Rho GTPases and RhoGDIs in the Regulation of Mast Cell Degranulation

by

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Abstract

Mast cells (MCs) are tissue resident innate immune cells. They are recognized for their immunoregulatory roles in the regulation of innate and adaptive immunity. Aberrant activation and responses of MCs result in inflammatory disorders like allergies. Thus, the biological pathways that control MC activation and responses are attractive targets for the treatment of MC-mediated inflammation. In this thesis, I present my data on the essential roles of Rho GTPases and their regulator, RhoGDI, during FcεRI-activation of RBL-2H3, a rat basophil leukemia cell line widely used as model MCs, and bone marrow-derived MCs (BMMCs).

We demonstrated that Rho GTPase subfamily proteins regulate unique events during the activation of MCs. Rac drives the formation of actin rich peripheral extensions and RhoA is involved in granule trafficking via microtubule extension. Inhibition of either Rho protein by pharmacological interference, significantly prevents MC degranulation. Further, we also characterized the distinct properties of RhoGDIs in the regulation of Rho GTPases. RhoGDIs may hold the key for strategic regulation of spatial and temporal Rho signaling. Here, we showed that RhoGDI subfamily members possess distinct functional and biochemical properties in their regulation of Rho proteins. These distinctions of functions could be an evolutionary conserved feature of RhoGDIs. We found that RhoGDI2 shows distinct localization to membrane compartments. This discrete localization in MCs could result in spatial regulation of Rho GTPases, which in turn, may impact MC degranulation. Further work using reduced expression of RhoGDIs in MCs can reveal the non-redundant and essential roles of RhoGDIs during FcεRI-activation of MCs.

Preface

Portions of Chapters 2 and 3 of this thesis were published as:

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List of Abbreviations	
APCs	Antigen presenting cells
Akt	Protein kinase B
Arp2/3	Actin related protein 2/3
BMMCs	Bone marrow mast cells
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DH	Dbl homology
DHR-2	Dock-homology region-2
DIAPH1	Diaphanous Homologue 1
DMEM	Dulbecco's modified eagle medium
DOCK	Dedicator of cytokinesis
ER	Endoplasmic reticulum
ERM	Ezrin, radixin, and moesin
FBS	Fetal bovine serum
Gab2	Grb2-associated binder-like protein 2
GDFs	GDI dissociation factors
GDP	Guanosine diphosphate
GPCRs	G protein coupled receptors
Grb2	Growth-factor-receptor-bound protein 2
GTP	Guanosine triphosphate
HMM	Hidden markov model
Ig	Immunoglobulin
IL	Interleukin

IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITAMs	Immunoreceptor tyrosine-based activation motifs
KD	Knockdown
KO	Knockout
LAT	Linker for activation of T cells
LPS	Lipopolysaccharide
MCPs	Mast cell progenitors
MCs	Mast cells
MEM	Minimum essential media
MPPs	Multipotent progenitors
MUG	4-Methylumbelliferyl-beta-D-galactopyranoside
NEAA	Non-Essential Amino Acids
p75 NTR	p75 neurotrophin receptor
PAK	p-21-activated kinase
PAMPs	Pathogen-associated molecular patterns
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PMSF	Phenylmethylsulphonyl fluoride
PRRs	Pathogen recognition receptors
PS	Phosphatidyl serine
PTMs	Post-translationally modifications
qPCR	Quantitative polymerase chain reaction
RBL-2H3	Rat basophil leukemia, mast cell model
RhoGAP	Rho GTPase activating protein

RhoGDI	Rho Guanine Dissociation Inhibitor
RhoGEF	Rho Guanine nucleotide exchange factor
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SLP-76	Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
TEMED	Tetra methyl ethylenediamine
T_H2	T helper 2
VDJ	Variable, diverse, and joining
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous

Chapter 1 Introduction

Chapter 1 Introduction

1.1 Mast cells

Mast cells (MCs) are tissue resident innate immune cells [1]. Their location in tissues at the interface between internal and external environments like skin, blood vessels, airways and gut make them sentinels of the immune system. They are recognized for their role in immune defense against pathogens and parasites [2], autoimmunity [3], inflammatory disorders [4], cancer [5], neurodegenerative diseases [6], and immune tolerance [7]. But, they are best known for their pro-inflammatory roles in allergies like asthma [8-13]. MCs are versatile regulators of the immune system and thus, have become an attractive therapeutic target.

MCs were first described by Paul Ehrlich in 1878. Ehrlich, in his doctoral dissertation, described granulate connective tissue cells and termed them 'mastzellen' due to their 'well-fed' appearance [14]. He incorrectly assumed that these granules contained nutrition that nourishes surrounding connective tissue. We now know that MC granules contain potent pre-formed immune mediators. Pre-formation of immune mediators allows for swift response against pathogens. Interestingly, these long-lived cells dynamically alter their granule content based on the microenvironmental cues allowing them to fine tune their response to stimuli [15]. Not surprisingly, MC dysfunction leads to inflammatory disorders like allergies, chronic inflammation and autoimmunity; hence, there is a need to understand the basic biology of MC activation and regulation of their immune response. This may lead to identification of novel druggable targets that can be used against MC-mediated disorders.

1.1.1 MC development, granularity and heterogeneity

MC origin and development has been a subject of inquiry for over a century [16-18]. Recently, with the use of novel, specific cell surface markers [19] and sophisticated murine MC models [20], MC lineages are being elucidated. MCs emerge as precursors from bone marrow; multipotent progenitors (MPPs) in the bone marrow, differentiate into either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). MC progenitors (MCPs) are thought to originate either from CMPs and/or MPPs but not CLPs [19]. MCPs use adhesion molecules such as integrins and chemokine receptors (e.g., CXCR2) to home into specific tissues where they fully mature in the presence of tissue-derived factors like stem cell factor (SCF) and Interleukin-3 (IL-3) [21-23]. Additionally, other cytokines can also influence the expression of distinct mediators leading to heterogenous population of MCs within the same tissue [24, 25].

The electron dense granules that give MCs their striking phenotype possess pre-formed mediators like biogenic amines (histamine, serotonin), proteases (tryptase, chymase-1, cathepsin G, granzyme B, and carboxypeptidase A3), lysosomal enzymes (β-glucuronidase, β-hexosaminidase, arylsulfatase), cytokines like TNFα, FGF, IL-4, and SCF, and proteoglycans like heparin and chondroitin sulfates (reviewed in [15]). MC activation results in the release of these mediators, through a process known as degranulation or granule exocytosis (**Figure 1.1**). This leads to rapid allergic responses, recruitment of leukocytes, and other inflammatory responses. Therefore, MCs can initiate and orchestrate complex immune responses. In addition to pre-formed mediators, MCs also release newly synthesized cytokines, chemokines and lipid mediators that contribute to late stage inflammation [8]. Long term MC degranulation may lead

to chronic inflammation resulting in tissue remodeling, scarring, organ dysfunction and, ultimately, death.

Based on their location, staining, granule content and reactivity to select secretagogues MCs have been classified in rodents as mucosal-type and connective tissue-type MCs [15, 24-27]. Human MCs also exhibit heterogeneity and are classified based on protease expression as tryptase-only, chymase-only and tryptase- and chymase-positive MC. However, tissue distribution of human MCs is not as clearly demarcated as in rodents [24, 28]. As previously mentioned, MC phenotypes are influenced by the microenvironmental conditions (cytokines, hormones, reactive radical species and adjacent cells) and trans-differentiation between the two phenotypes can occur [29]. This implies that the heterogeneity of MCs in tissues is much more diverse than merely two polarized phenotypes. Therefore, the responses of mature MCs are continuously refined by microenvironmental cues leading to fine-tuned immune responses.

1.1.2 MCs in health and diseases

Initially MCs were recognized for their role in inflammatory disorders like asthma and autoimmunity. However, we now recognize some of the protective immune responses mediated by MCs like pathogen defense, wound healing, angiogenesis, bone remodeling and peripheral immune tolerance [10, 12, 30]. The use of MC-deficient mice and their reconstitution with donor bone marrow derived MCs transfusions (MC knock-in model) has been a powerful tool to study MC function, *in vivo* [31]. For example, the importance of MCs in asthma, arthritis, experimental allergic encephalitis, experimental bullous pemphigoid, cancer progression, aortic

aneurysms, defense against bacteria and peripheral immune tolerance has been assessed by comparing outcomes in these models [31, 32].

Allergic immune response is an abnormal response directed against non-infectious environmental substances (i.e. allergens). Key features of this include involvement of allergen-specific immunoglobulin E (IgE), T helper 2 (T_H2) and IgE-primed MCs and their subsequent responses (**Figure 1.2**). Allergic asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness, bronchoconstriction, airway remodeling, excessive mucus production and increased collagen deposition [8]. During allergic inflammation, an innocuous allergen like pollen, may be picked up by antigen presenting cells (APCs) like dendritic cells (DCs) or macrophages. In the presence of IL-4 or IL-13, they induce the proliferation of T_H2 type adaptive immune response against the allergen [33]. T_H2 cells release their signature cytokines like IL-4, IL-5 to induce class switching of B cells, in which variable, diverse, and joining (VDJ) segments that were initially linked to another constant (C) region in the immunoglobulin heavy chain locus (for example, Cμ or Cγ) switch to the Cε region. This results in the production of allergen-specific IgE type antibodies [34]. Long lived IgE in turn makes its way to cells that possess the high affinity IgE receptor, FcεRI, like MCs, and prime them for any future encounters with the same allergen [35-37]. A secondary encounter would result in the rapid activation and response of sensitized cells.

MCs are found in high numbers within the airway smooth muscle (ASM) and bronchial epithelium in asthmatic patients [38-41]. MC mediators like histamine, proteases and cytokines are found in the interstitium of asthmatic lungs [39]. These mediators are known to cause

airway inflammation, bronchoconstriction, vasodilation and other characteristics of allergic asthma. Aberrant MC activation results in the release pre-formed and newly formed mediators that initiate, magnify and exacerbate inflammation in specific sites leading to abovementioned allergic symptoms [8]. Therefore, there is a renewed interest in targeting MC responses to mitigate symptoms of allergies.

1.1.3 FcεRI signaling in MCs

MCs express numerous surface receptors like pathogen recognition receptors (PRRs) that identify two classes of molecules: pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs), which are associated with components of host's cells that are released during cell damage [42, 43]. MCs abundantly express PRRs like toll-like receptors (TLRs), G protein coupled receptors (GPCRs), complement receptors, high affinity antibody receptors (FcεRI, FcγR), cytokine and chemokine receptors [44]. MC stimulation by ligands of these receptors result in a cascade of signaling events activate MCs to degranulate and release mediators. These events are tightly coordinated to regulate the persistence and the extent of activation.

Considerable information has been obtained from the study of FcεRI stimulation in MCs. This tetrameric receptor is comprised of the IgE-binding α chain, the membrane-spanning β chain, and a disulfide linked homodimer of the γ chains [45]. The extracellular domain has high affinity to the Fc-portion of IgE. When IgE-primed MCs are re-exposed to the original or a cross reactive bivalent or multivalent antigen, it results in the crosslinking of adjacent IgE -bound FcεRI and the consequent aggregation of surface FcεRI (**Figure 1.3**). When the FcεRI aggregation is of

sufficient strength and duration, it triggers downstream signaling events where the β chain amplifies the signal and the γ chains impart signaling competence [37, 46]. Both have immunoreceptor tyrosine-based activation motifs (ITAMs) which are phosphorylated on their tyrosine residues by Src family protein tyrosine kinases (Fyn and Lyn) and spleen tyrosine kinase (Syk) [47, 48]. Deficiency in Fyn [49, 50] or Syk [51] is demonstrated to severely inhibit MC degranulation. Once phosphorylated, novel binding sites are created on adaptor molecules, where other signaling proteins can bind and propagate signals required for MC effector responses.

Adaptors proteins like Grb2-associated binder-like protein 2 (Gab2), Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) and the linker for activation of T cells (LAT) are essential for propagation of FcεRI signal [52]. SLP-76 [53] and LAT [54] play an essential role in calcium signaling and cytoskeletal dynamics. LAT acts as a scaffold and recruits and activates growth-factor-receptor-bound protein 2 (GRB2), Src homology 2 (SH2)-domain-containing proteins, Rho guanine nucleotide exchange factor (RhoGEF) Vav [55] and phospholipase Cγ (PLC-γ) [54]. Complementarily, Gab2 activates phosphoinositide 3-kinase (PI3-K) and protein kinase C (PKC) [49]. Ultimately, these events trigger Ca2+ mobilization, cytoskeletal modifications, granule translocation, fusion and finally mediator release. However, distal Fc-signaling molecules and their specific roles in regulating MC degranulation are still unclear. Understanding how this network is coordinated will allow its disruption in a manner that could be therapeutically beneficial.

FcεRI-signaling in MCs results in formation of F-actin rich membrane ruffles on the dorsal surface and dynamic microtubule formation [56]. Stimulation of RBL-2H3 cells, a rat basophil leukemia cell line with typical mucosal mast cell morphology [57], and mouse bone marrow-derived MCs (BMMCs) have been used extensively to characterize these ruffles and tubule extension [58-66]. Nishida et al, proposed two discrete steps that result in MC degranulation: (1) calcium dependent F-actin disassembly and (2) calcium independent microtubule formation [67]. They concluded that F-actin acts as an actin barrier that prevents the granule fusion to plasma membrane, thereby negatively regulating degranulation and microtubule formation is required for granule translocation to plasma membrane. Therefore, molecules regulating F-actin disassembly and microtubule formation must play a key role in MC degranulation.

1.1.4 MC degranulation mechanisms and machinery

Degranulation or granule exocytosis refers to the loss of or release of granules and is most often associated with the explosive release of pre-formed mediators by cells like neutrophils, MCs and basophils [68, 69]. Degranulation occurs in four discreet steps: (1) granule translocation from the cytoplasm to target membrane (plasma membrane or other organelles) (2) vesicle tethering and docking (3) granule priming to make fusion-competent (4) granule fusion leading to fusion pore formation and ultimately release. These processes are tightly regulated by receptor-controlled mechanisms [69]. The degranulation machinery includes but is not limited to Ras superfamily members, their regulators and effectors, kinases/phosphatases, SNAREs and cytoskeletal elements.

While initial therapeutic strategies aiming to restrict mast cell activation are largely focused on blocking the activation of the IgE receptor and its early signaling events [70], targeting the late stage signaling steps can be a suitable alternative strategy. The latter approach would block the consequences of activation by many different receptor types that converge into the same degranulation pathway. Therefore, the machinery involved in granule mobilization, fusion and release have become attractive targets for therapies targeting MC-mediated inflammatory disorders.

1.2 Rho GTPases

Ras superfamily of GTPases regulate multitude of cellular processes (reviewed in [71, 72]). They number over 160 in mammals and fall into five major groups: Ras, Rho, Rab, Arf and Ran. Since the identification and characterization of the first member, Ha-Ras, as an oncogene [73], these GTP-binding proteins have been recognized to regulate almost every aspect of cell signaling. They possess a conserved backbone of 5 G-boxes involved in GTP-binding and GTPase activity [74]. They act as molecular switches that cycle between two conformational states: one bound to GTP ('active' state), the other bound to GDP ('inactive' state) (**Figure 1.4**). In the GTP-bound state, they recognize target proteins and generate a specific cellular response. For example, when cells are stimulated by growth factor ligands (e.g., epidermal growth factor) they activate Ras-Raf-Mek-Erk1/2 pathway that promotes cell proliferation. Not surprisingly, dysregulated Ras superfamily protein signaling can affect cell growth, survival and function [75, 76].

Rho GTPases belong to the Ras superfamily of GTPases and were initially discovered for their role in regulating actin cytoskeleton [77]. They are now recognized to participate in a host of

cellular processes like proliferation, apoptosis, adhesion, motility, differentiation, gene expression, microtubule dynamics and vesicular trafficking [78-80]. Immune cells like MCs depend on the activity of Rho GTPases to regulate their proliferation, differentiation, production and release of immune mediators during immune responses [81]. Timely recruitment, activation and responses of MCs are critical to protect the host. Therefore, Rho GTPases play an indispensable role in host immunity. On the other hand, dysfunction of Rho GTPases leads to aberrant immune responses that are detrimental to the host. For example, missense mutation on 1 allele of the *Rac2* gene in humans result in severely compromised pathogen defense [82]. Understanding the specific characteristics, regulation and the roles of Rho GTPases in MCs can validate them as targets for MC-mediated inflammatory disorders.

