11 cells are the late stage megakaryocytic cells that can later mature into platelets (van der Vuurst et al., 1997). These cells contain markers and growth factors similar to platelets and synthesize most of the proteins found in platelets. CHRF 288–11 cells are round in shape approximately 15 to 20 micron in diameter with granular cytoplasm and oval nucleus having 2 -3 nucleoli (Fugman, Witte, Jones, Aronow, & Lieberman, 1990). They have been used as a model for studying platelet signal transduction processes.

2. Hypothesis

It has been shown that Cdc42 is a CaM modulated protein. Cdc42 interacts with CaM in platelet cell lysates and plays an important role in Cdc42 activation in the platelets (Elsaraj & Bhullar, 2008). According to CaM target database analysis amino acids 151 – 163 is the region in Cdc42 that interacts with CaM (Figure 7). However, no study has been done to confirm the region in Cdc42 that binds to CaM in CHRF cells (a megakaryocytic cell line) and its role in regulating Cdc42 function. The fact that CaM regulates Cdc42 led us to test the hypothesis that CaM requires basic amino acids within 151-163 amino acid region of Cdc42 for its binding and that CaM plays significant role in regulating the function of Cdc42.

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MQTIKCVVVG DGAVGKTCLL ISYTTNKFPS EYVPTVFDNY AVTVMIGGEP
0000000000 0000000000 0000000000 0000000000 0000000000

YTLGLFDTAG QEDYDRLRPL SYPQTDVFLV CFSVVSPSSF ENVKEKWVPE
0000000000 0000000000 0000000000 0000000000 0000000000

ITHHCPKTPF LLVGTQIDLR DDPSTIEKLA KNKQKPITPE TAEKLARDLK
0000000000 0000000000 0000000000 0000000000 0001234678

AVKYVECSAL TQKGLKNVFD EAILAALEPP EPKKSRRCVL LTSLQSPFCT
9999999999 9998764321 0000000000 0000000000 0000012333

AGVGIILKAM FKSNRRL
3333333333 3333321
```

Fig 7: Calmodulin database analysis for Cdc42 showing the CaM binding domain: Normalized scores (0 to 9) are shown below the sequence indicating scoring based on the evaluation criteria. A consecutive string of high values indicates the location of a putative CaM binding site. Calmodulin target database used: http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.

3. Objectives

(i) To determine the CaM binding region in Cdc42:

(a) Determine whether the Cdc42 M (Δ151-163) interacts with CaM from CHRF cell lysate.

(b) Determine whether the Cdc42 M (Δ151-163) interact with pure CaM.

(c) Determine specific amino acids in region 151 – 163 that play a role in interaction of Cdc42 with CaM.

(ii) To determine the functional importance of CaM binding domain of Cdc42 GTPase.

(a) Determine the effects of mutations on Cdc42 activation.

(b) Determine GTP binding ability of Cdc42 mutants using *in vitro* loading of GTPγS.

4. Materials and Methods

4.1 Materials

GST-Cdc42WT and GST-Cdc42 mutant (amino acids 151 -163 deleted) bacterial expression plasmids were previously generated (Elsaraj & Bhullar, 2008). CHRF cells and GST-Pak 5 bacterial expression plasmids were kindly provided by Dr. Bing Xu and Dr. Olga Villamar – Cruz respectively. CaM was purchased from BioWorld. Leupeptin, fetal bovine serum, thrombin, lysozyme, glutathione agarose beads and polybrene were from Sigma Aldrich. Isopropyl 1–thio–β–D-galactopyranoside (IPTG), trypsin and RPMI 1640 medium were purchased from GIBCO. Primers were obtained from Invitrogen. Restriction enzymes and 1Kb DNA ladder were from New England Biolabs. Takara DNA ligation kit was purchased from Clontech Laboratories. Quick-change lightning mutagenesis kit was purchased from Agilent Technologies. Gel purification kit and Maxi prep kit were from Qiagen. Mini prep kit was obtained from Geneaid. Monoclonal Cdc42 antibody and monoclonal CaM antibody were purchased from BD Transduction. Anti HA antibody was purchased from Santa Cruz Biotechnologies. Horseradish peroxidase-conjugated secondary goat anti-mouse antibody was from Thermoscientific. Prestained low range SDS PAGE molecular weight standards and horseradish peroxidase-conjugated secondary goat anti- rabbit antibody were from Bio Rad Laboratories. PVDF membrane, ECL kit and Amersham hyperfilms were purchased from GE Healthcare.

4.2 Protein expression and purification

GST and GST fusion proteins, GST-Cdc42 wild type, GST-Cdc42 mutants and GST-Pak5 were expressed in *E. coli* DH5α cells and purified using glutathione agarose beads. In brief, 50µl of bacterial stock was inoculated into 5ml LB media supplemented with 50µg/ml ampicillin and incubated overnight at 37°C with shaking. Next day, 500µl to 1000µl of the culture was transferred into 200ml LB media, supplemented with 50µg/ml ampicillin and incubated overnight at room temperature with shaking. The following day, bacterial culture was incubated with 0.5mM IPTG for 2 hrs at room temperature. After 2 hrs, bacterial culture was centrifuged at 6,000 rpm for 20 min at 4°C. Cell pellet collected was re-suspended in 16ml NETT buffer consisting of 20mM Tris-HCl (pH 7.5), 1mM EDTA, 100mM NaCl and 1%Triton X-100. Cells were glass homogenized, followed by addition of 16mg lysozyme and 1mM PMSF. Cell suspension was incubated at room temperature for 10 min. After incubation, cells were sonicated twice for 30 sec. The lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant containing the GST fusion protein was incubated with glutathione agarose beads (prepared in NT buffer in 1:1v/v) for 30 min at 4°C, to isolate recombinant protein. The beads were washed 3 times with NETT buffer and 2 times with NT buffer containing 20mM Tris-HCl (pH 8.0) and 100mM NaCl to remove unbound proteins. 20µl of the beads were used to check the purity of the final preparation using SDS-PAGE followed by Coomassie staining.

4.3 Preparation of Glutathione Agarose Beads

0.1 g glutathione agarose beads were suspended in 1.5 ml of water. Excess water was removed by centrifugation. Finally 1.5ml NT buffer was added. Beads were allowed to stand for 5 min before use.

4.4 Cell Culture

CHRF 288-11 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (v/v) and 1% Penicillin Streptomycin at 37°C and 5% CO_2 in 75 cm^2 flasks.

4.5 Binding assay – Pure Calmodulin

Purified GST or GST-Cdc42 wild type or GST-Cdc42 mutants bound to glutathione agarose beads were taken in 500µl of MOPS buffer consisting of 30mM MOPS (pH 7.2), 1% NP-40 and 100mM KCl. Reaction conditions were buffer alone plus 20µg pure CaM or buffer plus 5mM Ca^{2+} and 20µg pure CaM added to tubes containing 100µl of GST-Cdc42 wild type or GST-Cdc42 mutant beads. Reaction mixture were incubated for 2 hrs at 4°C with shaking. After 2 hrs, the beads were washed three times with MOPS buffer. 35µl of Laemelli sample buffer was added to the beads and samples were heated at 100°C for 5 min to elute the proteins. Eluted proteins were subjected to 12% SDS-PAGE, transferred to PVDF membrane and western blot was performed using anti-CaM antibody (1:1000 dilution) and horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen antibody complex was visualized using enhanced chemiluminescence.

4.6 Binding assay – Endogenous Calmodulin

CHRF cells were washed with phosphate buffer saline (consisting of 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4) and lysed in 500µl MOPS buffer containing protease inhibitor cocktail (consisting of leupeptin 5µg/µl, aprotinin 2µg/µl and 1mM PMSF) by sonication twice for 30 sec. The cell lysate was centrifuged at 14,000g for 10 min at 4°C and supernatant was collected. After centrifugation, the supernatant was incubated with 100µl of GST-Cdc42 wild type or GST-Cdc42 mutant beads for 2 hrs at 4°C with shaking. Reaction conditions were lysate alone or lysate plus 5mM calcium. After 2 hrs, the beads were washed 3 times with MOPS buffer. 35µl of Laemelli sample buffer was added and samples were heated at 100°C for 5 min to elute the proteins. Eluted proteins were subjected to 12% SDS-PAGE, transferred to PVDF membrane and western blot was performed using anti-CaM antibody (1:1000 dilution) and horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen antibody complex was visualized using enhanced chemiluminescence.

4.7 Mutagenesis

Cdc42 mutants Cdc42K153A and Cdc42K163A were generated using Quick Change Lightning Site Directed Mutagenesis Kit. The full length GST-Cdc42 was used as the template. Mutagenic primers were obtained from Invitrogen. The primers used to mutate Cdc42K153A were CGTGACCTGAAGGCTGTGGCGTATGTGGAGTGTTCTGC (forward) and GCAGAACACTCCACATACGCGACAGCCTTCAGGTCACG (reverse). The primer used to mutate Cdc42K163A were

GGAGTGTTCTGCACTTACACAGGCAGGCCTAAAGAATGTATTTGAATTTGAC (forward) and GTCAAATACATTCTTTAGGCCTGCCTGTGTAAGTGCAGAACACT CT (reverse). PCR reaction cycling parameters were as follows: initial cycle of denaturation was performed for 20 min at 95°C, followed by 18 cycles of denaturation at 95°C for 20 sec. Annealing was done at 60°C for 10 sec and elongation reaction performed at 68°C for 30 sec/Kb of the plasmid. Final elongation was done at 68°C for 5 min. Both the mutants were sequenced using MICB DNA sequencing service to continue the expected mutation.