1.2.1 Subfamily members and function

Rho GTPases possess a conserved Rho-specific insert located in the GTPase domain and it is involved in the binding to effectors and regulators [83]. Twenty-two mammalian Rho GTPase genes are encoded in the human genome [84]. They are: three related Rho isoforms A, B, and C; three related Rac isoforms 1, 2, and 3; and the more distantly related Cdc42, RhoD, Rnd1, Rnd2, RhoE/Rnd3, RhoG, TC10 and TCL, RhoH/TTF, Chp and Wrch-1, Rif, RhoBTB1 and 2, and Miro-1 and 2. Most of these, except for a few atypical members, cycle between ‘on’ and ‘off’ states. In their GTP-bound state, they bind to a wide variety of effector proteins to regulate distinct cellular processes [85]. For example, RhoG was demonstrated to play an essential role in macrophage phagocytosis induced by Fcγ receptor (FcγR) and complement receptor 3 (CR3) activation [86].

During infections, immune cells are recruited to the sites of inflammation by various sentinel cells like DCs, macrophages and MCs. They are activated by various PRRs, that recognize PAMPs and DAMPs, leading to calcium mobilization, cytoskeletal changes and subsequent release of immune mediators. Each of these steps are regulated by a multitude of proteins to ensure rapid but selective release of mediators to avoid collateral tissue damage. Rho GTPases have been implicated in almost every aspect of immune response including immune cell activation, proliferation, migration and response [81, 87]. They do this by the regulation of not only actin and microtubules but also calcium signaling [88], gene expression [89], adhesion [90] and other cell behaviours [91].

Rho subfamily members Rho, Rac and Cdc42, have well defined functions in stress fibre formation, lamellipodia extension and filopodia protrusion, respectively [85, 92] (**Figure 1.5**). Actin polymerization in eukaryotic cells is regulated by Arp2/3 and formins that coordinate actin severing and capping proteins [93]. Both Rac and Cdc42 regulate Arp2/3 complex via different effector proteins leading to these distinct morphological features. Cdc42 GTPases binds to Wiskott-Aldrich syndrome protein (WASP) family [94] and Rac GTPase to WASP-family verprolin-homologous (WAVE) family of proteins [95] to induce actin depolymerization, a critical step in degranulation [56, 67]. RhoA GTPase has been implicated in the regulation of actin via its effectors Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) and Diaphanous Homologue 1 (DIAPH1) [96]. In addition to actin, microtubules are also critical for immune responses. Using both stabilizers and destabilizers, microtubule dynamisms has been demonstrated to be required for immune mediator release [67, 97]. Like actin filaments, microtubules have an intrinsic polarity, with a minus end, usually anchored at the centrosome,

and a dynamic plus end, usually at the cell periphery that grows in response to environmental cues. Microtubules are required for cell polarity, distribution of intracellular organelles, proliferation and immune mediator release. New evidence points to a role for Rho proteins in microtubule extension; however, the mechanisms of action and participating effector proteins are still under investigation [98, 99].

Additional actin independent roles of Rho GTPases are also well characterized. Rho, Rac, and Cdc42 can activate JNK-p38-MAP kinase and NFκB pathways to regulate gene expression, survival and growth [79-81, 91, 100-102]. Rac has also been identified as an essential structural component of the NADPH oxidase complex and was found to promote ROS production in phagocytic cells [103]. The diverse but essential roles of Rho GTPases in the regulation of cell fate are unravelling. Consequently, targeting Rho signaling has already become an attractive target for several therapeutics like inflammatory disorders [104-106]. Therefore, it is important that we define the specific roles of subfamily members in immune cell function.

1.2.2 Structure and regulation of Rho GTPases

Structural investigations of Rho GTPases and their binding partners have given an insight into conformational changes, effector recognition and activation/inactivation mechanisms of Rho GTPases [83, 107-113]. Rho proteins possess a common G-domain fold, which consists of a six-stranded β-sheet surrounded by α-helices. In RhoA, the differences between GDP- and GTP-bound structural forms are confined to two segments referred to as switch I and switch II (residues 28-44 and 62-69 in human RhoA). The conformational changes between GTP- and GDP-bound RhoA is subtle and mostly seen in these switch regions. The changes are stabilized

by hydrogen bonds between the γ-phosphate group of GTP and the main-chain amide groups of the invariant Thr 37 (switch I) and Gly 62 (switch II). The GDP-bound forms of Rho GTPases lack these interactions and hence are unable to bind to effector proteins [111-113]. Further, they are post-translationally modified (PTMs), which is critical for their appropriate subcellular localization, activation, functions and stability [114, 115]. Nascent Rho proteins possess a carboxyl-terminal C*AAX* (where C represents cysteine, *A* is an aliphatic amino acid, and *X* is a terminal amino acid) box. This motif is clipped and lipidated either by the addition of farnesyl or geranylgeranyl isoprenoid groups. Pharmacological or genetic perturbation in prenylation of Rho proteins results in mislocalization and subsequent degradation of Rho proteins [114]. Furthermore, Rho proteins also undergo additional PTMs like phosphorylation and ubiquitylation that regulates their localization, functions and stability [116, 117].

In addition to PTMs, the critical nucleotide states of Rho GTPase is tightly regulated by three classes of proteins [118]: Rho guanine-nucleotide exchange factors (RhoGEFs), which activate Rho GTPases by promoting the exchange of GDP for GTP [119-121]; Rho GTPase-activating proteins (RhoGAPs), which enhance the intrinsic GTPase activity, leading to inactive GDP-bound state of Rho proteins [122]; and Rho guanine nucleotide-dissociation inhibitors (RhoGDIs), which sequester Rho GTPases in their GDP-bound inactive state and regulate their intracellular localization [123]. Dysregulation in the function of any of these regulators may lead to altered Rho signaling which in turn impacts cell fate. For example, many of the RhoGEFs are oncogenes that drive uncontrolled cell division via their aberrant activation of Rho GTPases [121]. Therefore, regulators of Rho GTPases are also viewed as attractive druggable targets with more

selective outcome and thus, there is a need for elucidating their specific roles and mechanisms of action in the regulation of Rho GTPases.

The first RhoGEF, DBL, was identified initially as an oncogene in human diffuse B-cell lymphoma [124]. There are 70 human Dbl family RhoGEFs that can activate specific Rho GTPases resulting in defined function. They all display a conserved catalytic Dbl homology (DH) domain, that is required for activation of Rho GTPase, and an adjacent regulatory pleckstrin homology (PH) domain [119, 125, 126]. Humans also encode another class of RhoGEFs,
the dedicator of cytokinesis (DOCK) family with 11 human members that are characterized by a conserved Dock-homology region-2 (DHR-2) that serves as the RhoGEF catalytic domain [119]. In addition to positive regulators like RhoGEFs, Rho GTPases are also regulated by negative regulators like RhoGAPs and RhoGDIs. These negative regulators prevent aberrant activation of Rho GTPases and in turn can limit negative cellular effects. RhoGAPs are defined by the presence of a conserved arginine residue termed the 'arginine finger', which, through homophilic interactions enhances intrinsic GTPase activities for Rho GTPases; this results in the hydrolysis of GTP and returning Rho GTPases to their GDP-bound 'off' state [122]. The size, distinct localization and binding preferences of the RhoGEF and RhoGAP families point to highly complex pathways regulating Rho family GTPases, but the true significance of this is still unclear.

1.3 RhoGDI

The third class of regulators, RhoGDIs, also control Rho signaling. RhoGDIs were originally discovered, as a passive regulator of Rho GTPases, for their ability to prevent GDP-dissociation

in Rho proteins and maintaining a pool of inactive GTPases in the cytosol [123, 127]. More recent work has demonstrated an active role in the regulation on GTPases where they were shown to modulate the extent of GTPase expression, crosstalk between subfamily members, membrane localization and activation states [116, 123, 127-130]. Recently, RhoGDI1 was demonstrated to interact directly with GTP-bound Rho proteins, RhoGEFs and RhoGAPs to control the duration of Rho signaling [131]. The essential roles of RhoGDIs in spatial and temporal regulation of Rho signaling are only starting to be discovered. Therefore, RhoGDIs are now being considered robust negative and positive regulator of Rho signaling. Clinical relevance of RhoGDIs and their roles in regulating Rho GTPases is yet to be fully unravelled and may establish them as a valid, targets for therapies driven by aberrant Rho signaling.

1.3.1 Subfamily members, structure and functions

Compared to the large RhoGEF and RhoGAP families, only three RhoGDIs have been described: (1) RhoGDI1, the archetypical member of the family, is the most abundant and ubiquitously expressed member (2) RhoGDI2 displays hematopoietic cell specific expression [132] and (3) RhoGDI3 also shows a more restricted expression in certain tissues such as the brain, pancreas, testis, lung and kidney [133]. Interestingly, RhoGDI subfamily members display distinct Rho GTPase binding preferences. RhoGDI1 binds to RhoA, Cdc42, Rac1 and Rac2 both *in vitro* and *in vivo* [83, 116, 130, 134, 135]; whereas RhoGDI2 binding partners have not been clearly defined. RhoGDI3 was shown to interact with RhoA, RhoB, Cdc42 and RhoG but not with Rac1 or Rac2 [136, 137]. What governs these distinct binding preferences are still under investigation. Structural insight into the RhoGDI family members may reveal conserved domains that drive these distinct properties. RhoGDIs comprise of two structurally distinct regions: An N-terminal

flexible domain (residues 1–69) and a C-terminal inflexible β-barrel domain (residues 70–204) (**Figure 1.6**) [83, 109, 133, 138]. Both domains contribute significantly to the binding and consequently the inhibitory actions of the molecule through protein–protein and protein–lipid interactions. Co-crystallization studies of RhoGDI1 with GDP-bound RhoA, Cdc42 and Rac1, as well as that of RhoGDI2 with Rac2(GDP), demonstrate the two distinct domains. The N-terminus adopts a well-ordered helix-loop-helix motif that forms extensive contacts, both polar and hydrophobic, with the switch regions of Rho GTPases. The N-terminus is thought to regulate GTP binding [83] and subcellular localization of Rho GTPase in complex with RhoGDIs [139]. Interestingly, RhoGDI subfamily members show the highest amount of divergence in their N-termini; however, its impact on their ability to regulate Rho GTPases are currently unknown. The C-terminus adopts an immunoglobulin-like fold comprising nine β-strands in two antiparallel sheets and an α-helix. The two sheets create a hydrophobic cavity that is lined with hydrophobic amino acid residues that interacts with the C-terminal lipid modification of Rho GTPases [83, 109, 110, 138]. The C-terminal domain, therefore, regulates cytosol/membrane partitioning of Rho proteins. However, whether it also regulates Rho GTPase interaction preferences is unclear.

Biological functions of RhoGDIs have been investigated using RhoGDI knockout mice [140-142]. RhoGDI1 knockout (KO) mice are viable but adult mice develop progressive renal defects, that ultimately lead to death. RhoGDI2 KO mice, on the other hand, do not display any severe phenotype and only minor defects are seen in haematopoiesis and superoxide generation during pathogen defense. Further research using knockout mice can elucidate potential redundant and non-redundant roles of subfamily members in homeostasis and diseased states.

The clearest evidence in terms of non-redundant roles of RhoGDIs are seen in cancers. Many cancers show varied expression of RhoGDI1 and RhoGDI2, however, their biological roles differ depending on the tumour type [136, 143-145]. For example, RhoGDI1 expression is upregulated in colorectal and ovarian cancers and it correlates with increased invasion and resistance to chemotherapy. In contrast, RhoGDI1 expression in brain and breast cancers gives a conflicting view of their role in tumorigenesis, survival and migration. The expression levels of RhoGDI2 are also severely altered in a range of cancers. Like RhoGDI1, biological roles of RhoGDI2 in cancer survival and invasiveness depends on the cancer type. The effects of the RhoGDIs on cancers are clearly complex and do not fit a simple explanatory model. Thus, RhoGDI subfamily members have emerged as robust regulators of cell fate via their regulation of Rho signaling.

1.3.2 Regulation of Rho GTPase dissociation from RhoGDIs

To elicit a specific function, like lamellipodia formation, select Rho GTPases (Rac) must dissociate from their complex with RhoGDI. Unlike Rab GTPases, where a GDI dissociation factors (GDFs) displaces RabGDI from their GTPase counterpart [146], a displacement factor has not been identified for Rho GTPases. Instead, several mechanisms that result in the selective dissociation of Rho GTPase from RhoGDIs have been proposed [116]. For example, during integrin mediated adhesion, phosphoinositide lipids can induce the dissociation of Rac1 from RhoGDI. Similarly, distinct protein-protein interactions can also induce the displacement of RhoGDIs. Ezrin, radixin, and moesin (ERM) proteins [147-150], p75 neurotrophin receptor (p75NTR) [151] and the Tyr kinase ETK [152] have all been reported to promote the displacement of Rho GTPases from RhoGDI.

In addition, phosphorylation and acetylation of RhoGDIs have emerged as key post-translational modifications that can selectively induce the dissociation of Rho GTPases [107, 116]. Phosphorylation of RhoGDIs decreases their affinity for Rho GTPases, promoting their release and making them available for activation. On the other hand, phosphorylation of Rho GTPases increases their affinity for RhoGDIs, promoting their sequestration and inactivation in the cytosol. For example, phosphorylation of RhoGDI1 on Ser 101 and Ser 174 by p-21-activated kinase (PAK1) reduces its affinity for Rac1 [153] whereas, phosphorylation of Rac1 and Cdc42 on Ser 71 by Akt results in the increased association with RhoGDI [154]. Thus, dissociation and re-association of Rho GTPases to RhoGDIs is tightly regulated. However, the biological implications of RhoGDI regulation are not fully understood. Therefore, further work is required to define the specific roles of dissociation factors in a disease context.

1.4 Regulation of MC activation and responses by Rho GTPases and RhoGDIs

Rho GTPases, central regulators of actin remodeling, have been implicated in MC degranulation. Constitutively active and dominant negative mutants of RhoA, Rac1, Rac2 and Cdc42 have been studied in RBL-2H3 (a model mast cell line) and BMMCs [58, 62, 155]. Rac and Cdc42 are thought to facilitate granule fusion by inducing actin depolymerization near 'degranulation zones' at the plasma membrane. Rac1 has also been thought to play a role in the formation of dorsal sub-plasma membrane actin rich membrane ruffles, whereas, Rac2 is thought to be required for calcium mobilization [156]. RhoA, on the other hand, has been implicated in the regulation of microtubule formation via the Gab2-Fyn-RhoA pathway [157]. Therefore, Rho GTPases regulate MC responses by controlling both microtubule-dependent

translocation of granules to the membrane, and the calcium-dependent degranulation. Recent research on RhoGDIs demonstrated that they also regulate the stability of Rho subfamily members via competitive binding [130]. Overexpression of one Rho protein may lead to the displacement of other family members from RhoGDI complexes, leading to their degradation. Therefore, conclusions drawn from overexpression of wildtype or mutant Rho proteins studies, which have been the standard tools to study Rho protein functions in different pathways, have to be revisited if RhoGDI proteins were in limited supply. The importance of controlling Rho GTPase function is manifested in how aberrant Rho signaling contributes to the severity of diseases such as cancer survival and metastasis, Alzheimer's disease, inflammation and allergies [87, 91, 102, 158, 159]. The lack of potent drugs that target Rho GTPases compel studies that look at the naturally occurring inhibitor of the Rho proteins, RhoGDI.

Although RhoGDIs are a highly conserved family of proteins, they may have non-redundant roles in cells. While our knowledge of RhoGDI function mostly stems from studies on RhoGDI1, new studies implicate distinct roles for other RhoGDI family members [116, 123, 127-130], which have been discovered in aggressive cancers. Hence, there is a need to define the specific roles of RhoGDIs and their subfamily members in the regulation of Rho GTPases. And finally, the biological roles of RhoGDIs in MC activation and responses have not been investigated and currently remains unknown. Hence, defining the roles of ubiquitous RhoGDI1 and hematopoietic cell specific RhoGDI2 in IgE-mediated responses of MCs might identify and validate a potential new target for drug discovery and development targeting allergies like asthma.