4.8 Subcloning and Packaging into Lentivirus

Cdc42WT, K153A and K163A mutants were subcloned into lentivirus vector Cpp2E using BamH1 and Sal1 restriction sites. Lentivirus vector Cpp2E containing HA tag and GST-Cdc42 wild type or GST-Cdc42 mutant plasmids were digested with restriction enzymes BamH1 and Sal1 for 15 min at 37°C. 1% agarose gel was run to separate the resulting DNA fragments. HA-Cpp2E and band containing Cdc42 wild type or Cdc42 mutants were isolated using Qiagen Gel Purification Kit. The purified DNA fragment were ligated using Takara ligation kit for 30 min at 16°C in a water bath. Ligated plasmids were transformed into DH5α *E. coli* competent cells. Transformed cells were plated on LB culture plates containing 100µg/ml ampicillin and incubated overnight at 37°C. Plasmids were extracted from the bacteria using Qiagen Maxi Kit and were packaged into lentivirus using the core facilities in the Department of Immunology, University Of Manitoba.

4.9 Infection of CHRF cells with lentivirus

CHRF cells (10^5 cells/sample) were suspended in RPMI 1640 medium. Cells were transfected with 1X dilution of Cdc42 wild type or Cdc42 mutant lentivirus using Polybrene (Sigma) as per the manufacturer's protocol. After 4 days of incubation at 37°C and 5% CO_2, the cells were lysed in lysis buffer and protein expression was checked using anti HA antibody.

4.10 Preparation of GST-Pak 5

GST-Pak5 (Pak binding domain) was generated using polymerase chain reaction. Primers used were ATG TTT GGG AAG AAA AAG AAA (forward) and TGG CAC AGC CTG TTA GCA GAT (reverse). Plasmids obtained were digested using EcoR1 and Sal1 for 2 hrs at 37°C and were ligated into pGEX-2T at 16°C for 30 min. Ligation product was transformed into DH5α *E. coli* competent cells. Transformed cells were plated on LB culture plates containing 100µg/ml ampicillin and incubated overnight at 37°C. Bacterial culture was grown and plasmids were extracted from the bacteria using Mini Prep Kit and the results were confirmed by sequencing using MICB DNA sequencing services. GST-Pak5 (PBD) was purified using glutathione agarose beads and the protein expression was checked by gel electrophoresis and Coomassie blue stain.

4.11 GST-Pak 5 pull down of active form of Cdc42 or Rac1

To confirm that GST-Pak5 shows higher binding to active form of Cdc42 as compared to active form of Rac1, *in vitro* loading of Cdc42 and Rac1 in CHRF cell lysates with GTPγS or GDPβS was done. CHRF cells were washed with PBS and lysed using lysis

buffer consisting of 50mM Tris-HCl (pH 7.4), 1% NP-40, 500mM NaCl, 2.5mM $MgCl_2$, 10mM NaF, 10% glycerol, 1mM sodium orthovandate and protease inhibitor cocktail by sonication twice for 30 sec. The lysate was centrifuged at 14,000g for 10 min at 4°C and supernatant was collected. After centrifugation, 10mM EDTA and 100μM GTPγS or 100μM GDPβS were added to the supernatant and mixed. The mixture was incubated for 15 min at 30°C. The samples were kept on ice to stop the reaction. After incubation 60mM $MgCl_2$ was added. The reaction mixture was incubated with 100μL GST-Pak5 beads for 2 hrs at 4°C with shaking. The beads were washed 3 times with binding buffer. Beads were then suspended in 35μl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blot was done using anti Cdc42 antibody or anti Rac1 antibody (1:1000) followed horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1: 5000). The antigen antibody complex was visualized using ECL.

4.12 Role of CaM in the basal activity of Cdc42

Cdc42 wild type or Cdc42 mutants packaged in lentivirus were transfected into CHRF cells using Polybrene. After 4 days, the cells were lysed in RIPA buffer consisting of 50mM Tris-HCl (pH 7.4), 1% Triton X-100, and 0.5% sodium deoxycholate, 0.1% SDS, 450mM NaCl, 10mM $MgCl_2$, 2.5mM EGTA and protease inhibitor cocktail by sonication twice for 30 sec. The lysate was centrifuged at 14,000g for 10 min at 4°C. 30μl of the lysate was suspended in 10μl of Laemmli's sample buffer to determine the level of endogenous Cdc42. The rest of the lysate was incubated with 70μl GST-Pak5 beads for 2 hrs at 4°C with shaking. The beads were washed 3 times with washing buffer consisting of 50mM Tris-HCl (pH 7.4), 10mM $MgCl_2$, 150mM NaCl, 1% Triton X-100

and 5mM EGTA. Beads were then suspended in 35µl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blot was done using anti HA antibody (1:1000) and horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1: 5000). The antigen antibody complex was visualized using ECL.

4.13 GTP loading of HA-Cdc42 mutants

To confirm that there is no change in the ability of Cdc42 mutants to exchange GDP for GTP as compared to wild type Cdc42, *in vitro* loading of HA-Cdc42WT, HA-Cdc42K153A and HA-Cdc42K163A in CHRF cell lysates with GTPγS or GDPβS was done. CHRF cells were washed with PBS and lysed using lysis buffer consisting of 50mM Tris-HCl (pH 7.4), 1% NP-40, 500mM NaCl, 2.5mM $MgCl_2$, 10mM NaF, 10% glycerol, 1mM sodium orthovandate and protease inhibitor cocktail by sonication twice for 30 sec. The lysate was centrifuged at 14,000g for 10 min at 4°C and supernatant was collected. After centrifugation, 10mM EDTA and 100µM GTPγS or 100µM GDPβS were added to the supernatant and vortexed. The mixture was incubated for 15 min at 30°C. The samples were kept on ice to stop the reaction. After incubation 60mM $MgCl_2$ was added. The solution mixture was incubated with 100µL GST-Pak5 beads for 2 hrs at 4°C with shaking. The beads were washed 3 times with binding buffer. Beads were then suspended in 35µl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blot was done using anti HA antibody (1:1000) and horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1:5000). The antigen antibody complex was visualized using ECL.

4.14 Statistical Analysis

Radiographs were scanned and bands quantified using Alpha view Flourchem FC2 AIC program. Output was normalized and one-way ANOVA was used to determine the statistical significance.

5. Results

5.1 Expression of GST-Cdc42 wild type and GST-Cdc42 M (Δ151-163)

To determine whether Cdc42 wild type and Cdc42 M (Δ151-163) interact with CaM, GST-Cdc42WT and GST-Cdc42 M (Δ151-163) were used. GST-Cdc42WT and GST-Cdc42 M (Δ151-163) were expressed in *E. coli* and the expression levels were checked before performing the experiment. As expected, the expressed proteins have molecular weight of ~47kDa and there was no significant proteolytic degradation observed (Figure 8).

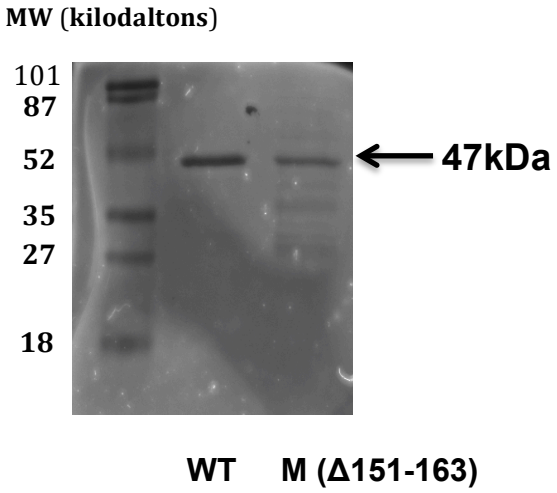


Figure 8: Expression of GST-Cdc42WT and GST-Cdc42 M (Δ151-163): GST-Cdc42WT and GST-Cdc42 M (Δ151-163) were expressed, isolated and purified from *E. coli* using glutathione agarose beads. Expression levels of the proteins were checked using 12% SDS-PAGE and Coomassie blue stain.

5.2 Cdc42 wild type and Cdc42 M (Δ151-163) interact with CaM.

CaM target database analysis has revealed that amino acids 151-163 of Cdc42 (AVKYVECSALTQK), is the CaM binding domain and has the potential to bind CaM (Elsaraj & Bhullar, 2008). To confirm this, Cdc42 wild type and Cdc42 M (Δ151-163) were expressed in *E. coli* and purified as GST fusion protein using glutathione agarose beads. GST-Cdc42 wild type and GST-Cdc42 M (Δ151-163) beads were incubated with CHRF cell lysate to examine the binding to endogenous CaM.

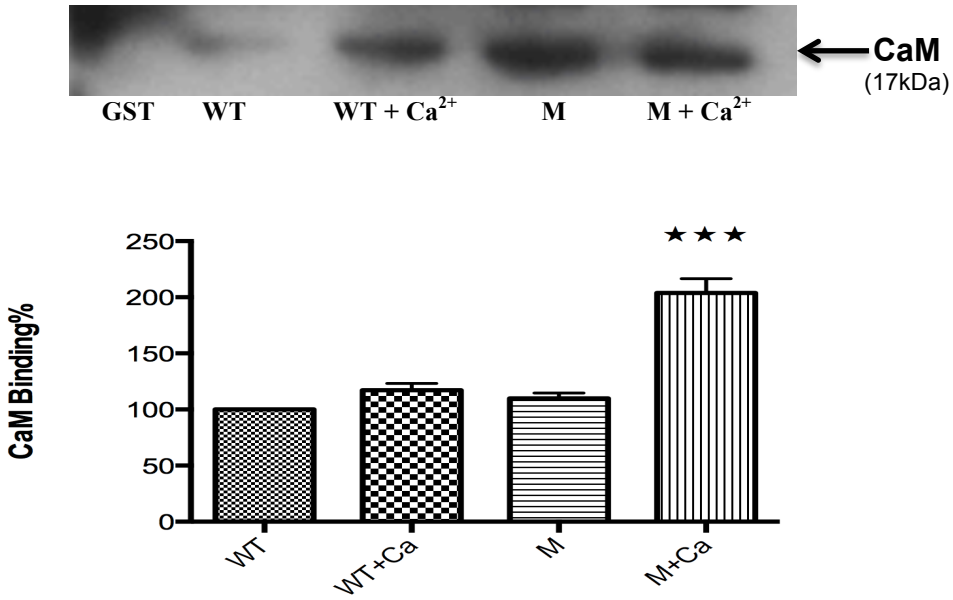


Figure 9: Deletion of putative CaM binding region of Cdc42 results in significant increase in its binding to CaM: GST-Cdc42 wild type and GST-Cdc42 M (Δ151-163) beads (100µl) were incubated with CHRF cell lysate prepared in MOPS buffer for 2 hrs at 4°C with shaking. Plain GST beads were used as negative control. The incubation conditions were: GST-Cdc42 wild type or GST-Cdc42 mutant beads with buffer alone or buffer plus 5mM Ca^{2+}. After incubation, beads were washed 3 times and bound proteins were eluted using Laemmli buffer. Western blot was done using anti CaM antibody. The antigen antibody complex was visualized using ECL. The experiment was repeated 3 times to confirm the results. Quantification was carried out using Alpha view Flourchem FC2 AIC program and p value < 0.001 were considered statistically significant.

As shown in Figure 9 Cdc42 wild type and Cdc42 mutant (amino acids 151-163 deleted), showed similar binding with endogenous CaM. However, Cdc42 M (Δ151-163) in the presence of calcium showed significantly increased binding to CaM as compared to wild type Cdc42. This suggests that amino acids 151-163 of Cdc42 are an important regulatory domain which when removed increases the binding of Cdc42 to CaM. Increase in CaM interaction with the deletion of amino acid 151 – 163 from Cdc42 shows that there could be an additional CaM binding site in another region of Cdc42 that becomes available upon deletion of the 151-163 amino acids region.

5.3 Cdc42 wild type and Cdc42 M (Δ151-163) interact with pure CaM.

In order to confirm that the interaction between Cdc42 wild type or Cdc42 mutant (amino acids 151-163 deleted) with Calmodulin in the previous experiment was direct, and no other protein is involved in the interaction, pure bovine brain CaM was used. GST-Cdc42 wild type or GST- Cdc42 M (Δ151-163) beads were incubated with 20µg of pure bovine brain CaM in MOPS buffer.

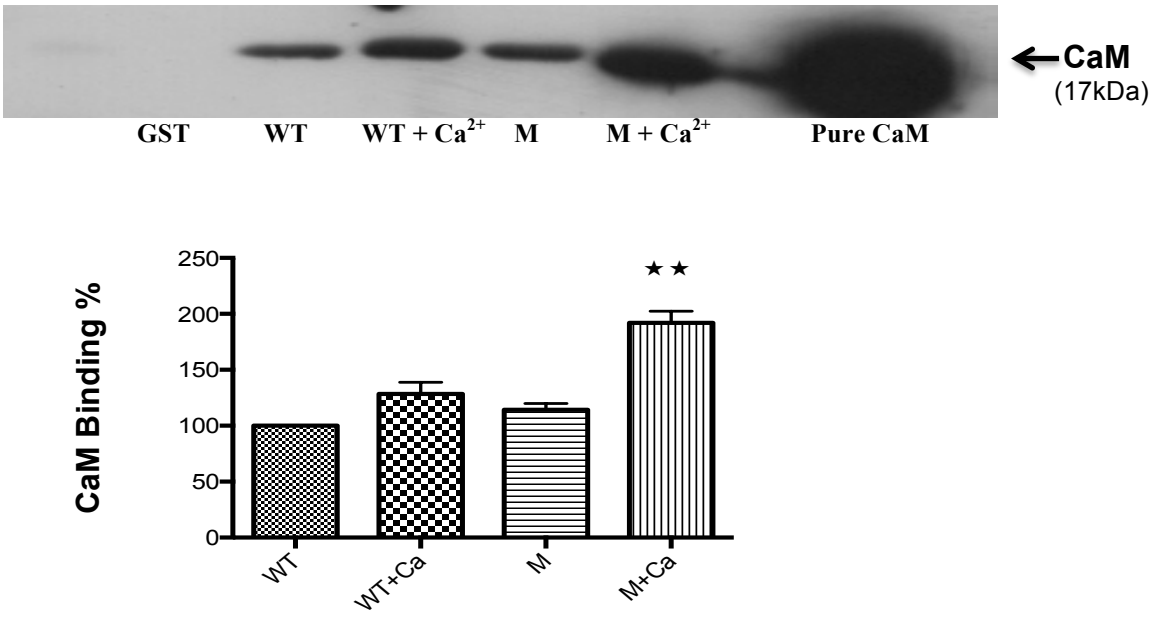


Figure 10: Cdc42 wild type or Cdc42 M (Δ151-163) bind to pure CaM: GST-Cdc42 wild type or GST-Cdc42 M (Δ151-163) beads (100µl) were incubated with pure bovine brain CaM (20µg) in MOPS buffer for 2 hrs at 4°C with shaking. The incubation conditions were with buffer alone and buffer plus 5mM Ca^{2+}. After 2 hrs, beads were washed 3 times and bound proteins were eluted in Laemmli's sample buffer by boiling the samples for 5 min. Western blot was done using anti CaM antibody. Experiment was repeated 3 times to confirm the results. Quantification was carried out using Aplha view Flourchem FC2 AIC program and p value < 0.01 were considered statistically significant.

The results were similar to the previous experiment. Both Cdc42 wild type and Cdc42 M (Δ151-163) showed binding to pure CaM indicating that there is direct interaction between Cdc42 wild type or Cdc42 M (Δ151-163) with CaM (Figure 10). The results also confirm that amino acid region 151 to 163 is an important regulatory domain that controls interaction between Cdc42 and CaM.

5.4 Identification of critical residues in Cdc42 GTPase CaM interacting domain.

As amino acids 151-163 are indicated to be the CaM binding region in full length Cdc42, we investigated role of basic amino acids within this region in CaM binding. So to identify specific amino acids in Cdc42 between 151 – 163 amino acid region that may interact with CaM, site directed mutagenesis of specific amino acids was done. The basic amino acids play important role in interaction between CaM and other proteins (Yamniuk & Vogel, 2004) Therefore two positively charged amino acids K153 and K163 were changed to alanine and the mutants Cdc42K153A and Cdc42K163A were generated. Cdc42 wild type, Cdc42K153A and Cdc42K163 were expressed as GST fusion proteins in *E. coli* and purified. The expression levels were assessed using SDS Page and Coomassie blue stain before performing the experiment. As expected, the expressed proteins have molecular weight of ~47 KDa (Figure 11).

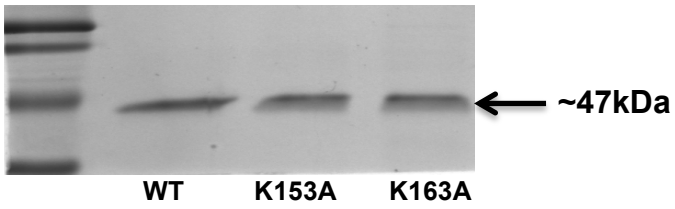


Figure 11: Expression of GST-Cdc42WT, GST-Cdc42K153A and GST-Cdc42K163A: GST-Cdc42WT, GST-Cdc42K153A and GST-Cdc42K163A were expressed, isolated and purified from *E. coli* using glutathione agarose beads. Expression levels of the proteins were checked using 12% SDS-PAGE and Coomassie blue stain.

GST-Cdc42 WT, GST-Cdc42K153A or GST-Cdc42K163A was incubated with pure bovine brain CaM in MOPS buffer. The results showed that there was no significant effect on Cdc42K153A interaction with CaM. However, the binding of Cdc42K163A to CaM was significantly reduced. The results indicate that amino acid K163 in Cdc42 is essential for interaction with CaM (figure 12).

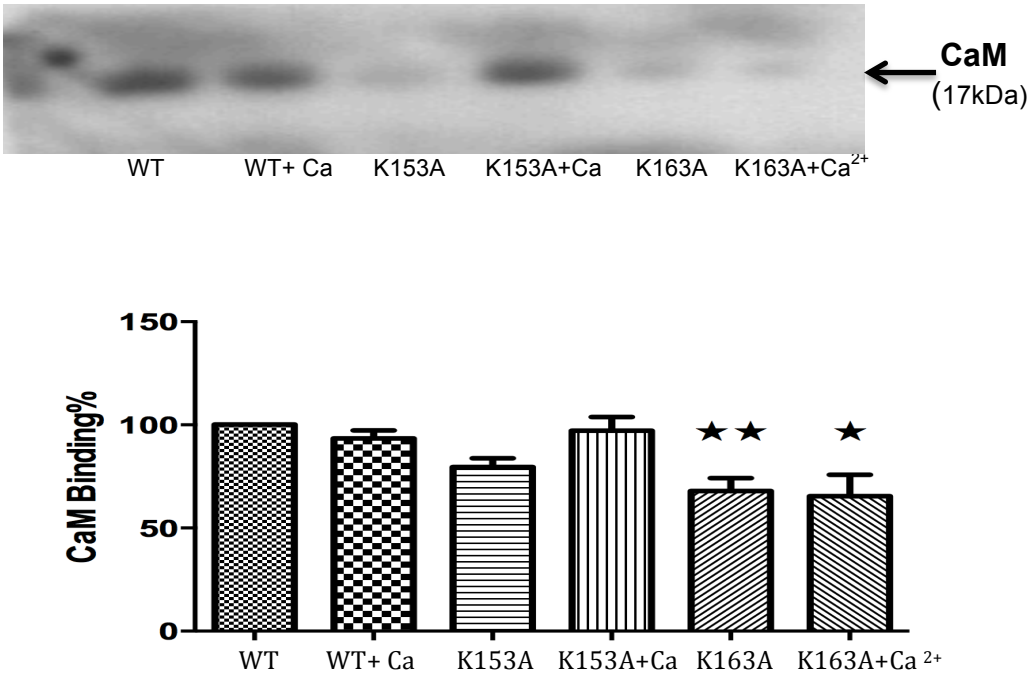


Figure 12: Binding of Cdc42WT, Cdc42K 153A and Cdc42K163A to pure CaM: GST-Cdc42 wild type or GST-Cdc42K153A or GST-Cdc42K163A (100µl) were incubated with pure bovine brain CaM (20µg) in MOPS buffer for 2 hrs at 4°C with shaking. The incubation conditions were with buffer alone and buffer plus 5mM Ca^{2+}. After 2 hrs, beads were washed 3 times and bound proteins were eluted in Laemmli's sample buffer by boiling the samples for 5 min. Western blot was done using anti CaM antibody. Experiment was repeated 3 times to confirm the results. Quantification was carried out using Alpha view Flourchem FC2 AIC program. **p value < 0.01 and *p value < 0.05 were considered statistically significant when compared to WT and WT+Ca^{2+} respectively.