1.5 Hypothesis and research plan

MCs have emerged as powerful regulators of both innate and adaptive immunity. Their versatility stems from the fact that they can rapidly but selectively respond to immune stimuli. The range of mediators that are released allows them to initiate, magnify and resolve complex immune responses. However, the cellular machinery involved in MC activation and responses are just coming to light. Previous research implicated RhoA, Cdc42, and Rac in degranulation in FcεRI stimulated RBL-2H3 cells, largely through genetic manipulation of their expression. Field et al. used mutant RBL cell lines to demonstrate that activation of Rho GTPases was critical for degranulation [160]. However, the use of genetic perturbation can confound results if cell adaptation occurs during growth and passage. Additionally, overexpression of mutant Rho proteins may lead to proteasome mediated degradation of endogenous Rho proteins. Therefore, we took the approach to acutely inhibit Rho proteins with drugs and to examine the effect within a few minutes of application, minimizing long-term effects. Pharmacologic approaches are often advantageous over genetic methods, which are clearly not feasible for studies that involve essential genes. Here, we hypothesized one or more Rho GTPase family members are critical for MC degranulation. We tested this hypothesis by monitoring Rho GTPase activation during FcεRI-mediated MC activation in RBL-2H3 and BMMCs. Using Rho specific inhibitors, we demonstrated that Rho signaling through Rac controls MC morphology, which transitions to an activated state to facilitate degranulation. RhoA may also participate in the morphology and granule movement during degranulation.

The fact that drugs that target Rho family of proteins affect MC activation and degranulation compel studies that look at the naturally occurring inhibitor of the Rho proteins, RhoGDI. RhoGDIs have emerged as robust regulators of spatial and temporal Rho signaling. New studies implicate distinct roles for other RhoGDI family members, however, the distinct characteristics of the subfamily members are not clearly defined. We hypothesized that although RhoGDIs are a highly conserved family of proteins, they may have non-redundant roles in the cell. Hence, we decided to define the distinct characteristics of RhoGDI subfamily members. Biochemical and bioinformatics analysis highlighted some of the key characteristics that define this family like their distinct Rho GTPase binding, subcellular localization, GTP exchange inhibitory capacities and evolutionary properties.

Finally, the distinct immunological roles of RhoGDI1 and RhoGDI2, the two expressed in MCs, are currently unknown. Based on our observations of distinct localization and ability to inhibit Rho GTPases, we hypothesize RhoGDI1 and RhoGDI2 may have non-redundant roles in regulating MC activation and immune responses. We are elucidating this by studying subcellular localization of RhoGDI1 and RhoGDI2 in resting and stimulated RBL-2H3 cells. Further work investigating the effects of knocking down the expression of RhoGDIs on MC degranulation will validate RhoGDIs as an attractive target to interfere with aberrant Rho signaling in MC-mediated inflammatory disorders.

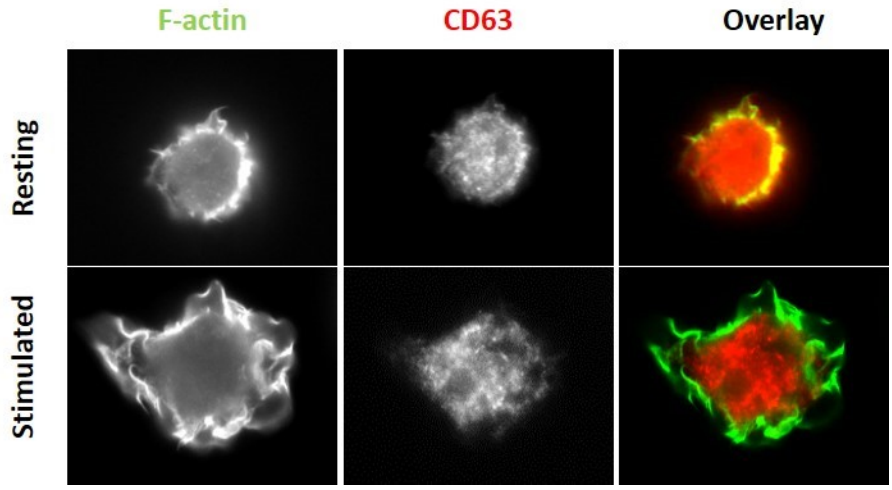


Figure 1.1 Bone marrow derived mast cells stained for F-actin and granules

Bone marrow derived MCs have been used extensively to characterize granule trafficking and morphological transitions in FcεRI -stimulated mast cells. Images represent F-actin and granules (CD63) in resting and antigen-stimulated BMMCs.

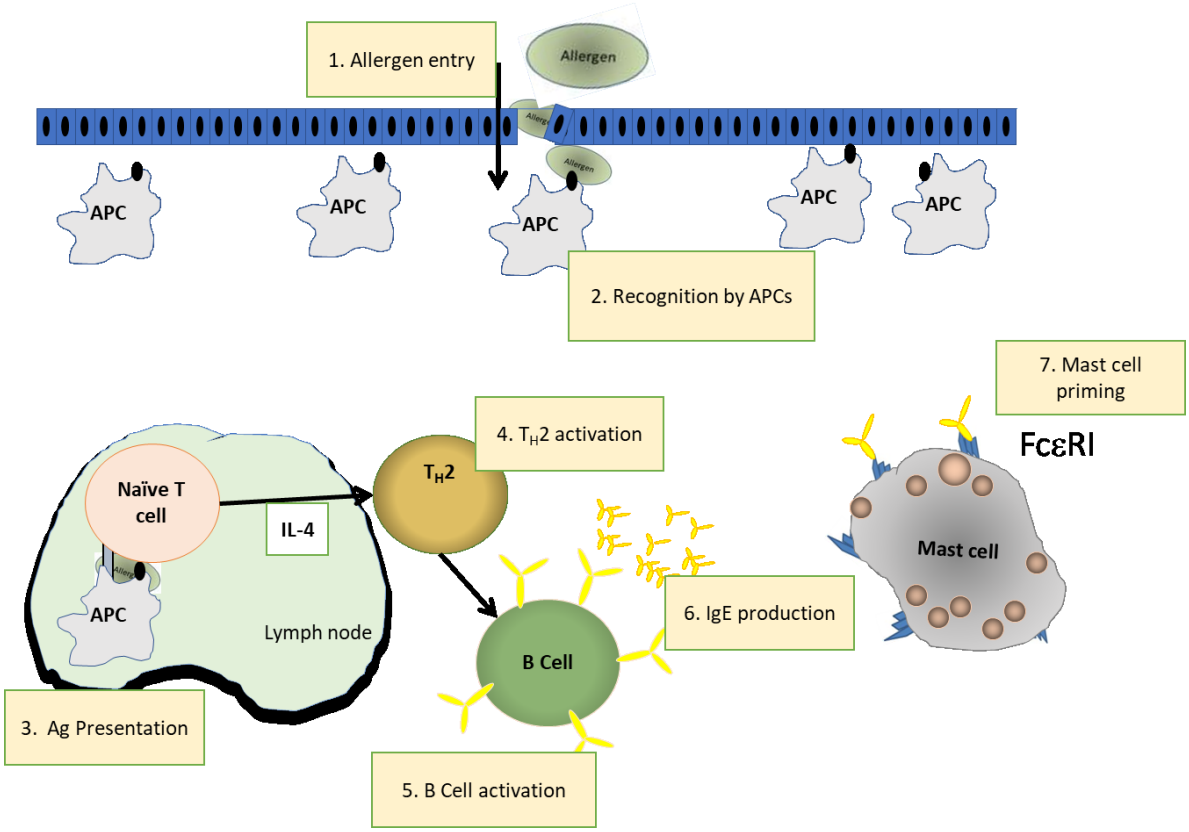


Figure 1.2 Schematic representation of steps in allergy development

(1) Allergies are initiated with the entry of innocuous allergens. (2) They are recognized by antigen presenting cells like DCs. (3) APCs migrate to nearby lymph nodes where they present processed antigens to naïve T cells. (4) Naïve T cells differentiate into T_H2 type resulting in the release of allergic cytokines. (5) T_H2 cells in turn induce B cell class switch to IgE type. (6) B cells produce IgE that deposit themselves on high affinity receptor, FcεRI. (7) Mast cells express FcεRI allowing them to be primed for subsequent encounters with similar antigens.

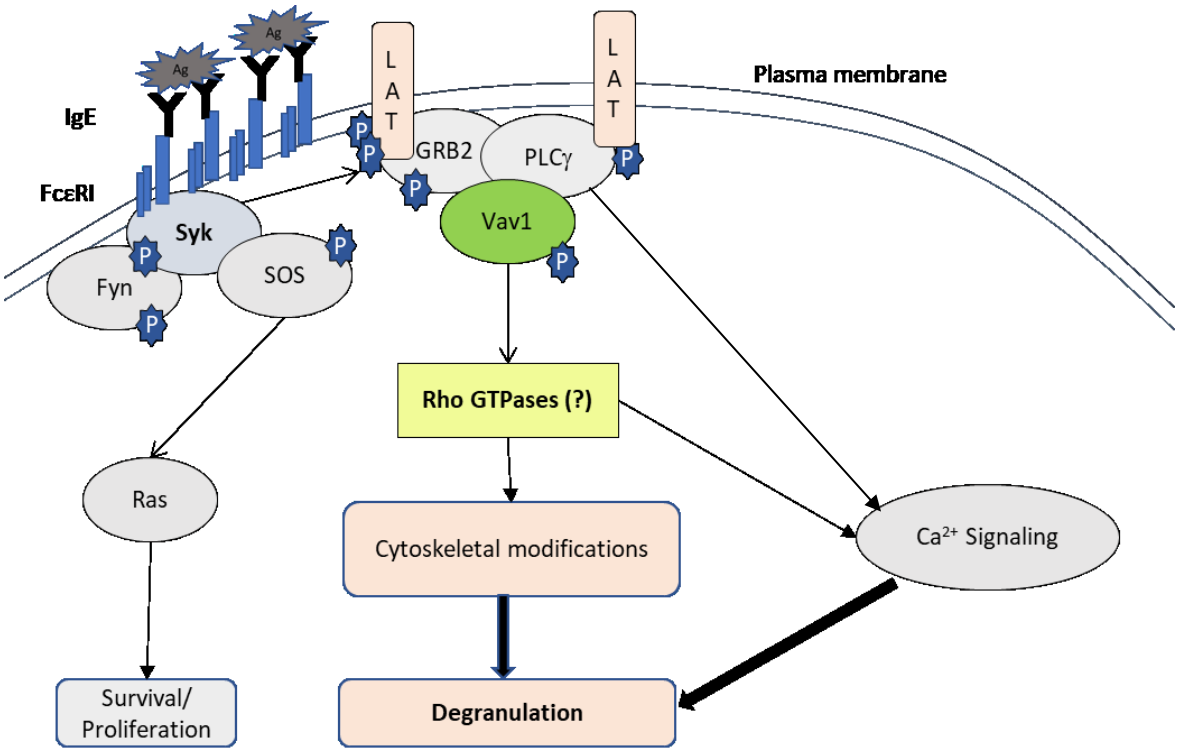


Figure 1.3 Schematic representation of FcεRI signaling during mast cell degranulation

Crosslinking multivalent antigen bound FcεRI receptors triggers a cascade of events that result in the activation of kinases, RhoGEFs and lipid metabolizing enzymes. Syk phosphorylates and activates scaffolding proteins like LAT, which in turn activates RhoGEFs (Vav1 in green). RhoGEFs are then able to activate Rho GTPases that ultimately regulate mast cell degranulation by controlling cytoskeletal modifications and calcium signaling. However, which Rho GTPases are essential for mast cell degranulation is unclear.

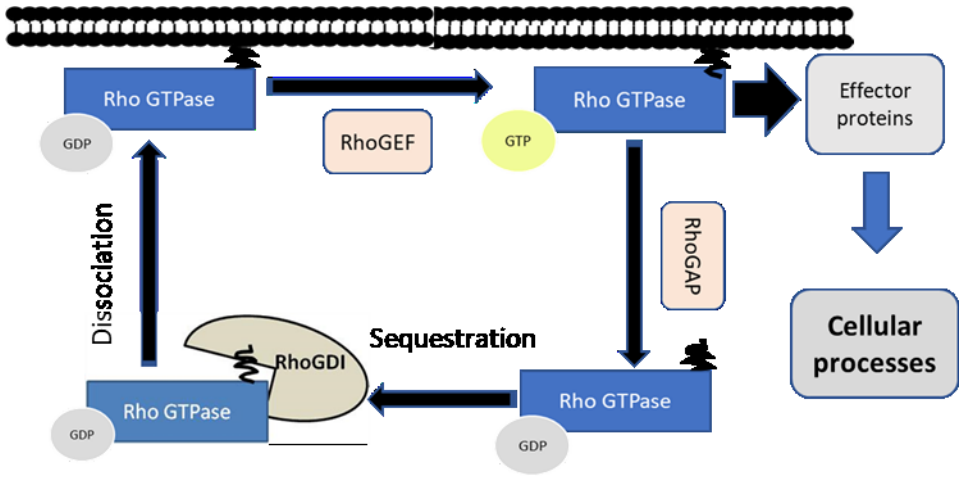


Figure 1.4 Schematic representation of Rho GTPase cycle

Rho GTPases are maintained in their 'off' GDP-bound state by RhoGDIs in the cytosol. Upon receiving upstream signal, they dissociate from Rho GTPases to allow RhoGEFs to catalyze the GTP exchange reaction. GTP-bound Rho proteins in turn function via effectors to regulate cellular processes. RhoGAPs catalyzes the GTP hydrolysis resulting in GDP-bound inactive Rho proteins, which are, once again sequestered by RhoGDIs.

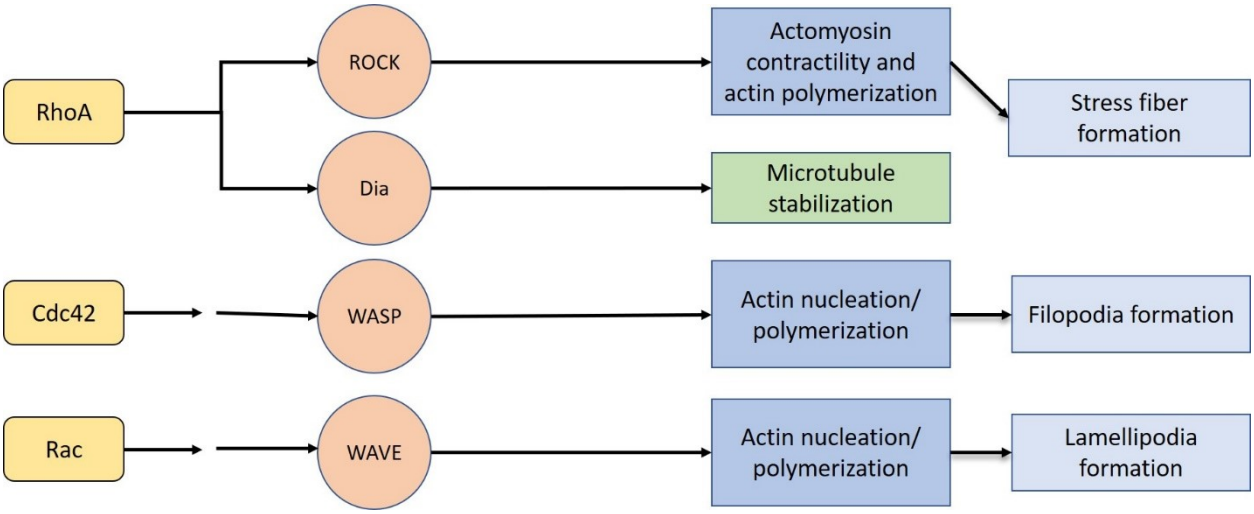


Figure 1.5 Distinct functions of the Rho GTPase subfamily members

In the GTP-bound 'ON' state, Rho GTPases interact with a number of effector proteins that result in distinct cellular behavior. The schematic representation of Rho GTPases and their downstream effectors that result in unique cell behavior. The well-studied Rho, Cdc42 and Rac GTPases regulate actin cytoskeleton to induce formation of stress fibers, filopodia and lamellipodia respectively.