5.5 Expression of GST-Pak5 (PaK binding domain/GTPase binding domain)

Pak – p21 activated kinase is the downstream effector of Cdc42 and Rac1 (Pandey et al., 2002). It is a serine threonine kinase that binds to active GTP bound forms of Cdc42 and Rac1 (Dan, Nath, Liberto, & Minden, 2002). There are a total of 6 Pak family members: Pak 1-6. Pak5 interacts preferentially with Cdc42 as compared to Rac1 (Pandey et al., 2002), through its GTPase binding domain (GBD) located in the amino terminal regulatory domain (Dan et al., 2002). In order to confirm this and to use Pak5 to preferentially pull the active form of Cdc42, GTPase binding domain of Pak5 was expressed as a GST fusion protein in *E. coli* and purified using glutathione agarose beads. Purity of the fusion protein was assessed before performing the experiment. As expected, the expressed protein has molecular weight of ~30kDa (Figure 13).

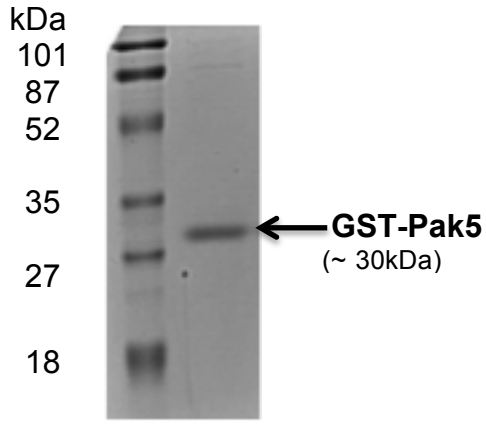


Fig 13: (a) Expression of GST-Pak5 (GTPase binding domain): GST-PaK5 GTPase binding domain was expressed, isolated and purified from *E. coli* using glutathione agarose beads. Expression levels of the proteins were checked using 12% SDS-PAGE and Coomassie blue stain.

(b) Amino acid sequence of GST-Pak5 (GTPase binding domain):
GST- EISGPSNFEHRVHTGFDPQEQKFTGLPQQWHSLLAD

5.6 GST- Pak5 pulls out GTP-Cdc42 from CHRF cell lysate

To confirm that GST-Pak5 shows preferential binding to the active form of Cdc42 as compared to the active form of Rac1, *in vitro* loading of Cdc42 and Rac1 in CHRF cell lysates with GTPγS or GDPβS was done. The results demonstrated that GST- Pak5 (GBD) binds to the active form of Cdc42 and it binds preferentially to GTP-Cdc42 as compared to GTP- Rac1 (Figure 14). GST-Pak1 was used as positive control in the experiment.

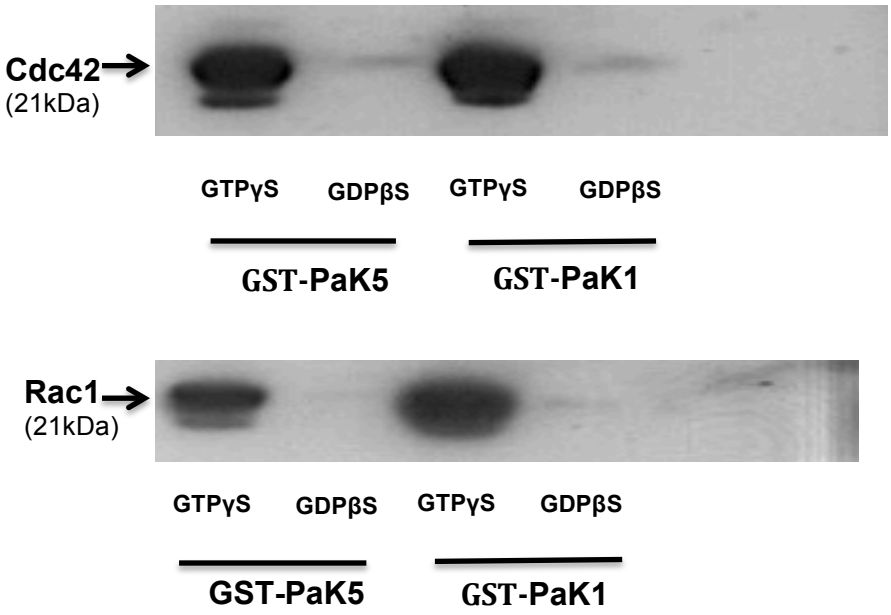
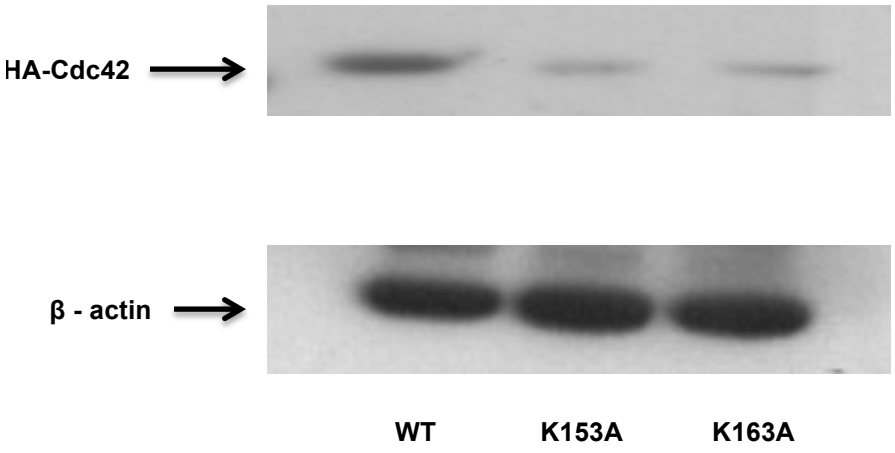


Fig 14: GST-Pak5 (GBD) pulls out GTP-Cdc42 preferentially over GTP-Rac1: CHRF cells were washed with PBS and lysed using lysis buffer by sonication for 30sec twice. The lysate was centrifuged at 14,000g for 10 min at 4°C. After centrifugation, 10mM EDTA and 100μM GTPγS or 100μM GDPβS were added to the supernatant. The mixture was incubated for 15 min at 30°C. After incubation 60mM $MgCl_2$ was added. The solution mixture was incubated with 100μL GST-Pak5 beads for 2 hrs at 4°C with shaking. Beads were then suspended in 35μl Laemmli's buffer and heated at 100°C for 5 mins. Western blot was done using anti Cdc42 antibody or anti Rac1 antibody (1:1000) and horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1: 5000). The antigen antibody complex was visualized using ECL.

5.7 Role of CaM in the basal activity of Cdc42

CaM plays important role in basal activity of Cdc42 (Xu & Bhullar, 2011). To investigate the effect of mutations in Cdc42 (where CaM binding is decreased) on its basal activity, wild type HA-Cdc42, HA-Cdc42K153A and HA-Cdc42K163A were transfected into CHRF cells using lentivirus vector. GST-Pak 5 was used to pull down the active form of the proteins. Results showed that Cdc42K153A and Cdc42K163A showed significantly decreased basal activity as compared to wild type Cdc42 in the cells (Figure 15).



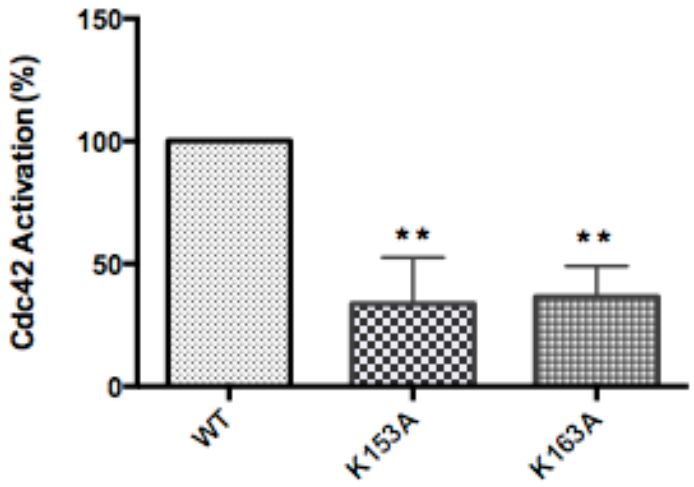


Figure 15: Role of CaM in basal activity of Cdc42: Cdc42 wild type or Cdc42 mutants packaged in lentivirus were transfected into CHRF cells. After 4 days, the cells were lysed using RIPA buffer consisting of 50mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate. 0.1% SDS, 450mM NaCl, 10mM $MgCl_2$, 2.5mM EGTA and protease inhibitor cocktail by sonication for 30 sec twice. The lysate was centrifuged at 14,000g for 10 min at 4°C. 30µl of the lysate was suspended in 10µl Laemmli's sample buffer to determine the level of endogenous Cdc42. The rest of the lysate was incubated with 70µl GST-Pak 5 beads for 2hrs at 4°C with shaking. The beads were washed 3 times with washing buffer consisting of 50mM Tris-HCl (pH 7.4), 10mM $MgCl_2$, 150mM NaCl, 1% Triton X-100 and 5mM EGTA. Beads were then suspended in 35µl Laemmli's buffer and heated at 100°C for 5 min. Western blot was done using anti HA antibody (1:1000) and horseradish peroxidase- conjugated secondary goat anti-mouse antibody (1: 5000). The antigen antibody complex was visualized using ECL. The experiment was repeated 3 times to confirm the results. Quantification was carried out using Alpha view Flourchem FC2 AIC program and p value < 0.01 were considered statistically significant.

5.8 GTP loading of Cdc42 Mutants: Cdc42K153A & Cdc42K163A

To confirm that point mutations in Cdc42: K153A and K163A did not result in any change in their ability to exchange GDP to GTP, experiment was done in which *in vitro* loading of HA-Cdc42WT, HA-Cdc42K153A and HA-Cdc42K163A in CHRF cell lysates with GTPγS and GDPβS was done. Active form of the proteins was pulled down using GST-Pak5. Results showed that there is no change in the ability of Cdc42K153A and Cdc42K163A to exchange GDP to GTP as compared to Cdc42WT.

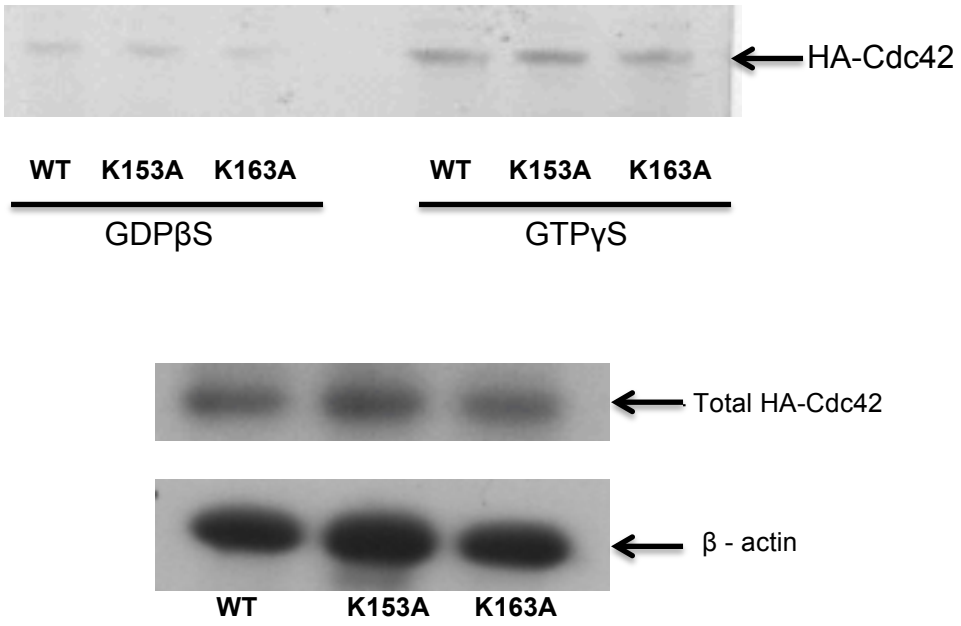


Figure 16: GTP loading of Cdc42 Mutants: CHRF cells were washed with PBS and lysed using lysis buffer consisting of 50mM Tris-HCl (pH 7.4), 1% NP-40, 500mM NaCl, 2.5mM $MgCl_2$, 10mM NaF, 10% glycerol, 1mM sodium orthovandate and protease inhibitor cocktail by sonication for 30sec twice. The lysate was centrifuged at 14,000g for 10 min at 4°C and supernatant was collected. After centrifugation, 10mM EDTA and 100μM GTPγS or 100μM GDPβS were added to the supernatant and were vortexed. The mixture was incubated for 15 min at 30°C. The samples were kept on ice to stop the reaction. After incubation 60mM $MgCl_2$ was added. The solution mixture was incubated with 100μL GST-Pak5 beads for 2 hours at 4°C with shaking. The beads were washed 3 times with binding buffer. Beads were then suspended in 35μl Laemmli's buffer and samples were heated at 100°C for 5 min. Western blot was done using anti HA antibody (1:1000)

6. Discussion and Conclusion

Cdc42 is a small G protein that belongs to the Rho family of Ras GTPase superfamily (Bishop & Hall, 2000). Cdc42 in its active form regulates various intracellular processes such as filopodia formation by actin cytoskeleton regulation, regulation of cell polarity by causing changes in Par6 and αPKC, chemotaxis, migration in macrophages, T cells and fibroblasts, intracellular trafficking, cell cycle regulation and cell division (Phillips et al., 2008). Active form of Cdc42 regulates various signaling pathways such as GPCR pathway, RTK pathway, proteoglycan pathway, etc. and activates various downstream effector proteins most of which contain CRIB motif (Bishop & Hall, 2000), (Cerione, 2004). As mentioned in introduction Cdc42 plays dual role in cancer progression with pro oncogenic and anti-oncogenic properties (Etienne-Manneville & Hall, 2002). Cdc42 plays significant role in cancer progression due to deregulation of roles of Cdc42 in cell proliferation, cell polarity, migration and survival, etc. (Etienne-Manneville & Hall, 2002) Cdc42 expression levels are increased in breast cancer, brain tumors, testicular cancer, cutaneous melanoma (Arias-Romero & Chernoff, 2013). However, Cdc42 acts as a tumor suppressor in liver as knockdown of Cdc42 in liver results in hepatomegaly and hepatocellular carcinoma (Arias-Romero & Chernoff, 2013).

CaM is a calcium modulating protein and is present ubiquitously in all eukaryotic cells (Kumar et al., 2013). It has no catalytic function of its own but acts as an adaptor protein and transduces the calcium signal to different target proteins. Thus, CaM plays important role in regulating various physiologic functions in the cell such as cell cycle

regulation, intercellular communication between cells, platelet aggregation, cell survival, etc. (Chin & Means, 2000). CaM interacts with various CaM binding proteins in the cell due to different orientation of the N and C terminal lobes of CaM (Dagher et al., 2011). CaM interacts with RalA and RalB and mediates the thrombin-induced activation of RalGTPases (Clough et al., 2002). CaM interacts specifically with KRasB and not KRasA, HRas or NRas (Villalonga et al., 2001). Calmodulin inhibits calcium dependent exocytosis by Rab3A (Coppola et al., 1999).

CaM interacts with Cdc42 in the platelets and plays significant role in its activity in the cell. CaM antagonist, W7, when added to platelets causes an increase in the level of Cdc42 activation. Therefore, CaM is proposed to keep Cdc42 in its inactive state in platelets (Elsaraj & Bhullar, 2008). However, W7 when added to the CHRF cell lysate decreased the basal activity of Cdc42 in cells indicating that CaM plays important role in maintaining basal activity of Cdc42 in CHRF 288–11 cells (Xu & Bhullar, 2011).. However, the interaction between CaM and Cdc42 in CHRF cells has not been analyzed before. In this study we analyzed if CaM interacts directly with Cdc42 in CHRF cells. We also analyzed the putative CaM binding region in Cdc42 and the role of specific amino acid residues K153 and K163 in this region for role in interaction with CaM. We also analyzed the role of CaM in the basal activation of Cdc42.

Small GTPase Cdc42 is a CaM modulated protein. CaM binding domain in CaM regulated proteins does not share sequence homology and is 16 – 30 amino acids

long consisting of basic and bulky hydrophobic groups (C. Fischer et al., 2013). According to CaM target database analysis the CaM binding domain in Cdc42 ranges from amino acids 151 -163. However, the deletion of amino acids 151 – 163 from Cdc42 did not decrease binding to CaM. On the contrary, there was significant increase in the binding of CaM to Cdc42 in the presence of calcium (Figure 9). Similar situation has been explained previously for HRas and RalB small GTPases**.** It has been shown that full length HRas does not bind to CaM whereas C–terminal truncated HRas interacts with CaM (Villalonga et al., 2001)**.** Similarly in case of RalB, C–terminal region has been shown to have inhibitory effect on interaction with CaM (Clough et al., 2002)**.** This shows that there could be another CaM binding domain in the N terminal region of Cdc42 that is expressed on deletion of the putative CaM binding region. However, according to CaM target database analysis for Cdc42 there is only one CaM binding site located at the C- terminal region (Figure 7). Hence it is possible that the removal of large portion of protein (amino acids 151 – 163) resulted in change in its 3 dimensional structure, leading to the formation of new ligand binding site having higher affinity for CaM as compared to putative CaM binding domain in full length Cdc42. Similar situation has been explained for protein syntaxin in C. elegans, in which two point mutations L165A, E166A resulted in the open configuration of the protein thus allowing it to interact with synaptobrevin protein (Richmond, Weimer, & Jorgensen, 2001). Moreover, C terminal truncated Cdc42 does not exist physiologically in the cell. Therefore the focus of my research was to analyze the CaM binding domain in full length Cdc42. In full length Cdc42, individual amino acids in the potential CaM binding region (amino acids 151 – 163) became the focus of study.

Therefore, Cdc42 mutants were constructed with point mutations rather than deletion mutant.