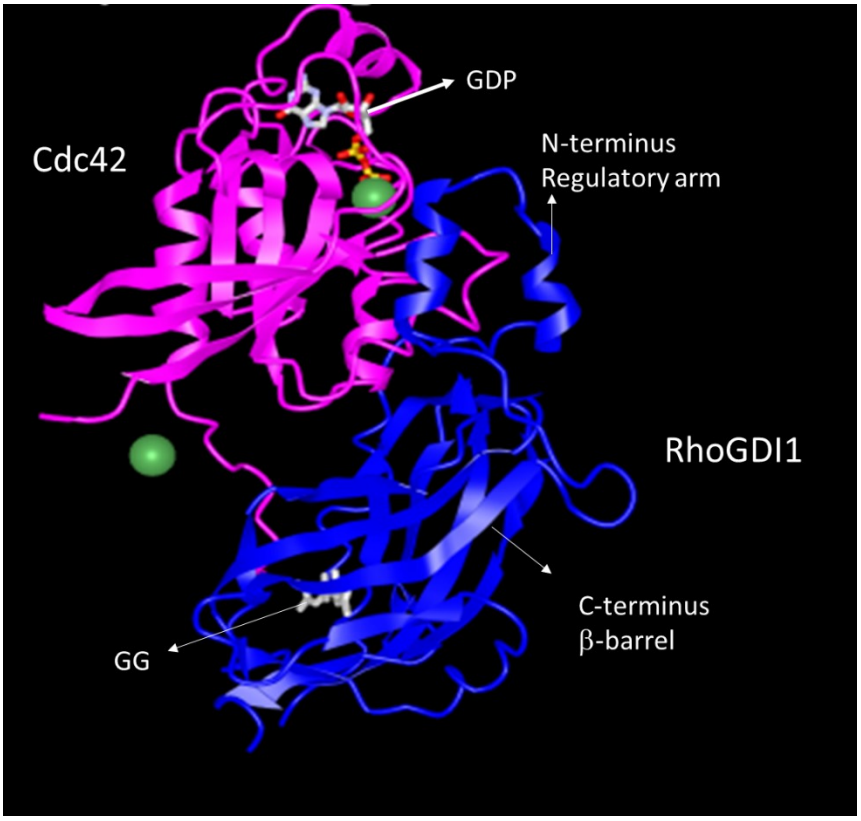


Figure 1.6 Example structure of RhoGDI1 in complex with Cdc42

Figure represents the crystal structure of prenylated Cdc42 (pink) in complex with RhoGDI1 (blue). These structures are reproduced from the NCBI Molecular Modeling Database (ID: 12765). RhoGDIs interact with Rho GTPases at both N- and C- termini. N-terminus may act as regulatory arm to dictate preferential interaction. GDP, Guanine diphosphate, GG, geranylgeranyl; N, amino terminus; C, carboxy terminus.

Chapter 2 Methods and Materials

Chapter 2: Materials and methods

For our study, various chemicals, reagents, enzymes and commercial kits were used according to manufacturer's protocol unless specifically stated otherwise. Workplace Hazardous Materials Information System (WHMIS) and the University of Alberta environmental health and safety office recommendations and protocols were followed for all reagent handling.

2.1 Reagents

Table 1 - Following Reagents were used in this study:

Chemicals/Reagent	Supplier
0.25% Trypsin – EDTA	Thermo Fisher Scientific (Invitrogen)
30% acrylamide/bis (29:1)	Thermo Fisher Scientific (Invitrogen)
4',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific
4-Methylumbelliferyl-beta-D-galactopyranoside (MUG)	Sigma-Aldrich
6x DNA loading dye	FroggaBio
Acetic acid, glacial	Fisher chemical
Agarose	Thermo Fisher Scientific
Ammonium persulphate	BDH Chemicals
Ampicillin	Thermo Fisher Scientific
Anti-DNP-BSA IgE	Sigma-Aldrich
Bovine serum albumin (BSA)	Thermo Fisher Scientific
Bromophenol blue	Thermo Fisher Scientific
Calcium chloride	Sigma-Aldrich
Chloramphenicol	Thermo Fisher Scientific
DH5α competent E. coli	Thermo Fisher Scientific
Dithiothreitol (DTT)	Sigma-Aldrich
DNP-BSA	Thermo Fisher Scientific
dNTPs	Sigma-Aldrich
Dulbecco's modified eagle medium (DMEM)	Thermo Fisher Scientific (Invitrogen)
EDTA	BDH Chemicals
Ethanol	Fisher Chemical
Fetal bovine serum (FBS)	Thermo Fisher Scientific (Invitrogen)
Glucose	Fisher Chemical
Glutathione agarose	Sigma-Aldrich
Glycerol	Fisher Bioreagent
HEPES	Fisher Biotech
Hydrochloric acid	Fisher Chemical
Interleukin-3 (IL-3)	Sigma-Aldrich
Isopropanol	Fisher Chemical
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Thermo Fisher Scientific

Kanamycin	Thermo Fisher Scientific
Lipofectamine 2000	Thermo Fisher Scientific (Invitrogen)
Lysotracker green	Thermo Fisher Scientific
Lysotracker red	Thermo Fisher Scientific
Lysozyme	ICN Biomedicals
Magnesium chloride	BDH Chemicals
Mant-GTP	Thermo Fisher Scientific
Methanol	Sigma-Aldrich
Minimum essential medium (MEM)	Sigma-Aldrich
MOWIOL	Inhouse (A kind gift from Dr. Andrew Simmonds, University of Alberta)
Opti-MEM	Thermo Fisher Scientific (Invitrogen)
Oregon green 488 phalloidin	Thermo Fisher Scientific
Paraformaldehyde	Fisher Bioreagents
Penicillin/Streptomycin	Thermo Fisher Scientific (Invitrogen)
Phenylmethylsulphonyl fluoride (PMSF)	Bioshop Canada Inc
Phosphate buffered saline	Thermo Fisher Scientific (Invitrogen)
Potassium chloride	EM Sciences
Precision plus protein standard	Thermo Fisher Scientific (Invitrogen)
Prolong Gold antifade reagent	Thermo Fisher Scientific (Invitrogen)
Protein G sepharose	Thermo Fisher Scientific (Invitrogen)
Red stain	FroggaBio
Restriction enzymes	Thermo Fisher Scientific (Invitrogen)
Rhodium phalloidin	Thermo Fisher Scientific
Rosetta competent E. coli	Thermo Fisher Scientific
Sodium bicarbonate	Sigma-Aldrich
Sodium chloride	Fisher Bioreagent
Sodium dodecyl sulphate (SDS)	Fisher Bioreagent
Sodium Fluoride	Sigma-Aldrich
Sodium hydroxide	Fisher Chemicals
Sodium Vanadate	Sigma-Aldrich
Stabl3 competent E. coli	New England Biolabs
Sucrose	Sigma-Aldrich
T4 DNA ligase	Thermo Fisher Scientific
Tetracycline	Thermo Fisher Scientific
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific
Tris Base	Thermo Fisher Scientific
Triton X-100	Thermo Fisher Scientific (Invitrogen)
Tryptone	Fisher Bioreagents
Tween-20	Thermo Fisher Scientific (Invitrogen)
Yeast extract	Fisher Bioreagents

Table 2 - Small molecule inhibitors used in this study

Drug name	Target	Supplier
Cytochalasin B	F-actin destabilizer	Sigma-Aldrich
EHop-016	Rac1-Vav2 inhibitor	Tocris Biosciences
EHT-1864	Rac1 and Rac2 inhibitor	Tocris Biosciences
HA-1100	Rho-associated protein kinase (ROCK) inhibitor	Sigma-Aldrich
ML-141	Cdc42 inhibitor	Tocris Biosciences
Nocodozole	Microtubule stabilizer	Tocris Biosciences
NSC-23766	Rac-Trio inhibitor	Tocris Biosciences
Rhosin	RhoA inhibitor	Tocris Biosciences

Table 3 - Commercially available kits that were used in this study

Commercial Kits	Supplier
Annexin V detection kit (FITC)	Sigma-Aldrich
Plasmids DNA maxi prep	Qiagen
Plasmid DNA mini prep	Qiagen
Qiaquick gel extraction kit	Qiagen
Rho activation assay GLISA – Cdc42	Cytoskeleton
Rho activation assay GLISA – Rac1	Cytoskeleton
Rho activation assay GLISA – Rho	Cytoskeleton

Table 4 - Commonly used buffers and solutions

Buffers/Solutions	Composition
1x TAE	40 mM Tris, 20 mM acetate and 1 mM EDTA
Bacteria lysis buffer	10 mM HEPES (pH 7.5), 120 mM NaCl, 5 mM MgCl2, 0.5% Triton X-100
Blocking buffer	2% BSA in PBS
Cell fixation buffer	4% (w/v) paraformaldehyde, PBS and 0.25 M sucrose
GEF buffer	20 mM Tris-Cl (pH 7.5), 60 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.05 mg/ml BSA, 10% (v/v) glycerol
H-buffer	20 mM HEPES pH 7.5, 60 mM NaCl, 5 mM MgCl2
HEPES Tyrode's buffer (HTB)	25 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 1 g/L glucose, and 1 g/L BSA
Lysis buffer	0.5% Triton X-100 PBS with protease inhibitor and phosphatase inhibitor cocktail
Permeabilization buffer	0.2% Triton X-100, PBS
Phosphate buffer saline (PBS)	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4

	and 1.47 mM KH_2PO_4 (pH 7.4)
Running buffer	25mM Tris-HCl, 190 mM glycine and 0.1 % (w/v) SDS
5X SDS PAGE loading buffer	250 mM Tris–HCl (pH 6.8), 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 40% (v/v) glycerol and 20% (v/v) β-mercaptoethanol
Transfer buffer	25 mM Tris-HCl (pH 7.6). 192 mM glycine, 20% methanol, 0.1 % (w/v) SDS
Western wash	1x PBS, 0.05% Tween 20

2.2 Cell culture and generation of bone marrow derived mast cells (BMMCs)

The following cell lines and primary cells were used in this thesis: RBL-2H3 (Rat basophil leukemia cell line, ATCC), HEK-293T (Human embryonic kidney cells, a kind gift from Dr. Thomas Simmen) and bone marrow derived mast cells (BMMCs). Cells were grown in recommended medium with 10% heat inactivated FBS, 0.1 mg/ml penicillin and 100 units/ml streptomycin in a 5% CO_2 incubator set at 37^{o}C. Cells were split every 2–3 d with 0.25% (wt/v) trypsin/1 mM EDTA.

BMMCs were derived by cytokine differentiation, as previously described [156]. Briefly, mice tibia and fibulae were isolated from wildtype mice and washed with sterile PBS. The isolated bone ends were clipped, and 5 ml of PBS was injected on one side forcing bone marrow cells to emerge from the other side which were collected. Cells were pelleted by centrifugation at 300g for 10 min at 4^{o}C and resuspended in 10 ml of warm RPMI 1640 supplemented with cytokines (20 ng/ml Interleukin-3 (IL-3), 1 X Non-Essential Amino Acids (NEAA), 1X sodium pyruvate, 10% heat inactivated FBS, 100 units/ml penicillin and streptomycin. Cells were incubated in T75 flasks for four days at 37^{o}C and 5% CO2. After the fourth day post isolation, 10 ml of fresh cytokine supplemented RPMI 1640 was added to the original flask and incubated for an additional four days. Every four days thereafter, the media was hemi-depleted (50% removed)

and the suspended cells were transferred to a new T75 flask to which 10 ml of fresh supplemented RPMI 1640 was added. BMMCs were maintained at concentrations ranging between 1 - 2 x 10^6 cells/ml. Cells used in experimental assays were cultured in this way for a minimum of four weeks prior to use in experiments.

2.3 Antibodies

Primary and secondary antibodies used in immunoblots and immunofluorescence assays are listed below:

Table 5 - Primary antibodies used in Immunoblots

Antibody (Raised in)	Dilution	Source	Catalogue number
Calnexin (Rabbit)	1:2000	Kind gift from Dr. Thomas Simmen, University of Alberta, Canada	NA
Cdc42 (Mouse)	1:1000	Santa Cruz Biotechnology	8401
GAPDH (Mouse)	1:1000	Santa Cruz Biotechnology	47724
GLUT1 (Mouse)		GeneTex Inc	GTX15309
GST (Mouse)	1:5000	Sigma-Aldrich	G7781
phospho-Syk Y525/526 (Rabbit)	1:2000	Cell signaling technologies	2711
Rac (Rabbit)	1:1000	Santa Cruz Biotechnology	517424
Rac1(Rabbit)	1:1000	EMD Millipore	05-389
Rac2 (Rabbit)	1:1000	In-house (raised against C-terminus Rac2 in rabbits)	NA
RhoA (Mouse)	1:1000	Santa Cruz Biotechnology	418
RhoGDI1 (Mouse)	1:1000	Santa Cruz Biotechnology	373724
RhoGDI1 (Rabbit)	1:1000	Santa Cruz Biotechnology	360
RhoGDI2 (Mouse)	1:1000	Santa Cruz Biotechnology	365663
RhoGDI2 (Rabbit)	1:1000	AbCam	181252
RhoGDI3 (Mouse)	1:1000	Santa Cruz Biotechnology	393690
Sucrose dehydrogenase A (Rabbit)	1:2000	Cell signaling technologies	5839
Total Syk (Rabbit)	1:2000	Cell signaling technologies	2712
VDAC1 (Mouse)	1:1000	AbCam	14734
β-tubulin (Rabbit)	1:2000	AbCam	15568

Table 6 - Secondary antibodies used in immunoblots

Antibody	Dilution	Source
Alexa Fluor 680 goat anti-rabbit	1:10000	Molecular probes (Invitrogen)
Alexa Fluor 750 goat anti-mouse	1:10000	Molecular probes (Invitrogen)

Table 7 - Primary antibodies used in immunofluorescence

Antibody	Dilution	Source
Calnexin	1:100	In house (Michalak lab)
CD63	1:100	BD Biosciences
Complex II	1:100	AbCam
Complex II (SDHA)	1:100	Cell signaling technologies
GLUT1	1:100	GeneTex Inc
Protein disulfide-isomerase (PDI)	1:100	Thermo Fisher Scientific
RhoGDI1	1:100	Santa Cruz Biotechnology
RhoGDI2	1:100	Santa Cruz Biotechnology
β-tubulin	1:100	AbCam

Table 8 - Secondary antibodies used in immunofluorescence

Antibody	Dilution	Source
Alexa fluor 546 goat anti-rabbit	1:250	Molecular probes (Invitrogen)
Alexa fluor 488 donkey anti-rabbit	1:250	Molecular probes (Invitrogen)
Alexa fluor 546 goat anti-mouse	1:250	Molecular probes (Invitrogen)
Alexa fluor 488 donkey anti-mouse	1:250	Molecular probes (Invitrogen)
Alexa fluor 405 donkey anti-rabbit	1:250	Molecular probes (Invitrogen)

2.4 Construction of plasmids

The following plasmids were constructed by cloning genes of interest byPCR amplification of commercially available cDNA (GE Healthcare, Little Chalfont, UK) into the indicated vectors:

2.4.1 Generation of tagged RhoGDIs

Table 9 – RhoGDI constructs and primers

Plasmid name	Vector	5’ oligo	3’ oligo
RhoGDI1-GFP	eGFP N1	CCGGAATTCATGGC TGAGCAGGAGCCCA	AATGGATCCTCAGT CCTTCCAGTCCTTCT
RhoGDI2-GFP	eGFP N1	CCGGAATTCATGACT GAAAAAGCCCCAGA	AATGGATCCTCATTC TGTCCACTCCTTCTT

RhoGDI3-GFP	eGFP N1	CCGGAATTCATGCT GGGCCTGGACGCGT	AATGGATCCTCAGT CCTTCCAGTCCTGGC
GFP-RhoGDI1	eGFP C1	CCGGAATTCATGGC TGAGCAGGAGCCCA	AATGGATCCTCAGT CCTTCCAGTCCTTCT
GFP-RhoGDI2	eGFP C1	CCGGAATTCATGACT GAAAAAGCCCCAGA	AATGGATCCTCATTC TGTCCACTCCTTCTT
GST-RhoGDI1	pGEX-4T1	CCGGGATCCATGGC TGAGCAGGAGCCCA	AATGAATTCTCAGT CCTTCCAGTCCTTCT
GST-RhoGDI2	pGEX-4T1	CCGGGATCCATGACT GAAAAAGCCCCAGA	AATGAATTCTCATTC TGTCCACTCCTTCTT
GST-tRhoGDI3	pGEX-4T1	CCGGGATCCGACAA GGAGGGTGGGCCGC	AATGAATTCCAGTC CTTCCAGTCCTGGC

2.4.2 Generation of CRISPR plasmids

The following guide RNAs were cloned into LentiCRISPR V2 (LC V2) plasmid, using the Zhang lab protocol [161]. The empty vector was cut using Bsmb1 restriction enzyme and annealed oligos were ligated into it. The oligos are designed based on the target site sequence (20bp) and needs to be flanked on the 3' end by a 3bp NGG PAM sequence. Benchling software (https://benchling.com) was used to generate oligos that had high specificity and low off-target effects. Control guide RNA was cloned based on control sequences available on Addgene. Plasmids were sequenced to confirm appropriate insertion of the sgRNA.