CaM is acidic in nature and basic amino acids play an important role in its interaction with other proteins (Xu, Chelikani, & Bhullar, 2012). Therefore the two basic amino acids K153 and K163 in CaM binding region were mutated to alanine. Unlike the removal of amino acids 151 – 163 that could have changed the 3 dimensional configuration of the protein, alanine being non polar and small in size is proposed to have no effect on the charge and configuration of the protein. We also confirmed that Cdc42K153A and Cdc42K163A are GTP binding proteins by performing *in vitro* loading of the proteins in CHRF cell lysate with GTPγS. As compared to wild type Cdc42, Cdc42K163A showed significantly reduced binding to CaM, whereas Cdc42K153A showed reduced but non-significant decrease in its interaction with CaM (Figure 12). This indicates that in addition to the basic amino acids that are important for the interaction of Cdc42 with CaM, there are other factors that also play important role. Similar situation has been presented before in the case of KRas interaction with CaM. In KRasR164D, the binding to CaM was not significantly affected as compared to wild type whereas in KRasR161D the binding to CaM was completely abolished. This was due to the reason that KRasR161D was unable to farnesylate and localize to plasma membrane (Lopez-Alcala et al., 2008). Moreover RalA GTPase requires isoprenylation to interact with CaM (Sidhu, Elsaraj, Grujic, & Bhullar, 2005). This shows that in addition to basic amino acids, post translational modifications also play an important role in the interaction of small GTPases with CaM. This could be one of

the reasons in case of Cdc42K153A for not demonstrating significant decrease in its interaction with CaM.

Previously in our lab it has been shown that CaM has inhibitory effect on the thrombin induced activation of Cdc42 in platelets but has no significant role in CHRF cells (Xu & Bhullar, 2011). However it has been shown that CaM plays an important role in maintaining basal activity of Cdc42 in CHRF cells (Xu & Bhullar, 2011). Therefore the mutant forms of Cdc42, Cdc42K153A and Cdc42K163A, which show changes in binding to CaM were analyzed for their basal activity. Pak5 is a member of Pak family consisting of Pak1 - 6, which binds specifically to the active form of Cdc42 (Dan et al., 2002). Therefore GST Pak5 was used to pull down the active form of Cdc42 from CHRF cell lysates. Cdc42 mutants Cdc42K153A and Cdc42K163A; both showed significant decrease in the basal activity in the cell as compared to wild type Cdc42. Although Cdc42 mutant K153A showed little decrease in its interaction with CaM in the absence of calcium, it showed significant decrease in the basal activity. This indicates that even a small but non-significant change in CaM binding to Cdc42 affects its basal activation. Thus, indicating that CaM plays significant role in maintaining the basal activity of Cdc42. The decrease in basal activity in the Cdc42 mutants was not due to any changes in their ability to load GTP/GDP.

In conclusion, this study shows that CaM interacts with and regulates the basal activity of Cdc42 in CHRF cells. Amino acid K163 in Cdc42 plays an important role in interaction with CaM and is required for CaM mediated basal activation of Cdc42. In

addition, this study also provides evidence that there could be an additional CaM binding region which is not detected by the CaM database analysis and require further analysis to identify its location in Cdc42.

7. Future Directions

1. Determine additional CaM binding site in Cdc42 GTPase.

The research conducted here has discovered that CaM binds to C terminal region of Cdc42 in CHRF cells and plays important role in its basal activity. Further studies are still needed to determine if there are other CaM binding sites present towards the N terminal of Cdc42. Previously it has been shown that RalA and RalB contain N terminal calcium independent CaM binding domain and C terminal calcium dependent CaM binding domain (Clough et al., 2002). Truncated Cdc42 constructs can be used to identify additional CaM binding sites.

2. Determine the interaction between Cdc42 and CaM using molecular modeling techniques.

To further confirm the binding between Cdc42 and CaM, Cdc42 M (Δ151-163), Cdc42K153A and Cdc42K163A can be analyzed for their interaction with CaM by using molecular modeling methods and/or FRET (Fluorescence resonance energy transfer) analysis.

3. Determine the posttranslational modification of Cdc42K153A and Cdc42K163A and its effect on CaM binding.

Cdc42 contains conserved CAAX motif at its C terminal region that undergoes isoprenylation by addition of 20-carbon geranylgeranyl moiety to the cysteine. Ras converting enzyme 1 cleaves the AAX peptide, followed by carboxymethylation of

geranylgeranylated cysteine residue (Hoffman et al., 2000). As discussed before the difference in the binding capacity of Cdc42K153A and Cdc42K163A to CaM could be due to differences in posttranslational modifications. Therefore this can be analyzed by using *in vitro* transcription-translation system in the presence of isoprenyl substrate - geranylgeranyl pyrophosphate to assess any differences in the isoprenylation status of different mutants.

8. Refrences

Allen, W. E., Zicha, D., Ridley, A. J., & Jones, G. E. (1998). A role for Cdc42 in macrophage chemotaxis. *J Cell Biol, 141*(5), 1147-1157.

Arias-Romero, L. E., & Chernoff, J. (2013). Targeting Cdc42 in cancer. *Expert Opin Ther Targets, 17*(11), 1263-1273. doi: 10.1517/14728222.2013.828037

Auer, K. L., Contessa, J., Brenz-Verca, S., Pirola, L., Rusconi, S., Cooper, G., . . . Dent, P. (1998). The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. *Mol Biol Cell, 9*(3), 561-573.

Babu, Y. S., Bugg, C. E., & Cook, W. J. (1988). Structure of calmodulin refined at 2.2 A resolution. *J Mol Biol, 204*(1), 191-204.

Benninger, Y., Thurnherr, T., Pereira, J. A., Krause, S., Wu, X., Chrostek-Grashoff, A., . . . Relvas, J. B. (2007). Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development. *J Cell Biol, 177*(6), 1051-1061. doi: 10.1083/jcb.200610108

Berchtold, M. W., & Villalobo, A. (2014). The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. *Biochim Biophys Acta, 1843*(2), 398-435. doi: 10.1016/j.bbamcr.2013.10.021

Bishop, A. L., & Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem J, 348 Pt 2*, 241-255.

Bos, J. L., Rehmann, H., & Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. *Cell, 129*(5), 865-877. doi: 10.1016/j.cell.2007.05.018

Casey, P. J., & Seabra, M. C. (1996). Protein prenyltransferases. *J Biol Chem, 271*(10), 5289-5292.

Cerione, R. A. (2004). Cdc42: new roads to travel. *Trends Cell Biol, 14*(3), 127-132. doi: 10.1016/j.tcb.2004.01.008

Chin, D., & Means, A. R. (2000). Calmodulin: a prototypical calcium sensor. *Trends Cell Biol, 10*(8), 322-328.

Chou, J. J., Li, S., Klee, C. B., & Bax, A. (2001). Solution structure of Ca(2+)-calmodulin reveals flexible hand-like properties of its domains. *Nat Struct Biol, 8*(11), 990-997. doi: 10.1038/nsb1101-990

Clapham, D. E. (2007). Calcium signaling. *Cell, 131*(6), 1047-1058. doi: 10.1016/j.cell.2007.11.028

Clough, R. R., Sidhu, R. S., & Bhullar, R. P. (2002). Calmodulin binds RalA and RalB and is required for the thrombin-induced activation of Ral in human platelets. *J Biol Chem, 277*(32), 28972-28980. doi: 10.1074/jbc.M201504200

Coppola, T., Perret-Menoud, V., Luthi, S., Farnsworth, C. C., Glomset, J. A., & Regazzi, R. (1999). Disruption of Rab3-calmodulin

interaction, but not other effector interactions, prevents Rab3 inhibition of exocytosis. *EMBO J, 18*(21), 5885-5891. doi: 10.1093/emboj/18.21.5885

Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., . . . Gutkind, J. S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell, 81*(7), 1137-1146.

Cox, A. D., & Der, C. J. (2010). Ras history: The saga continues. *Small GTPases, 1*(1), 2-27. doi: 10.4161/sgtp.1.1.12178

Csepanyi-Komi, R., Levay, M., & Ligeti, E. (2012). Small G proteins and their regulators in cellular signalling. *Mol Cell Endocrinol, 353*(1-2), 10-20. doi: 10.1016/j.mce.2011.11.005

Dagher, R., Peng, S., Gioria, S., Feve, M., Zeniou, M., Zimmermann, M., . . . Kilhoffer, M. C. (2011). A general strategy to characterize calmodulin-calcium complexes involved in CaM-target recognition: DAPK and EGFR calmodulin binding domains interact with different calmodulin-calcium complexes. *Biochim Biophys Acta, 1813*(5), 1059-1067. doi: 10.1016/j.bbamcr.2010.11.004

Dan, C., Nath, N., Liberto, M., & Minden, A. (2002). PAK5, a new brain-specific kinase, promotes neurite outgrowth in N1E-115 cells. *Mol Cell Biol, 22*(2), 567-577.

De Vries, L., Zheng, B., Fischer, T., Elenko, E., & Farquhar, M. G. (2000). The regulator of G protein signaling family. *Annu Rev Pharmacol Toxicol, 40*, 235-271. doi: 10.1146/annurev.pharmtox.40.1.235

Dovas, A., & Couchman, J. R. (2005). RhoGDI: multiple functions in the regulation of Rho family GTPase activities. *Biochem J, 390*(Pt 1), 1-9. doi: 10.1042/BJ20050104

Drivas, G. T., Shih, A., Coutavas, E., Rush, M. G., & D'Eustachio, P. (1990). Characterization of four novel ras-like genes expressed in a human teratocarcinoma cell line. *Mol Cell Biol, 10*(4), 1793-1798.