Table 10 – CRISPR plasmids used in this study

CRISPR plasmid	5’ guide RNA	3’ guide RNA
RhoGDI1 LC V2	CACCGTGTGACACTAGAGACCACAG	AAACCTGTGGTCTCTAGTGTCACAC
RhoGDI2 LC V2	CACCGCCACTGGAAGCTCAGAACAG	AAACCTGTTCTGAGCTTCCAGTGGC
RhoGDI3 LC V2	CACCGCAGCATGCTCTTCTTCCCAG	AAACCTGGGAAGAAGAGCATGCTGC
Scrambled LC V2	CACCGTATTACTGATATTGGTGGG	AAACCCCACCAATATCAGTAATAC

2.5 Other plasmids

Table 11 -The following plasmids were bought or cloned in house:

Plasmids	Vector	Source
eGFP	pEGFP-C1/N1	Addgene
gag-pol	pUMCV	Addgene
GST-Cdc42	pGEX-2T1	Inhouse
GST-CRB	pGEX-2T1	Inhouse
GST-PAK	pGEX-2T1	Inhouse
GST-Rac1	pGEX-2T1	Addgene
GST-Rac2	pGEX-2T1	Inhouse
GST-RhoA	pGEX-2T1	Inhouse
His_6-DH-PH Dbl	pET-15d	Kind gift by Dr. KL Rossman (University of North Carolina, Chapel Hill)
His_6-DH-PH Itsn1	pET-15d	Kind gift by Dr. KL Rossman (University of North Carolina, Chapel Hill)
His_6-DH-PH TrioN	pET-15d	Inhouse
LentiCRISPR V2	Custom	Addgene
pET11d	custom	Inhouse (kind gift by Dr. Paul LaPointe, University of Alberta)
pET15d	custom	Addgene
pGEX-4T1	Custom	Addgene
psPAX2	Custom	Addgene
pVSVg	Custom	Addgene
RhoGDI1 shRNA (4 variants)	pRS-GFP	Origene
RhoGDI2 shRNA (4 variants)	pRS-GFP	Origene
Scrambled shRNA (4 variants)	pRS-GFP	Origene

2.6 Protein expression and purification

Tagged- proteins like GST-Rho GTPases (RhoA, Cdc42, Rac1 and Rac2, -CAAX box removed), GST-RhoGDIs (RhoGDI1, RhoGDI2 and RhoGDI3) constructs were generated in pGEX-2T1; His_6-DH-PH domains (RhoGEF domains) were cloned from cDNA into pET15b for protein production and purification. A 28 amino acid N-terminally truncated RhoGDI3 construct was generated since full-length produced an insoluble protein. E.coli (Rosetta strain) containing expression plasmids were grown in LB with 0.04 mg/ml chloramphenicol and other appropriate antibiotics,

until they reached an OD of 0.5. IPTG was used to induce the expression of tagged proteins. Bacteria were lysed in lysis buffer by freeze-thaw and sonication and the cleared supernatant containing proteins were aliquoted and stored in -80^{o}C.

Proteins were further purified by affinity purification, as previously described [162]. Clear bacterial lysates containing tagged-proteins were passaged over GST-affinity columns to allow binding of GST-proteins to the resin. Purified proteins were then eluted by adding 10 mM reduced glutathione, that competitively displaces the immobilized glutathione binding interaction with GST, allowing the fusion protein to emerge from the affinity column. Purified proteins (10 μg) were run on SDS-PAGE gel and stained with Coomassie blue to ascertain purity. To cleave GST-tag off proteins, proteins were incubated with thrombin (1U) for 30 mins and 1 mM PMSF was used to quench the reaction.

2.7 Transient transfections, generation of stable cell lines and CRISPR knockout clones

Transient transfections were done with tissue culture cells that were grown on No. 1.5 glass coverslips in 6 well dishes. Glass coverslips were surface sterilized using 70% ethanol. Depending on the type of experiment (microscopy or generation of stable line) cells were plated and transfected with appropriate plasmids using Lipofectamine 2000 or Lipofectamine 3000, following the manufacturer's protocols. Cells were cultured approximately for 18-24 hr to allow for protein expression/ knockdown and were further processed depending on the experiments.

To generate stable knockdown cells, 4 different shRNA variants (Origene, USA), were purchased against RhoGDI1, RhoGDI2 and scrambled shRNA control. To generate retroviral particles,

shRNA, gag-pol and VSVg plasmids were co-transfected into early passage HEK-293T cells. Cell supernatant containing viruses was collected at 48 and 72 hr, post transfection. To transduce, target cells (RBL-2H3) were plated in 10 cm dishes and allowed to grow to 60-70% confluency. Viral supernatant was added along with polybrene (8 μg/ml), allowed to infect for 6 hr and media was replaced with fresh growth media. 24 hr post-infection, media with puromycin (0.01 mg/ml) was added and puro-resistant colonies were selected and screened for knockdown efficiency by western blotting and RT-PCR.

To generate RhoGDI CRISPR knockout cells, guide RNA against RhoGDIs were cloned into the empty vector (LentiCRISPR V2) following Zhang lab protocol. These plasmids along with pPax2 and VSVg were co-transfected to early passage HEK-293T cells. Lentiviral particles were collected 48 and 72 hr post transfection and was used to infect RBL-2H3 cells. CRISPR KO colonies were selected with puromycin, expanded and efficiency was checked using immunoblotting and RT-PCR.

2.8 Protein binding studies

Protein-protein interaction studies were done by pulldown assays, as previously described [163, 164]. Briefly, GST-proteins were immobilized on glutathione agarose beads (Sigma-Aldrich). Samples containing target proteins were incubated with the beads for 30 min to allow binding of proteins. Excess proteins were washed in H-buffers and beads were processed for immunoblot.

2.9 Immunoblotting and analysis

Following protein separation by SDS-PAGE, proteins were transferred to nitrocellulose membrane at 300 mA for two hr. Membrane was then blocked in 2% BSA for at least 30 mins before being incubated with appropriate primary antibodies. Following 1.5 hr incubation, membranes were washed thrice with PBS-Tween 20 and incubated with fluorescent secondary antibodies for 45 min. Membranes were washed again in PBST (2x), distilled water (2x) and scanned on a Licor Odyssey scanner (Licor Biotechnology, Lincoln, NE). Odyssey software was also used to quantify immunoblots. Band intensities were quantified by manually drawing rectangle around the region of interest which automatically corrected for background based on the median pixel intensity in a 3-pixel region along the upper and lower edges of the drawn rectangle. Quantified intensities were exported to Excel and statistical analysis was performed.

2.10 Immunofluorescence microcopy

Typically following required treatments, cells were washed twice with warmed PBS and fixed with 4% (wt/v) paraformaldehyde for 20 min. Subsequently, cells were washed in PBS, and permeabilized with 0.2% (v/v) Triton X-100 in PBS to allow antibodies to access intracellular structures. Prior to incubation with primary antibodies, cells were blocked with 2% BSA for 30 min. Usually, cells were double/triple labelled using antibodies raised in different specifies and secondary antibodies conjugated to different fluorophores. Coverslips were mounted on clear glass slides with prolong gold anti-fade reagent or homemade MOWIOL.

2.10.1 Widefield microscopy

Fixed-cell fluorescence microscopy was performed on RBL-2H3 cells, BMMCs and HEK-293T cells to visualize cytoskeleton, granules and subcellular localization of proteins. Images were

taken on a Zeiss Observer Z1 wide-field microscope (Carl Zeiss, Oberkochen, Germany) with a 63X objective (1.4 NA) and processed using Axiovision 4.8 software (Carl Zeiss).

2.10.2 Spinning disc confocal microscopy

To perform colocalization studies, fixed cells stained with appropriate antibodies were imaged in a spinning disc confocal system (Ultraview ERS; PE Biosciences) and Axioimager M2 (Zeiss) with a 63X Plan-Apochromat oil lens (1.4 NA, Zeiss), and Volocity acquisition software (PE Biosciences). Images were taken every 0.166 μm step (z-stack). Analysis was performed using Huygens Professional (SVI), Imaris (Bitplane), and Prism (Graphpad).

2.10.3 Live cell microscopy

Live-cell, brightfield DIC and fluorescence microscopy was performed on RBL-2H3 cells to visualize cell morphology and granule dynamics during FcεRI stimulation. 500,000 cells were plated in 35 mm and 50 mm disposable plastic petri dishes with the optical quality of glass. Cells were stained with LysoTracker green for granule-tracking experiments. Cells were imaged during Ag stimulation with a temperature- (37°C) and CO_2 (5%)- controlled, live-cell chamber to ensure ideal environmental conditions. Imaging was performed on a PerkinElmer (Waltham, MA, USA) Ultra-VIEW VoX spinning-disk, confocal microscope with a 363 objective (1.4 NA) and brightfield DIC with a 1 s exposure or brightfield and a 488 laser set to 10% power and 100 ms exposure. Images were taken every 10 s and processed with Velocity 6.0 software (Spirent Communications, Crawley, West Sussex, United Kingdom), which contained a particle-tracking module, and exported as .wmv (Windows media video) files at 10 frames/s.

2.11 Degranulation assay

To monitor MC degranulation, the release of β-hexosaminidase from IgE-FcεRI stimulated RBL-2H3 cells and BMMCs was measured [64]. RBL-2H3 cells and BMMCs were plated in 24-well plates at a density of $2x10^5$ cells/well and grown overnight. They were sensitized with 120 ng/ml anti-DNP-BSA IgE antibody for 2 hr. Following sensitization, cells were washed twice with HTB and stimulated with multivalent DNP-BSA to β-hexosaminidase activity. Cleavage of MUG by β-hexosaminidase releases the fluorescent product methylumbelliferone, which was detected with a Synergy-4 fluorometer set to em/ex of 360/460 nm (BioTek Instruments, Winooski, VT, USA). Fluorescence is directly proportional to degranulation, which was calculated as the percentage of β-hexosaminidase in the supernatant, divided by β-hexosaminidase from 0.5% Triton X-100–lysed cells (released/total). Data were analyzed by nonpaired, 2-tailed Student's t test to indicate a significant difference between two means ± SEM.

2.12 Flow cytometry and analysis

To monitor granule degranulation by a different method in RBL-2H3 cells and BMMCs, flow cytometric approach was used [165]. Upon degranulation, granule inner membrane containing phosphatidyl serine (PS) is exposed on the outer surface of cells. Therefore, MC degranulation can be monitored by staining stimulated cells with fluorescently tagged-annexin V (FITC-annexin V) that binds to PS. Sensitized and stimulated RBL-2H3 cells and BMMCs fixed with 4% (wt/v) paraformaldehyde and stained with FITC-annexin V. A P4 gate was set for annexin V–negative cells, which included ~95% of cells in the resting sample, and the P5 gate was set for

annexin V–positive cells. The FACSDiva analysis program (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze data. Data were analyzed by nonpaired, 2-tailed Student's t test.

2.13 Rho activation assays

The levels of activated (GTP-bound) RhoA, Rac1, and Cdc42 were tested in resting and in Ag-stimulated RBL-2H3 cells and BMMCs by G-LISA assay, according to the manufacturer's protocol. Briefly, resting and stimulated RBL-2H3 and BMMC lysates were incubated in 96-well dish precoated with probes that specifically recognize the activated forms of RhoA, Rac1, and Cdc42. Constitutively active forms of Rho proteins (provided in the kit) were used as positive controls. After incubating for 30 min, wells were washed and incubated with primary Abs that recognize RhoA, Rac1, and Cdc42, followed by an HRP-conjugated secondary Ab. The levels of activated Rho proteins were determined by luminescence intensity of the HRP reagent using a Synergy-4 fluorometer (BioTek).

Rho activation assays were also performed by pull-down assay, as previously described [166]. Briefly, cell lysates were incubated with GST-CBD (which contains the Cdc42/Rac-binding domain from PAK1) or GST-RBD (which contains the Rho-binding domain from rhotekin) immobilized on 10 μl packed glutathione agarose. Samples were incubated for 30 min to allow binding of activated Rac1, Rac2, Cdc42, or RhoA, washed in H-buffer, and analyzed by immunoblot.

2.14 Cell fractionation and analysis

Cell fractionation was used to identify subcellular localization of RhoGDIs with respect to markers of organelles like plasma membrane, ER, mitochondria and cytosol. Briefly, tissue

culture cells were grown in 15 cm plates to confluency, washed twice with PBS and lysed in PBS supplemented with protease and phosphatase inhibitor cocktail by freeze/thaw method. Cell lysates were centrifuged for 5 min at 300*g* to remove cell debris. An aliquot of clear supernatant was used for a loading control and the rest was layered on top of a 10 ml linear sucrose gradient (ranging from 5 - 40%) and centrifuged using an SW41Ti rotor (Beckman Coulter, Mississauga, ON, Canada) at 234,326g_{max} for 3 hr at 4°C. 1 ml fractions were collected, TCA precipitated and analysed by immunoblot.

2.15 RhoGEF-mediated GTP exchange assay

Nucleotide exchange activity was assayed by monitoring the relative increase in fluorescence of the fluorescent GTP analog, mant-GTP, upon binding a GTPase as previously described. GEF assays contained 1 μM of purified GST-Rho protein, 150 nM mant-GTP in a total volume of 2 ml of GEF buffer. Baseline readings were taken for 5 min, then 50 nM purified RhoGEF DH-PH domain was added, and reactions were monitor for a further 20 min. Fluorescence measurements were taken using a PTI fluorimeter (ex/em = 360/440 nm +/- 5 nm) with a temperature-controlled cuvette holder set to 20°C. GEF activity was calculated as the initial rate of fluorescence increase relative to buffer control. To inhibit RhoGEF-mediated GTP exchange, Rho GTPases were pre-incubated with 5 μM of purified GST-RhoGDIs for 5 min. Statistical analysis was performed using a two-tailed Student's t-test. Error bars represent standard error of the mean.

2.16 Comparative genomics and analysis

Similarity searches were run to identify orthologues of the human RhoGDI proteins in sequence data from a sampling of animals. Human RhoGDI1, RhoGDI2 and RhoGDI3 were used as query

sequences in the Basic Local Alignment Search Tool (BLAST 2.2.29+), with the BLASTp algorithm used to search amino acid sequences, and the tBLASTn algorithm used to search nucleotide sequences. A reciprocal best hit approach was taken, with the criteria that identified sequences must retrieve the human sequences when used as a query for searching human protein sequences. An E-value cut-off of 0.05 for both searches, and in the reverse search, the original query must be retrieved with an E-value at least two orders of magnitude lower than sequences that do not correspond to the original human RhoGDI query. Amino acid sequences identified using BLASTp were aligned using MUSCLE v3.8.31 [167] with default parameters. Redundant sequences were removed and the alignment was used to construct a hidden markov model (HMM). This HMM was used to identify all RhoGDI sequences in the protein sequence data, including some that may have been missed by the BLASTp search results using the HMMer3 software package 3.1b2 (http://hmmer.org) [168]. Similar E-value and reverse-blast criteria were applied, but sequences were retained if they retrieved any human RhoGDIs in a reverse BLASTp search. Where orthologues could not be identified in specific organisms, or amino acid sequences are not available (*L. erinacea*), nhmmer was used to search scaffold sequences with an HMM constructed from RhoGDI coding sequences and any identified sequences were used to search the NCBI nr nucleotide database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the most similar homologues.