Elsaraj, S. M., & Bhullar, R. P. (2008). Regulation of platelet Rac1 and Cdc42 activation through interaction with calmodulin. *Biochim Biophys Acta, 1783*(5), 770-778. doi: 10.1016/j.bbamcr.2008.01.022

Etienne-Manneville, S. (2004). Cdc42--the centre of polarity. *J Cell Sci, 117*(Pt 8), 1291-1300. doi: 10.1242/jcs.01115

Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature, 420*(6916), 629-635. doi: 10.1038/nature01148

Feltham, J. L., Dotsch, V., Raza, S., Manor, D., Cerione, R. A., Sutcliffe, M. J., . . . Oswald, R. E. (1997). Definition of the switch surface in the solution structure of Cdc42Hs. *Biochemistry, 36*(29), 8755-8766. doi: 10.1021/bi970694x

Fischer, C., Kugler, A., Hoth, S., & Dietrich, P. (2013). An IQ domain mediates the interaction with calmodulin in a plant cyclic

nucleotide-gated channel. *Plant Cell Physiol, 54*(4), 573-584. doi: 10.1093/pcp/pct021

Fischer, R., Wei, Y., Anagli, J., & Berchtold, M. W. (1996). Calmodulin binds to and inhibits GTP binding of the ras-like GTPase Kir/Gem. *J Biol Chem, 271*(41), 25067-25070.

Flinn, H. M., & Ridley, A. J. (1996). Rho stimulates tyrosine phosphorylation of focal adhesion kinase, p130 and paxillin. *J Cell Sci, 109 (Pt 5)*, 1133-1141.

Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shekter, L. R., . . . Hamm, H. E. (1998). Molecular basis for interactions of G protein betagamma subunits with effectors. *Science, 280*(5367), 1271-1274.

Fritz, G., Brachetti, C., Bahlmann, F., Schmidt, M., & Kaina, B. (2002). Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *Br J Cancer, 87*(6), 635-644. doi: 10.1038/sj.bjc.6600510

Fugman, D. A., Witte, D. P., Jones, C. L., Aronow, B. J., & Lieberman, M. A. (1990). In vitro establishment and characterization of a human megakaryoblastic cell line. *Blood, 75*(6), 1252-1261.

Geiser, J. R., van Tuinen, D., Brockerhoff, S. E., Neff, M. M., & Davis, T. N. (1991). Can calmodulin function without binding calcium? *Cell, 65*(6), 949-959.

Gilman, A. G. (1987). G proteins: transducers of receptor-generated signals. *Annu Rev Biochem, 56*, 615-649. doi: 10.1146/annurev.bi.56.070187.003151

Gomez Del Pulgar, T., Valdes-Mora, F., Bandres, E., Perez-Palacios, R., Espina, C., Cejas, P., . . . Lacal, J. C. (2008). Cdc42 is highly expressed in colorectal adenocarcinoma and downregulates ID4 through an epigenetic mechanism. *Int J Oncol, 33*(1), 185-193.

Heasman, S. J., & Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol, 9*(9), 690-701. doi: 10.1038/nrm2476

Hendrickson, W. A. (2005). Transduction of biochemical signals across cell membranes. *Q Rev Biophys, 38*(4), 321-330. doi: 10.1017/S0033583506004136

Hoffman, G. R., Nassar, N., & Cerione, R. A. (2000). Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. *Cell, 100*(3), 345-356.

Hoshino, M., & Nakamura, S. (2003). Small GTPase Rin induces neurite outgrowth through Rac/Cdc42 and calmodulin in PC12 cells. *J Cell Biol, 163*(5), 1067-1076. doi: 10.1083/jcb.200308070

Hudmon, A., & Schulman, H. (2002). Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J, 364*(Pt 3), 593-611. doi: 10.1042/BJ20020228

Hurowitz, E. H., Melnyk, J. M., Chen, Y. J., Kouros-Mehr, H., Simon, M. I., & Shizuya, H. (2000). Genomic characterization of the human heterotrimeric G protein alpha, beta, and gamma subunit genes. *DNA Res, 7*(2), 111-120.

Jaffe, A. B., & Hall, A. (2005). Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol, 21*, 247-269. doi: 10.1146/annurev.cellbio.21.020604.150721

Jilkine, A., Maree, A. F., & Edelstein-Keshet, L. (2007). Mathematical model for spatial segregation of the Rho-family GTPases based on inhibitory crosstalk. *Bull Math Biol, 69*(6), 1943-1978. doi: 10.1007/s11538-007-9200-6

Johnson, D. I. (1999). Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol Mol Biol Rev, 63*(1), 54-105.

Kahn, R. A., & Gilman, A. G. (1984). Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J Biol Chem, 259*(10), 6228-6234.

Kahn, R. A., & Gilman, A. G. (1986). The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J Biol Chem, 261*(17), 7906-7911.

Kamai, T., Yamanishi, T., Shirataki, H., Takagi, K., Asami, H., Ito, Y., & Yoshida, K. (2004). Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. *Clin*

Cancer Res, 10(14), 4799-4805. doi: 10.1158/1078-0432.CCR-0436-03

Konstantinopoulos, P. A., Karamouzis, M. V., & Papavassiliou, A. G. (2007). Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nat Rev Drug Discov, 6*(7), 541-555. doi: 10.1038/nrd2221

Kovalevskaya, N. V., van de Waterbeemd, M., Bokhovchuk, F. M., Bate, N., Bindels, R. J., Hoenderop, J. G., & Vuister, G. W. (2013). Structural analysis of calmodulin binding to ion channels demonstrates the role of its plasticity in regulation. *Pflugers Arch, 465*(11), 1507-1519. doi: 10.1007/s00424-013-1278-0

Kozma, R., Ahmed, S., Best, A., & Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol, 15*(4), 1942-1952.

Kumar, V., Chichili, V. P., Tang, X., & Sivaraman, J. (2013). A novel trans conformation of ligand-free calmodulin. *PLoS One, 8*(1), e54834. doi: 10.1371/journal.pone.0054834

Lakowski, T. M., Lee, G. M., Okon, M., Reid, R. E., & McIntosh, L. P. (2007). Calcium-induced folding of a fragment of calmodulin composed of EF-hands 2 and 3. *Protein Sci, 16*(6), 1119-1132. doi: 10.1110/ps.072777107

Li, R., Debreceni, B., Jia, B., Gao, Y., Tigyi, G., & Zheng, Y. (1999). Localization of the PAK1-, WASP-, and IQGAP1-specifying regions of Cdc42. *J Biol Chem, 274*(42), 29648-29654.

Lopez-Alcala, C., Alvarez-Moya, B., Villalonga, P., Calvo, M., Bachs, O., & Agell, N. (2008). Identification of essential interacting elements in K-Ras/calmodulin binding and its role in K-Ras localization. *J Biol Chem, 283*(16), 10621-10631. doi: 10.1074/jbc.M706238200

Machesky, L. M., & Insall, R. H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr Biol, 8*(25), 1347-1356.

Madaule, P., & Axel, R. (1985). A novel ras-related gene family. *Cell, 41*(1), 31-40.

McCudden, C. R., Hains, M. D., Kimple, R. J., Siderovski, D. P., & Willard, F. S. (2005). G-protein signaling: back to the future. *Cell Mol Life Sci, 62*(5), 551-577. doi: 10.1007/s00018-004-4462-3

Milligan, G., & Kostenis, E. (2006). Heterotrimeric G-proteins: a short history. *Br J Pharmacol, 147 Suppl 1*, S46-55. doi: 10.1038/sj.bjp.0706405

Moyers, J. S., Bilan, P. J., Zhu, J., & Kahn, C. R. (1997). Rad and Rad-related GTPases interact with calmodulin and calmodulin-dependent protein kinase II. *J Biol Chem, 272*(18), 11832-11839.

Murad, F. (2011). Nitric oxide: the coming of the second messenger. *Rambam Maimonides Med J, 2*(2), e0038. doi: 10.5041/RMMJ.10038

Murtaugh, T. J., Rowe, P. M., Vincent, P. L., Wright, L. S., & Siegel, F. L. (1983). Posttranslational modification of calmodulin. *Methods Enzymol, 102*, 158-170.

Nobes, C. D., & Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol, 144*(6), 1235-1244.

O'Day, D. H., & Myre, M. A. (2004). Calmodulin-binding domains in Alzheimer's disease proteins: extending the calcium hypothesis. *Biochem Biophys Res Commun, 320*(4), 1051-1054. doi: 10.1016/j.bbrc.2004.06.070

O'Neil, K. T., & DeGrado, W. F. (1990). How calmodulin binds its targets: sequence independent recognition of amphiphilic alpha-helices. *Trends Biochem Sci, 15*(2), 59-64.

Oldham, W. M., & Hamm, H. E. (2007). How do receptors activate G proteins? *Adv Protein Chem, 74*, 67-93. doi: 10.1016/S0065-3233(07)74002-0

Oldham, W. M., & Hamm, H. E. (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol, 9*(1), 60-71. doi: 10.1038/nrm2299

Olofsson, B. (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal, 11*(8), 545-554.

Olwin, B. B., & Storm, D. R. (1985). Calcium binding to complexes of calmodulin and calmodulin binding proteins. *Biochemistry, 24*(27), 8081-8086.

Orlando, K., & Guo, W. (2009). Membrane organization and dynamics in cell polarity. *Cold Spring Harb Perspect Biol, 1*(5), a001321. doi: 10.1101/cshperspect.a001321

Osmani, N., Vitale, N., Borg, J. P., & Etienne-Manneville, S. (2006). Scrib controls Cdc42 localization and activity to promote cell polarization during astrocyte migration. *Curr Biol, 16*(24), 2395-2405. doi: 10.1016/j.cub.2006.10.026

Paduch, M., Jelen, F., & Otlewski, J. (2001). Structure of small G proteins and their regulators. *Acta Biochim Pol, 48*(4), 829-850.