Amino acid sequences identified using HMMer were aligned using MUSCLE v3.8.31 [168] with default parameters, adjusted manually when necessary, and trimmed to retain only positions of conserved sequence similarity. To identify an appropriate model of sequence evolution, ProtTest v3.4.2 was used [169], and for every analysis the model LG4X with the gamma rates

distribution was used. Maximum likelihood phylogenetic analysis using RAxMLv8.2.8 [170] was performed with 100 non-parametric bootstraps using the default faster hill climbing method (-f b, –b, –N 100). Bayesian phylogenetic analysis was performed using MrBayes version v3.2.6 [171]. For MrBayes, over 10 million Markov chain Monte Carlo generations were run, to convergence indicated by average standard deviations of splits frequencies of 0.01 or lower (with a burn-in of 25%). All phylogenetic analyses were performed using the CIPRES webservice [172].

2.17 qPCR

To determine the expression of RhoGDI1, RhoGDI2 and GAPDH in RBL-2H3 cells, we performed qPCR. Freshly extracted RNA was transcribed into complementary DNA (cDNA) sequences by reverse transcriptase, followed by the amplification of the newly synthesized cDNA by standard PCR procedure. Briefly, RNA was extracted from RBL-2H3 by TRIzol®-chloroform extraction and first strand cDNA was synthesized by superscript II using gene specific primers. Following this, specific gene of interest was quantified using appropriate primers, first strand cDNA (0.1, 1, 10 ng) and Bio-Rad iQ™ SYBR® Supermix.

Chapter 3 Effect of Rho Drugs on Mast Cell Activation and Degranulation

Chapter 3: The effect of Rho drugs on mast cell activation and degranulation

3.1 Abstract

MCs are tissue-resident immune cells that possess potent proinflammatory mediators contained in cytoplasmic granules. Stimulation triggers degranulation, a process that mobilizes granules to dock and fuse to the plasma membrane, releasing mediators. MC degranulation has an important role in immunity but can also intensify inflammation and contribute to allergic disorders. Hence, it is important to understand signaling pathways that regulate MC degranulation. Here, we examined the role of Rho proteins in regulating MC activation leading to degranulation. RBL-2H3 cells and bone marrow-derived MCs (BMMCs) were stimulated through aggregation of FcεRI receptors. Stimulated cells showed a large increase in the levels of activated Rac and, to a lesser extent, RhoA. Drugs were used to acutely inhibit the function of specific Rho proteins. The Rac inhibitor EHT-1864 and the RhoA inhibitor Rhosin inhibited degranulation. Microscopic characterization showed that, upon stimulation, RBL-2H3 cells formed surface ridges that grew into large protrusions reminiscent of circular dorsal ruffles, which flattened into large lamellipodia. LysoTracker-labeled cells showed granules stream into peripheral protrusions. EHT-1864 reduced granule motility, whereas Rhosin increased motility; both drugs affected the formation of peripheral protrusions. These results showed that, in response to stimuli, Rho proteins control discrete cytoskeletal remodeling processes that are needed for granule exocytosis. Rac is required to stimulate the remodeling of MCs, triggering actin-mediated flattening of the cell periphery to create an active degranulation zone, whereas RhoA controls the streaming of highly motile granules into the active zone

3.2 Background

Aberrant MC degranulation contributes to many inflammatory diseases, most notably allergy and hypersensitivity disorders. High-affinity IgE receptor FcεRI-signaling has been implicated in allergies like asthma [11, 52]. MC granules, which contain potent pro-inflammatory immune mediators are rapidly released when IgE primed MCs are stimulated by a bi/multivalent antigen (Ag). Unregulated MC degranulation in asthmatic lungs, over a sustained period, leads to chronic inflammatory symptoms like fibrosis, reduced lung capacity and ultimately organ dysfunction [10, 173, 174]. Therefore, there is a need to elucidate the complex network of protein signals, downstream of FcεRI, that may lead to the identification of novel therapeutic targets to prevent MC degranulation.

Binding of multivalent antigens to FcεRI-bound IgE induces receptor aggregation, triggering MC activation, which leads to granule translocation, calcium mobilization and finally release of granule content. FcεRI regulates degranulation by transmitting signals via intracellular ITAMs, kinases, lipid mediators and GTPases. Vav1, a RhoGEF, is activated downstream of Src signaling, which in turn activates Rho GTPases. Indeed, a role for multiple classes of Rho GTPases in degranulation has been established [56, 60, 62, 155, 156, 175]. As central regulators of actin-related morphologies, Rho, Rac and Cdc42 have unique roles in mediating actin remodeling. RhoA stimulates the formation of stress fibers, whereas Cdc42 and Rac stimulate the formation of branched F-actin structures present in filopodia and lamellipodia [85]. MCs undergo rapid morphologic changes upon stimulation, which is an integral part of transitioning to an activated

state and may facilitate the degranulation mechanism. Rac is involved in the formation of actin-rich dorsal membrane ruffles, which we have shown to form in MCs [156]

Here, we examined the role of Rho proteins in MC activation and responses. Previous research indicates that Rho proteins may be generally involved in MC degranulation [58-60, 62, 155, 156, 160]. We examined whether one or more Rho GTPase family members are critical for degranulation using the availability of new drugs that acutely inhibit the function of specific Rho proteins. The Rac inhibitor EHT-1864 [176], the Cdc42 inhibitor ML-141 [177], the Rho inhibitor Rhosin [178], the Rac1-Vav2 inhibitor EHop-016 [179], and the TrioN/Tiam1- Rac1 inhibitor NSC-23766 [180] were all examined for their effect on MC activation and degranulation. Our studies show that Rac is the primary Rho GTPase involved in the regulation of MC morphologic transitions during activation, leading to degranulation. Drugs that target RhoA signaling also showed slight negative effects on MC morphology and responses. Based on our results, we propose that Rho signaling would be an effective target for anti-inflammatory therapy in disorders that involve MCs.

3.5 Results

3.5.1 Monitoring *in vitro* MC activation and responses

For this study, we used the rat basophilic leukemia cell line, RBL-2H3, which has been a widely used model of mucosal MCs [66] and mouse BMMCs to study FcεRI-stimulated degranulation. MC activation was monitored by several methods. Syk, a hematopoietic kinase that transmits membrane-proximal activation signals from Fc receptors is a good indicator of MC activation. Phosphorylation of Syk is essential for the transmission of signal [51, 181, 182]. Thus, phospho-Syk and total-Syk levels were measured in Ag stimulated RBL-2H3. Phospho-Syk (Y525/526) showed rapid increase in levels within 2.5 mins, sustained it for 20 mins and showed reduced levels by 40 min, post stimulation (**Figure 3.1 A**).

MC response (degranulation) was assayed by measuring the relative amount of β-hexosaminidase released (**Figure 3.1 B**) and FITC-annexin V staining of Ag-stimulated cells (**Figure 3.2**). Optimum concentration of Ag (DNP-BSA) was determined by performing degranulation assay with varying concentrations of Ag. Maximum β-hexosaminidase activity was observed when sensitized RBL-2H3 and BMMCs were stimulated with 27 ng/ml DNP-BSA. This informed our future stimulation experiments. BMMCs and RBL-2H3 cells showed a maximum of 53 and 37% degranulation (β-hexosaminidase activity), respectively, when stimulated with 27 ng/ml of the Ag (DNP-BSA).

To measure degranulation by flow cytometry, annexin V staining of stimulated RBL-2H3 and BMMCs was quantified (**Figure 3.2**). When MCs are stimulated, the release of granule content results in the exposure of phosphatidylserine on the external surface of the cell, which can be

quantified by FITC–annexin V staining [165]. Stimulated RBL-2H3 cells showed a 12.1-fold increase in annexin V staining compared with resting cells when not pretreated with a drug.

3.5.2 Effect of Rho drugs on MC activation and degranulation

To establish the effects of targeting Rho GTPases in MC activation and degranulation, we used specific small molecule drugs. The effect of acute inhibition of Rho GTPases was examined by preincubating RBL-2H3 cells and BMMCs with Rho drugs. Rhosin, ML-141, and EHT-1864 are direct inhibitors of RhoA, Cdc42, and Rac, respectively [176-178]; in addition, the Rac-GEF–specific inhibitors NSC-23766 (TrioN and Tiam1) [180] and EHop-016 (Vav2) [179] and the Rho kinase inhibitor HA- 1100 [183] were tested. EHT-1864 and Rhosin were the only drugs that reproducibly showed substantial inhibition of β-hexosaminidase release in both RBL-2H3 cells (**Figure 3.3 A)** and BMMCs (**Figure 3.3 B)**.

The effects of Rho drugs on MCs were also monitored by flow cytometry (**Figure 3.3 C and D**). The percentage of cells that appeared in a P5-stimulated cell gate in each of the drug-treated samples was compared to the untreated, stimulated samples. EHT-1864 was the only drug that caused a significant (74%) reduction in annexin V staining compared with untreated cells, which confirms that inhibition of Rac affects degranulation. EHop-016-treated RBL-2H3 cells also showed a slight, but statistically significant (29%), decrease in annexin V staining. The drugs ML-141, Rhosin, NSC-23766, and HA-1100 did not show a statistically significant effect. For BMMCs, cells outside a P4 resting cell gate were also quantified. BMMCs treated with EHT-1864, Rhosin, and EHop-016 all showed a slight decrease in annexin V staining, which confirms that inhibition

of Rac, Rho, and possibly Vav2 affect degranulation in BMMCs. The drugs ML-141, Rhosin, NSC-23766, and HA-1100 did not show a statistically significant effect.

3.5.3 Analysis of Rho activation in MC

Our goal here was to elucidate the role of Rho GTPases during regulated exocytosis in MCs. To identify which Rho proteins are expressed by MCs, RhoA, Rac1, Rac2 and Cdc42 were probed for in RBL-2H3 and BMMCs lysates (**Figure 3.4 A**); All the tested Rho proteins were detected in the lysates. Additionally, FcεRI- proximal signaling was also investigated by probing for p-Syk (Y525/526) in the presence of Rho drugs (**Figure 3.5 E**). None of the Rho drugs tested affected the levels of Syk phosphorylation after 5 min and they were comparable to DMSO treated control. This indicates that Rho drugs do not interfere with early FcεRI-signaling.

To examine the role of Rho proteins in MC stimulation and degranulation, we performed Rho protein activation assays by GLISA (G-protein ELISA). To determine Rho proteins activated by Ag stimulation, RBL-2H3 cells and BMMCs were lysed after 5 min of stimulation and the GTP-bound “on” state was affinity isolated with probes made from Rho-binding domains of downstream effectors. Rac1-GTP levels increased 2.6-and 1.9-fold in stimulated RBL-2H3 cells and BMMCs, respectively, compared with resting cells (**Figure 3.4 B**). There was a slight increase in Cdc42-GTP levels in stimulated BMMCs cells but no change in RBL-2H3 cells, with high levels detected in both resting and stimulated cells (**Figure 3.4 C**). The high basal Cdc42 activity in RBL-2H3 cells was likely due to the fact that these cells rapidly divide, which requires active production of Cdc42-GTP. RhoA-GTP levels increased 2.5- and 6.1-fold in stimulated RBL-2H3 and BMMCs, respectively, compared with resting cells; however, the overall levels were

significantly lower compared with the standardized control (**Figure 3.4 C**). However, in all cases, pre-treatment of MCs with Rho inhibitors blocked the activation of cognate Rho protein when cells were stimulated. Our results demonstrate that Rac and RhoA are activated for degranulation in Ag-stimulated MCs when combined with the observation that degranulation is reduced in EHT-1864- and Rhosin-treated cells.

Rho protein activation can be similarly assayed by affinity pulldown with Rac and Rho activation probes, which we used to show their differential timing of activation (**Figure 3.5**). To identify whether Rac activation is indicative of the activation of Rac1, Rac2, or both, lysates from stimulated RBL-2H3 cells were incubated with an immobilized Rac-activation probe, and the bound fraction was immunoblotted for Rac1 and Rac2 with specific Abs. Both Rac1-GTP and Rac2-GTP were detected 5 min after stimulation; whereas, RhoA-GTP levels were detected after 20 min of stimulation. EHT-1864 inhibited the generation of both Rac1-GTP and Rac2-GTP, 5 min post-stimulation (**Figure 3.5 B and C**). Rhosin inhibited the generation of RhoA-GTP, 20 min post stimulation (**Figure 3.5 D**). This also shows that Rho drugs specifically inhibited only their target and no other closely-related members.

3.5.4 Effect of Rho drugs on MC morphology, cytoskeleton

Rho proteins control cell shape changes through regulation of cytoskeletal remodeling [56, 78, 90, 91]. We imaged living cells by brightfield DIC microscopy and documented the morphologic transitions that occurred in RBL-2H3 cells during the FcεRI stimulation leading to degranulation. Still images from videos show the flattening of rounded cells within 10 min of stimulation (Fig. 4A, upper panels). Cells first formed small surface ruffles, then larger surface ridges (**Figure 3.6**

A, asterisk), which culminated in large, peripheral protrusions and flattened cells (**Figure 3. 6 A**, arrow). These large protrusions were morphologically similar to CDRs observed in growth factor stimulated epithelial cells [184, 185]. The formation of CDRs was dependent on Rac activation. EHT-1864, which effectively blocked degranulation, also inhibited peripheral protrusion, cell flattening, and the formation of CDRs but did not inhibit minor surface ruffling or the formation of ridges. The development of surface ridges was dependent on Rho activation. Rhosin-treated cells formed surface ruffles but rarely formed ridges or CDRs (**Figure 3.6 B**).

We further explored cytoskeletal changes by immunofluorescence microscopy. RBL-2H3 cells were fixed after 10 min of stimulation, and microtubules, F-actin, and granules were labeled with anti-β-tubulin Abs, Oregon green 488-phalloidin, and anti-CD63 antibodies, respectively (**Figure 3.7 A**). F-actin was enriched in surface ruffles and intensely stained peripheral projections of flattened cells (**Figure 3.7 A**, upper panels). We also observed changes in the pattern of microtubule staining when cells were activated, which showed quite prominent track formation in peripheral projections. Whereas granules were randomly distributed in resting cells, they accumulated near the plasma membrane of peripheral protrusions.

Stimulated RBL-2H3 cells pretreated with either EHT-1864 or Rhosin were similarly processed for immunofluorescence. In both cases, cell spreading was significantly reduced compared with untreated cells, peripheral projections did not form, and F-actin showed punctate cytoplasmic staining (**Figure 3.7 A**, middle and lower panels). Granules remained dispersed throughout the cytoplasm. Microtubule tracks in the cell body remained in EHT-1864-treated cells (**Figure 3.7 A**, middle panels) but were noticeably absent in Rhosin-treated cells (**Figure 3.7 A**, lower

panels). Pretreatment with the ROCK inhibitor HA-1100 did not block peripheral projections or microtubule organization (data not shown). These results suggest that cytoskeletal elements are uniquely controlled by Rho proteins during activation to drive the exocytosis process. Rac inhibition affected actin distribution in stimulated cells but did not affect microtubule patterns, whereas RhoA inhibition affected microtubule organization, which is mediated by a downstream effector that is unlikely to be ROCK. Both drugs reduced peripheral membrane spreading and the coincident distribution of granules to the cell periphery. This further defines the role of Rac and Rho in MC degranulation.