Pandey, A., Dan, I., Kristiansen, T. Z., Watanabe, N. M., Voldby, J., Kajikawa, E., . . . Mann, M. (2002). Cloning and characterization of PAK5, a novel member of mammalian p21-activated kinase-II subfamily that is predominantly expressed in brain. *Oncogene, 21*(24), 3939-3948. doi: 10.1038/sj.onc.1205478

Park, J. B. (2001). Regulation of GTP-binding state in RalA through Ca2+ and calmodulin. *Exp Mol Med, 33*(1), 54-58. doi: 10.1038/emm.2001.10

Phillips, M. J., Calero, G., Chan, B., Ramachandran, S., & Cerione, R. A. (2008). Effector proteins exert an important influence on the signaling-active state of the small GTPase Cdc42. *J Biol Chem, 283*(20), 14153-14164. doi: 10.1074/jbc.M706271200

Richmond, J. E., Weimer, R. M., & Jorgensen, E. M. (2001). An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. *Nature, 412*(6844), 338-341. doi: 10.1038/35085583

Rihet, S., Vielh, P., Camonis, J., Goud, B., Chevillard, S., & de Gunzburg, J. (2001). Mutation status of genes encoding RhoA, Rac1, and Cdc42 GTPases in a panel of invasive human colorectal and breast tumors. *J Cancer Res Clin Oncol, 127*(12), 733-738.

Roberts, P. J., Mitin, N., Keller, P. J., Chenette, E. J., Madigan, J. P., Currin, R. O., . . . Der, C. J. (2008). Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. *J Biol Chem, 283*(37), 25150-25163. doi: 10.1074/jbc.M800882200

Schwamborn, J. C., & Puschel, A. W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat Neurosci, 7*(9), 923-929. doi: 10.1038/nn1295

Sidhu, R. S., Elsaraj, S. M., Grujic, O., & Bhullar, R. P. (2005). Calmodulin binding to the small GTPase Ral requires isoprenylated Ral. *Biochem Biophys Res Commun, 336*(1), 105-109. doi: 10.1016/j.bbrc.2005.08.053

Sinha, S., & Yang, W. (2008). Cellular signaling for activation of Rho GTPase Cdc42. *Cell Signal, 20*(11), 1927-1934. doi: 10.1016/j.cellsig.2008.05.002

Smrcka, A. V. (2008). G protein betagamma subunits: central mediators of G protein-coupled receptor signaling. *Cell Mol Life Sci, 65*(14), 2191-2214. doi: 10.1007/s00018-008-8006-5

Soderling, T. R. (1999). The Ca-calmodulin-dependent protein kinase cascade. *Trends Biochem Sci, 24*(6), 232-236.

Stengel, K., & Zheng, Y. (2011). Cdc42 in oncogenic transformation, invasion, and tumorigenesis. *Cell Signal, 23*(9), 1415-1423. doi: 10.1016/j.cellsig.2011.04.001

Stevens, F. C. (1983). Calmodulin: an introduction. *Can J Biochem Cell Biol, 61*(8), 906-910.

Swulius, M. T., & Waxham, M. N. (2008). Ca(2+)/calmodulin-dependent protein kinases. *Cell Mol Life Sci, 65*(17), 2637-2657. doi: 10.1007/s00018-008-8086-2

Szczur, K., Xu, H., Atkinson, S., Zheng, Y., & Filippi, M. D. (2006). Rho GTPase CDC42 regulates directionality and random movement via distinct MAPK pathways in neutrophils. *Blood, 108*(13), 4205-4213. doi: 10.1182/blood-2006-03-013789

Takahashi, K., Sasaki, T., Mammoto, A., Takaishi, K., Kameyama, T., Tsukita, S., & Takai, Y. (1997). Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the

activation of the Rho small G protein. *J Biol Chem, 272*(37), 23371-23375.

Takai, Y., Sasaki, T., & Matozaki, T. (2001). Small GTP-binding proteins. *Physiol Rev, 81*(1), 153-208.

Tang, Y., Olufemi, L., Wang, M. T., & Nie, D. (2008). Role of Rho GTPases in breast cancer. *Front Biosci, 13*, 759-776.

Thurnherr, T., Benninger, Y., Wu, X., Chrostek, A., Krause, S. M., Nave, K. A., . . . Relvas, J. B. (2006). Cdc42 and Rac1 signaling are both required for and act synergistically in the correct formation of myelin sheaths in the CNS. *J Neurosci, 26*(40), 10110-10119. doi: 10.1523/JNEUROSCI.2158-06.2006

Touchot, N., Chardin, P., & Tavitian, A. (1987). Four additional members of the ras gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. *Proc Natl Acad Sci U S A, 84*(23), 8210-8214.

Tuteja, N. (2009). Signaling through G protein coupled receptors. *Plant Signal Behav, 4*(10), 942-947.

Valentijn, L. J., Koppen, A., van Asperen, R., Root, H. A., Haneveld, F., & Versteeg, R. (2005). Inhibition of a new differentiation pathway in neuroblastoma by copy number defects of N-myc, Cdc42, and nm23 genes. *Cancer Res, 65*(8), 3136-3145. doi: 10.1158/0008-5472.CAN-04-2469

van der Vuurst, H., van Willigen, G., van Spronsen, A., Hendriks, M., Donath, J., & Akkerman, J. W. (1997). Signal transduction through trimeric G proteins in megakaryoblastic cell lines. *Arterioscler Thromb Vasc Biol, 17*(9), 1830-1836.

van Nieuw Amerongen, G. P., & van Hinsbergh, V. W. (2001). Cytoskeletal effects of rho-like small guanine nucleotide-binding proteins in the vascular system. *Arterioscler Thromb Vasc Biol, 21*(3), 300-311.

Vega, F. M., & Ridley, A. J. (2008). Rho GTPases in cancer cell biology. *FEBS Lett, 582*(14), 2093-2101. doi: 10.1016/j.febslet.2008.04.039

Villalonga, P., Lopez-Alcala, C., Bosch, M., Chiloeches, A., Rocamora, N., Gil, J., . . . Agell, N. (2001). Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates its downstream signaling. *Mol Cell Biol, 21*(21), 7345-7354. doi: 10.1128/MCB.21.21.7345-7354.2001

Vogel, H. J. (1994). The Merck Frosst Award Lecture 1994. Calmodulin: a versatile calcium mediator protein. *Biochem Cell Biol, 72*(9-10), 357-376.

Walsh, M. P. (1983). Calmodulin and its roles in skeletal muscle function. *Can Anaesth Soc J, 30*(4), 390-398.

Wang, K. L., Khan, M. T., & Roufogalis, B. D. (1997). Identification and characterization of a calmodulin-binding domain in Ral-A, a Ras-related GTP-binding protein purified from human erythrocyte membrane. *J Biol Chem, 272*(25), 16002-16009.

Wang, K. L., & Roufogalis, B. D. (1999). Ca2+/calmodulin stimulates GTP binding to the ras-related protein ral-A. *J Biol Chem, 274*(21), 14525-14528.

Wennerberg, K., Rossman, K. L., & Der, C. J. (2005). The Ras superfamily at a glance. *J Cell Sci, 118*(Pt 5), 843-846. doi: 10.1242/jcs.01660

Wilson, M. A., & Brunger, A. T. (2000). The 1.0 A crystal structure of Ca(2+)-bound calmodulin: an analysis of disorder and implications for functionally relevant plasticity. *J Mol Biol, 301*(5), 1237-1256. doi: 10.1006/jmbi.2000.4029

Xu, B., & Bhullar, R. P. (2011). Regulation of Rac1 and Cdc42 activation in thrombin- and collagen-stimulated CHRF-288-11 cells. *Mol Cell Biochem, 353*(1-2), 73-79. doi: 10.1007/s11010-011-0776-7

Xu, B., Chelikani, P., & Bhullar, R. P. (2012). Characterization and functional analysis of the calmodulin-binding domain of Rac1 GTPase. *PLoS One, 7*(8), e42975. doi: 10.1371/journal.pone.0042975

Yamniuk, A. P., & Vogel, H. J. (2004). Calmodulin's flexibility allows for promiscuity in its interactions with target proteins and peptides. *Mol Biotechnol, 27*(1), 33-57. doi: 10.1385/MB:27:1:33

Yang, Z. (2002). Small GTPases: versatile signaling switches in plants. *Plant Cell, 14 Suppl*, S375-388.

Yao, R., Wang, Y., Lubet, R. A., & You, M. (2002). Differentially expressed genes associated with mouse lung tumor progression. *Oncogene, 21*(37), 5814-5821. doi: 10.1038/sj.onc.1205422

Yap, K. L., Ames, J. B., Swindells, M. B., & Ikura, M. (1999). Diversity of conformational states and changes within the EF-hand protein superfamily. *Proteins, 37*(3), 499-507.

Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., & Ikura, M. (2000). Calmodulin target database. *J Struct Funct Genomics, 1*(1), 8-14.

Yasuda, S., Oceguera-Yanez, F., Kato, T., Okamoto, M., Yonemura, S., Terada, Y., . . . Narumiya, S. (2004). Cdc42 and mDia3 regulate microtubule attachment to kinetochores. *Nature, 428*(6984), 767-771. doi: 10.1038/nature02452

Yu, Y. Y., Dai, G., Pan, F. Y., Chen, J., & Li, C. J. (2005). Calmodulin regulates the post-anaphase reposition of centrioles during cytokinesis. *Cell Res, 15*(7), 548-552. doi: 10.1038/sj.cr.7290324

Zhang, S., Schafer-Hales, K., Khuri, F. R., Zhou, W., Vertino, P. M., & Marcus, A. I. (2008). The tumor suppressor LKB1 regulates lung cancer cell polarity by mediating cdc42 recruitment and activity. *Cancer Res, 68*(3), 740-748. doi: 10.1158/0008-5472.CAN-07-2989