We also examined the effect of Rho drugs on primary MC F-actin and granule distribution by immunofluorescence microcopy (**Figure 3.7 B**). BMMCs were stimulated for 10 min, fixed then F-actin, and granules were labeled with Oregon green 488-phalloidin and anti-CD63 antibodies, respectively. F-actin was remodeled from a ring in resting cells to peripheral ruffling in stimulated cells, with a coincident increase in granule staining at the cell periphery (**Figure 3.7**, no drug). The Rac and RhoA drugs, EHT-1864 and Rhosin, blocked the formation of this stimulated morphology, whereas the Cdc42 and ROCK drugs, ML-141 and HA-1100, had little to no effect. These results confirm roles for Rac and RhoA in MC degranulation, and that, RhoA works through a downstream effector complex that does not involve ROCK.

3.3.5 Effect of Rho drugs on granule motility in MCs

To examine the role of Rac and RhoA in the dynamics of granule movements, we looked at the effects of EHT-1864 and Rhosin treatment on RBL-2H3 cells stained with LysoTracker. Lyso-Tracker has been used to stain both lysosomes and secretory granules in MCs [186]. Two

regions of interest were defined: the cell body, which contains larger, static lysosomes and smaller, motile granules; and the peripheral extensions, which contain mostly small, motile granules (**Figure 3.8 A and B**). LysoTracker-stained granules showed random movement in the cell body and nondirectional movement in peripheral cell extensions in resting cells (**Figure 3.8**, resting). Upon stimulation, cell ruffles and peripheral extensions began to form (**Figure 3.8**, stimulated), and granule motility slightly decreased in those regions (17% reduction); 10 - 15 min after stimulation, cells flattened, CDRs formed, and granules appeared to stream into flattened regions. Rac-inhibited cells did not undergo flattening and did not form dorsal ruffles. After 30 min poststimulation, some cell flattening was observed, but granules were static and accumulated at the cell body-peripheral extension boundaries. The Rac inhibitor EHT-1864 reduced granule motility in the cell periphery by 64%, both before and during stimulation, compared to untreated cells (**Figure 3.8 B**). RhoA-inhibited cells showed an increase in granule motility by 74%, both before and during stimulation, compared with untreated cells. RBL-2H3 cells treated with Rhosin did not have a robust response upon stimulation (**Figure 3.8 B,** bottom panel), and after long periods of stimulation, cells spread slightly but did not flatten and polarize as observed in untreated cells. These results suggest that Rac may instigate cytoskeletal remodeling and flattening in the cell peripheral regions, whereas RhoA may facilitate granule capture and directional motility for exocytosis. This may facilitate granule exocytosis by controlling motility in peripheral ruffles to increase their fusion stochastically in these active zones.

3.3.6 Effect of cytoskeletal drugs on MC morphology, granule movement and degranulation

MC stimulation results in activation of Rho GTPases, which recruit a variety of effector complexes that include many cytoskeletal remodeling proteins [56]. Rac1 effectors regulate the local assembly/disassembly of F-actin, which promote lamellipodia formation, as observed during RBL-2H3 cell spreading (reviewed in Sit et al. [187]). Studies have implicated Rho signaling in the regulation of microtubule dynamics [98, 99, 157, 188-190]. Recent research demonstrated that microtubule remodeling stimulated by FcεRI signaling is required for MC degranulation [67]. Hence, we decided to examine the effect of targeting the cytoskeleton of RBL-2H3 cells with drugs that destabilize actin (cytochalasin B) and microtubules (nocodazole). Consistent with previous research in MCs, we observed cytochalasin B, which disrupts F-actin assembly, led to a 33.4% increase in degranulation (**Figure 3.9 A**). These results are consistent with the idea that disassembly of cortical F-actin is required for granule fusion to the plasma membrane. When we disrupted microtubule formation and stability using nocodazole, we observed a 54% decrease in response to Ag stimulation via the degranulation assay.

To further define the role of the cytoskeleton in the dynamics of granule movements, we examined LysoTracker-stained RBL- 2H3 cells treated with cytochalasin B and nocodazole to depolymerize F-actin and microtubules. LysoTracker-stained granules showed increased motility when actin was depolymerized and arrested motility when microtubules were depolymerized (**Figure 3.9 B and C**). In addition, nocodazole-treated cells caused a slow retraction of peripheral cell protrusions and accumulated granules in the cell body. These data support the idea that actin remodeling drives cell shape changes (e.g., flattening and ruffling)

that facilitate granule exocytosis, whereas microtubules facilitate granule movement into the cell periphery, which are the active zones for degranulation (see model in **Figure 3.10**).

3.3 Discussion

Aberrant MC degranulation has been implicated in several inflammatory disorders [6, 8, 10, 11]. Our goal here was to further understand the cellular regulation of FcεRI-mediated degranulation in MCs, with a focus on pharmacologic intervention. Previous research implicated RhoA [175], Cdc42, and/or Rac [58, 62, 160] in degranulation of secretory vesicles in RBL-2H3 cells, largely through genetic manipulation of their expression using overexpression of wild type or mutant Rho proteins. However, genetic manipulation can confound results due to cell adaptations. Therefore, our approach to acutely inhibit Rho signaling with specific inhibitors provides a more accurate picture of Rho signaling during MC responses. Additionally, some of the Rho proteins are essential genes and hence, a pharmacological approach of acute inhibition is advantageous. However, this approach is restricted to proteins that have small molecule, membrane-permeable inhibitors. In addition, the potential off-target effects must also be considered.

To monitor FcεRI stimulated MC activation and responses, we performed time point stimulation of RBL-2H3 and measured phospho-Syk levels, β-hexosaminidase release (**Figure 3.1)** and annexin V staining (**Figure 3.2**).

Our goal was to understand Rho signaling in activated MCs, so, we quantified the fold increase in GTP-Rho levels in stimulated RBL-2H3 and BMMCs (**Figures 3.4 and 3.5**). All Rho GTPases examined showed increased levels of activation in extracts of stimulated MCs. However, Rac-

GTP levels showed the most significant increase, with rapid activation kinetics. RhoA-GTP levels also increased but with a much smaller yield and slower activation kinetics compared with Rac. We showed selectivity of those compounds with Rho-activation assays (**Figure 3.5**). Several small molecule compounds that inhibit Rho GTPase have been identified recently, largely through function directed screens of compound libraries or in silico compound docking on protein structures. We used those compounds as "Rho drugs" to acutely inhibit Rho protein function and to characterize events leading to MC degranulation (**Figure 3.3**). The Rac inhibitor, EHT-1864, showed the greatest inhibition of MC degranulation, and blocked morphologic transitions in stimulated cells (**Figures 3.6 and 3.7**), resulting in granule accumulation in the cell body (**Figure 3.8**).

The RhoA inhibitor, Rhosin, also inhibited degranulation, although to a lesser degree than EHT-1864 (**Figures 3.3**). Rhosin inhibited morphologic transitions differently than EHT-1864; cells still flattened, and granules were mobilized into the flattened cell periphery (**Figures 3.6 and 3.7**). These results suggest that Rac and RhoA have distinct roles in regulating MC degranulation (**Figures 3.10**). Two additional Rac drugs were tested that affect specific Rac-GEF interactions. NSC-23766, which blocks the interaction with TrioN and Tiam1, had no significant effect on degranulation; however, EHop-016, which blocks the Vav2-Rac1 interaction, inhibited at high micromolar concentrations. This may be an off-target effect since EHop-016 reportedly inhibits Vav2 binding at low micromolar concentration (IC_{50} = 1.1 μM) [179]. There are three structurally related Vav GEFs, and it may be that another member is required. Vav1 is recruited to LAT, a nucleating site for multiprotein signaling complexes in leukocytes [191]. Vav1-deficient bone marrow MCs also exhibited reduced degranulation [192]. Vav1 activates RhoA, Rac, and

Cdc42, which makes it an intriguing candidate as a central upstream factor that may control the downstream activation of several Rho proteins.

The ROCK inhibitor HA-1100 was also examined but did not show any major effects. Therefore, RhoA must use a different effector to activate MC degranulation, and previously, mDia has also been shown to have a role in cytoskeletal reorganization during stimulation [56, 98, 157, 189, 190]. We interpret the role of Rho proteins as the rapid activation of Rac1 to trigger cell flattening, projecting granules into lamellipodia for degranulation, and subsequent activation of RhoA to halt granules in active exocytosis zones. Studies into temporal control of Rho regulators' signaling are needed to elucidate the coordination of this mechanism.

Rho proteins have a role in cytoskeletal dynamics that invoke cell movement and shape changes. Rac activation regulates actin dynamics that give rise to the formation of lamellae and cell spreading on the substratum. Research in neutrophils has demonstrated a role for Rac GTPases in the regulation of exocytosis in conjunction with actin remodeling [68, 69, 193-196]. The question is whether those events are linked, so that morphologic transitions drive degranulation, or whether Rac and Rho drive two independent events. When stimulated, RBL-2H3 MCs underwent a series of ordered morphologic transitions that occurred in conjunction with degranulation. Cells rapidly flattened and displayed small, surface ruffles and, after longer periods of stimulation, formed major, surface ruffles (**Figures 3.6**). These large membrane surface protrusions were morphologically similar to CDRs observed in growth factor-stimulated epithelial cells [184, 185, 197, 198]. In epithelial cells, the function of CDRs is proposed to be rapid internalization of integrins, which redistribute to newly forming focal adhesions at the

leading edge to facilitate cell migration. In MCs, CDRs may be similar to structures that take part in pinocytosis, which is deemed important for the removal of cortical actin and the reduction of an actin barrier to degranulation [67]. The formation of CDRs was dependent on Rac activation and coincided with the timing of peak degranulation. We did not observe the trafficking of granules into CDRs; however, because they formed above the focal plane, they may have eluded detection via 2-D confocal microscopy. Thus, it remains possible that CDRs generate sites of exocytosis.

Peripheral extensions seem to be where degranulation takes place in RBL-2H3 cells. Granule motility in peripheral extensions was significantly greater and, in conjunction with cell flattening, could facilitate granule docking and exocytosis at the plasma membrane. We used drugs that destabilize actin and microtubule polymers to interfere with cytoskeletal dynamics and examined the ability of MCs to respond to stimuli. We observed that targeting F-actin with cytochalasin resulted in increased degranulation, even though actin-driven morphologic changes were absent (**Figure 3.9**). The increase in degranulation may be the result of the depolymerization of cortical actin, which has been deemed a barrier to exocytosis, and the increase in granule motility we observed, which could increase docking events stochastically at the plasma membrane [199]. Granule motility in peripheral regions was significantly slowed by the Rac inhibitor EHT-1864 and the microtubule destabilizing drug nocodazole, both of which also inhibited degranulation (**Figures 3.3 and 3.9**). Nocodazole caused retraction of peripheral extension, which likely resulted in the inability to generate competent degranulation sites. Examination of cytoskeletal drugs clearly demonstrated the need for actin-driven morphologic changes (e.g. cell flattening), which may facilitate the fusion of granules to the plasma

membrane. Simultaneously, microtubule extensions may facilitate granule movement into the cell periphery, which are active zones for degranulation (**Figures 3.10)**. In conclusion, using Rho inhibitors, we found that Rho signaling through Rac controls MC morphology, which transitions to an activated state to facilitate degranulation. RhoA may also participate in the morphology and granule movement during degranulation.

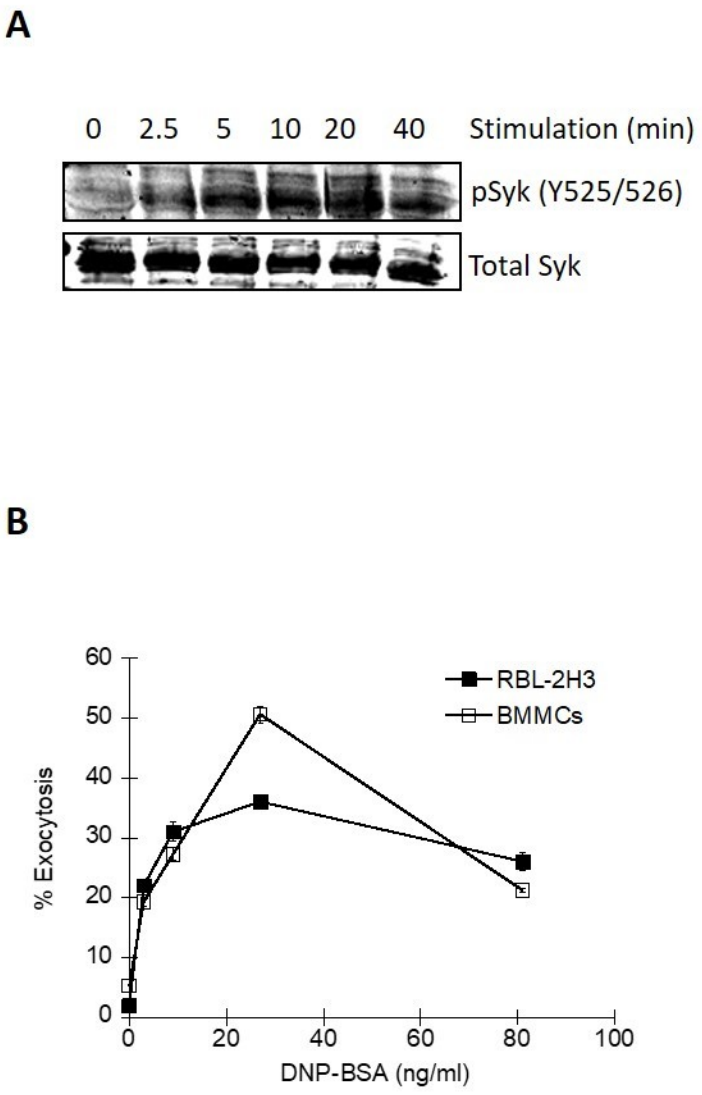


Figure 3.1 Monitoring MC activation and degranulation (A) RBL-2H3 cells were antigen-stimulated as in panel B. At the indicated time points, cells were lysed and analyzed by immunoblot for levels of phospho-Syk (Y525/526) and total Syk. (B) RBL-2H3 cells and BMMCs were sensitized by incubation with 0.5 mg/ml anti-DNP-IgE, then stimulated for 30 min with increasing concentration of $DNP_{\sim 30}$-BSA (Ag). MC degranulation was measured by levels of β-hexosaminidase activity in cell-free supernatants of stimulated cells compared to total released by detergent cell lysis. Peak exocytosis was stimulated with 27 ng/ml DNP-BSA (n=3).

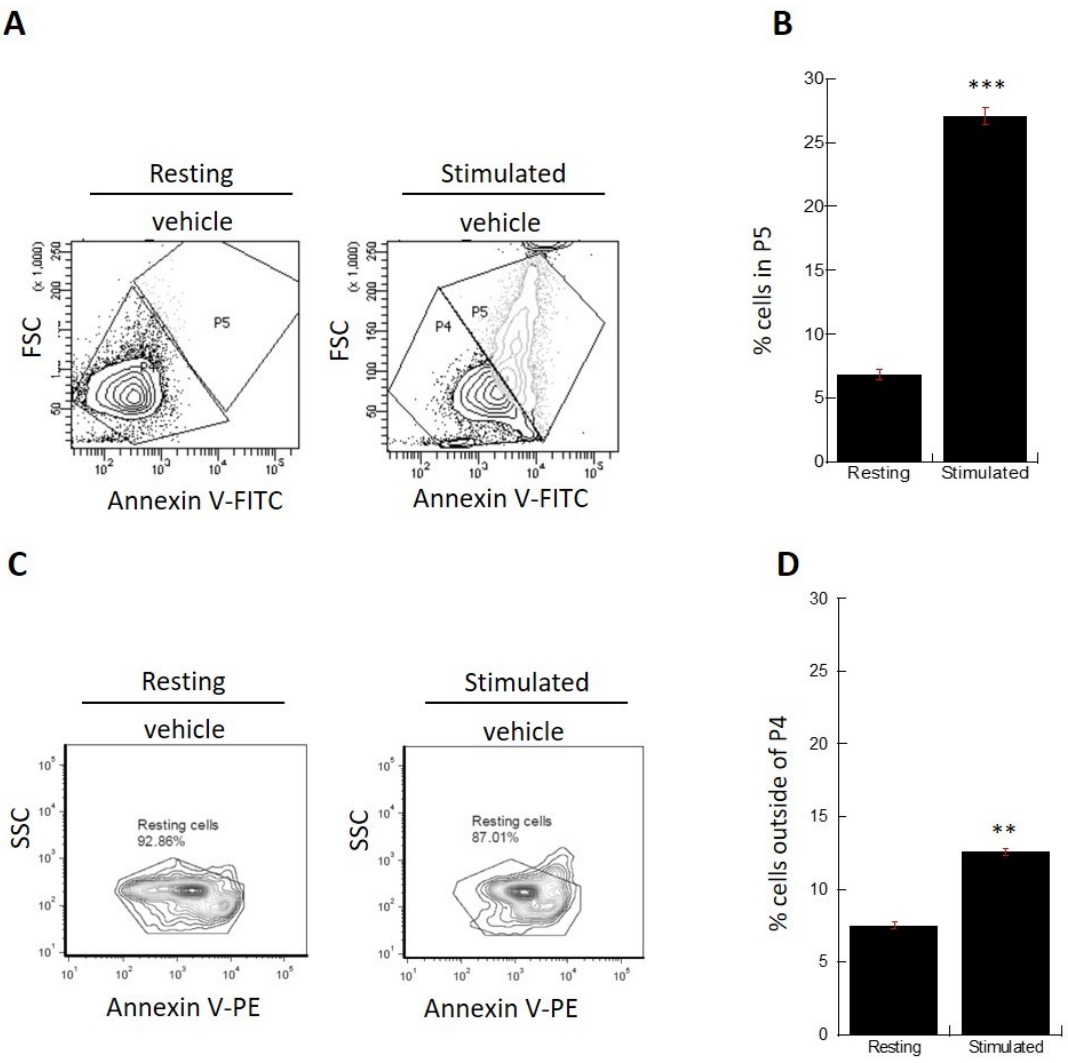


Figure 3.2 Measurement of degranulation by flow cytometry IgE-sensitized RBL-2H3 cells (A and B) or BMMCs (C and D) were stimulated with 27 ng/ml DNP-BSA. After 15 min, cells were fixed and stained with FITC–annexin V. A P4 gate was set that included 95% of the resting cells, and the cells outside the P4 gate after stimulation were quantified for exocytosis. (B) Percentage of FITC-annexin V–positive RBL-2H3 cells in a P5 gate for stimulated cells. (D) Percentage of BMMCs outside the P4 resting population of cells. A paired student t-test analysis was performed to determine statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001; n = 3).

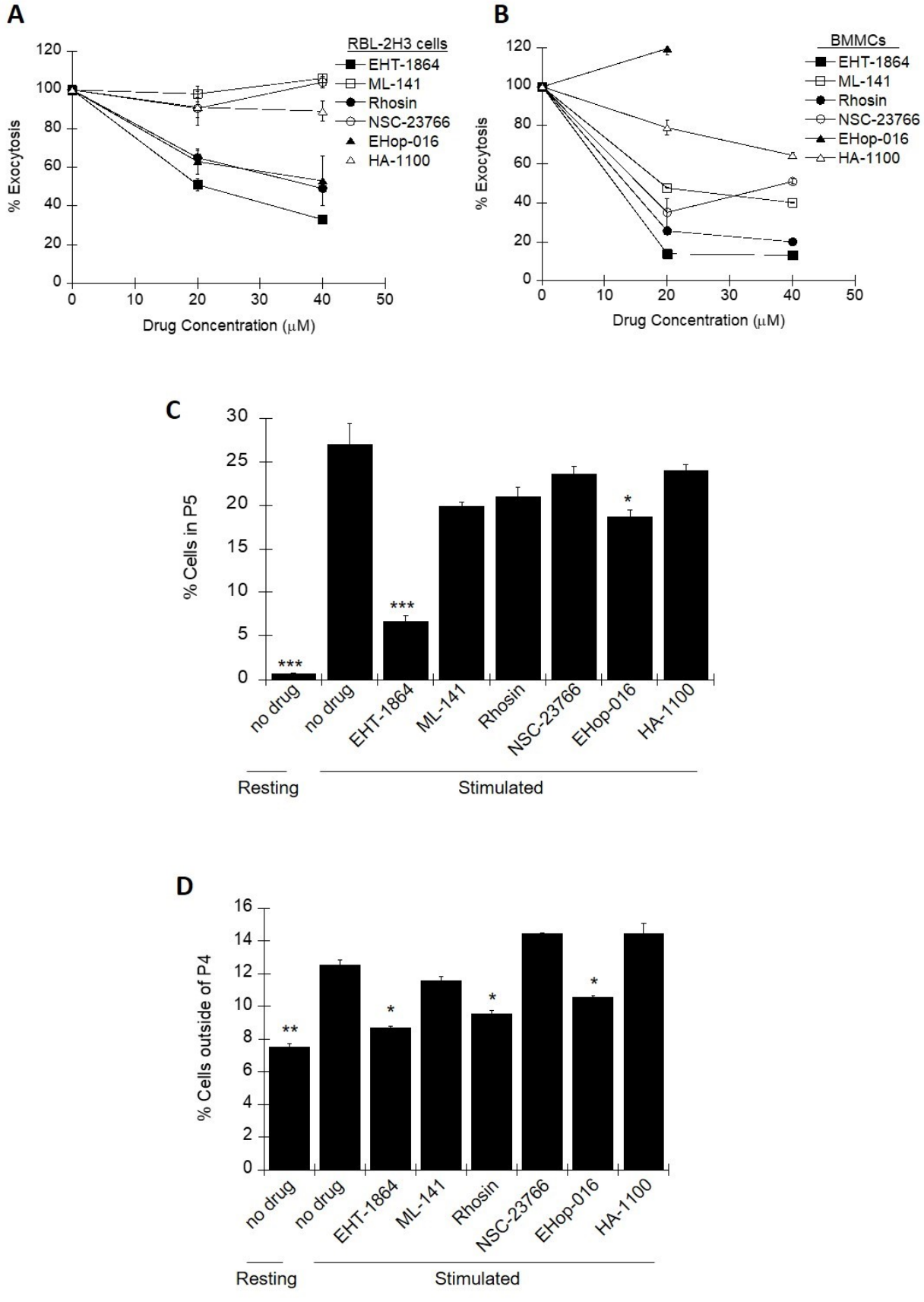

A
RBL-2H3 cells
EHT-1864
ML-141
Rhosin
NSC-23766
EHop-016
HA-1100
% Exocytosis
Drug Concentration (μM)
B
BMMCs
EHT-1864
ML-141
Rhosin
NSC-23766
EHop-016
HA-1100
% Exocytosis
Drug Concentration (μM)
C
% Cells in P5
***
***
*
no drug
no drug
EHT-1864
ML-141
Rhosin
NSC-23766
EHop-016
HA-1100
Resting
Stimulated
D
% Cells outside of P4
**
*
*
*
no drug
no drug
EHT-1864
ML-141
Rhosin
NSC-23766
EHop-016
HA-1100
Resting
Stimulated

Figure 3.3 Effect of Rho drugs on RBL-2H3 and BMMC degranulation (A) IgE-sensitized cells were pre-treated with 0, 20, and 40 μM of the indicated Rho drugs or DMSO for 30 min and then stimulated with 27 ng/ml DNP-BSA for 15 min. Values were normalized to DMSO treatment. (C and D) Percentage of FITC-annexin V– positive RBL-2H3 cells in a P5 gate for stimulated cells. (D) Percentage of BMMCs outside the P4 resting population of cells. A student t-test analysis was performed where sample groups were compared to DMSO treated stimulated group. *P < 0.05; **P < 0.01; ***P < 0.001; n = 3.

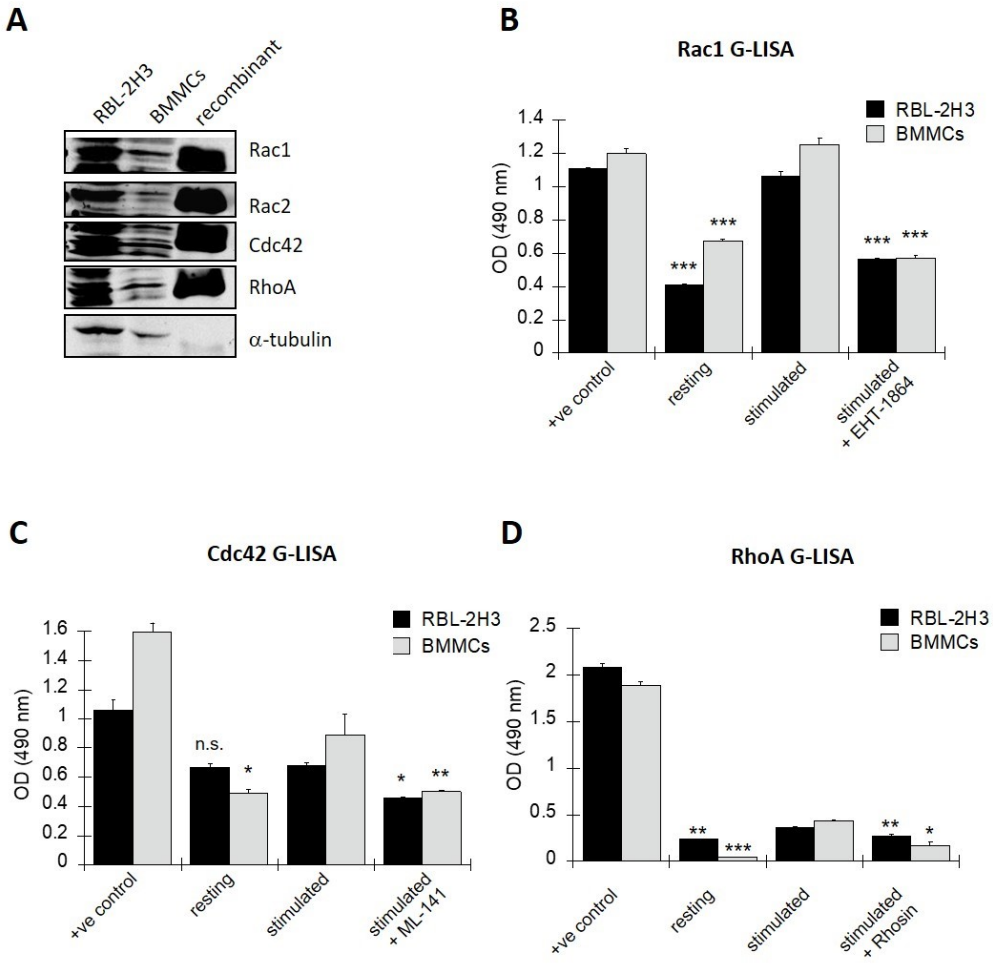


Figure 3.4 Analysis of Rho activation in stimulated mast cells. (A) Both RBL-2H3 and BMMCs were immunoblotted for Rho proteins and α-tubulin. Rac1, Rac2, RhoA, Cdc42 were detectable in lysates. (B-D) IgE-sensitized RBL-2H3 cells and BMMCs were stimulated for 10 min with 27 ng/ml DNP-BSA. Cells were lysed and immediately probed for levels of activated Rac1, Cdc42, and RhoA with a G-LISA kit (Cytoskeleton). Positive controls containing 2 ng of constitutively active Rho protein were included with each assay. (B) Quantified Rac1-GTP levels in RBL-2H3 cells and BMMCs that were pre-treated with either DMSO or EHT-1864 (40 μM) for 30 min. (C) Quantified Cdc42-GTP levels in RBL-2H3 cells and BMMCs that were pre-treated with either DMSO or ML-141 (20 μM) for 30 min. (D) Quantified RhoA-GTP levels in RBL-2H3 cells and BMMCs that were pre-treated with either DMSO or Rhosin (40 μM) for 30 min. Unpaired student t-test analyses was done comparing no drug treated stimulated group with sample groups *P < 0.05; **P < 0.01; ***P < 0.001; n = 3.

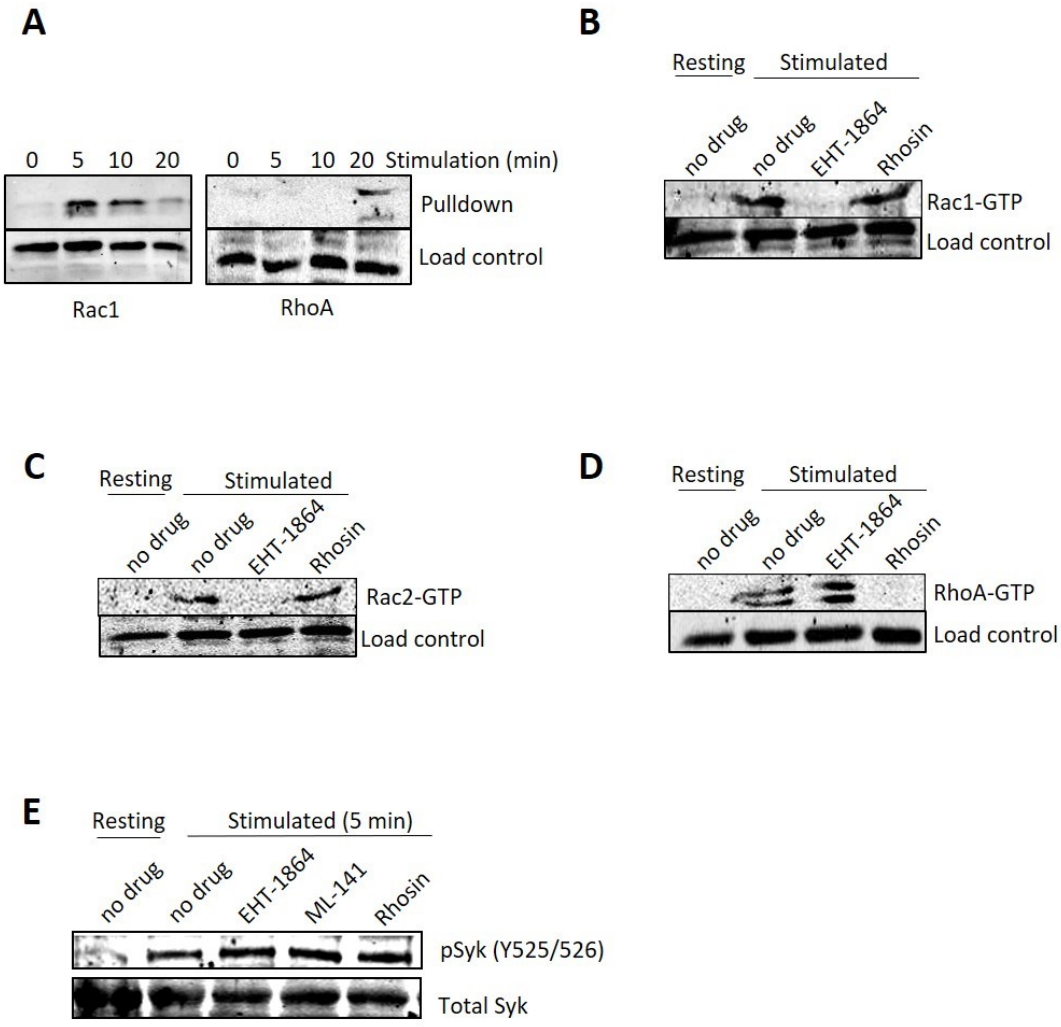


Figure 3.5 Analysis of Rho activation in stimulated MCs by affinity pull-down (A) Time course of Rac-GTP and RhoA-GTP generation, as determined by affinity pull-down assay using beads containing immobilized Rac and RhoA binding domains. (B-E) Cells were pre-treated with vehicle (no drug) or 40 μM of the indicated drug for 30 min and then stimulated with 27 ng/ml DNP-BSA for 5 min. Rac activation assays performed on RBL-2H3 cells pre-treated with DMSO (no drug) or EHT-1864 (40 mM). Immunoblot analysis revealed elevated levels of (B) Rac1-GTP and (C) Rac2-GTP 5 min after stimulation. EHT-1864 treatment inhibited Rac1 and Rac2 activation. (D) Elevated levels of RhoA-GTP detected after 20 min stimulation were inhibited by Rhosin. (E) Syk phosphorylation in RBL-2H3 cells was unaffected by pre-incubation with Rho drugs.

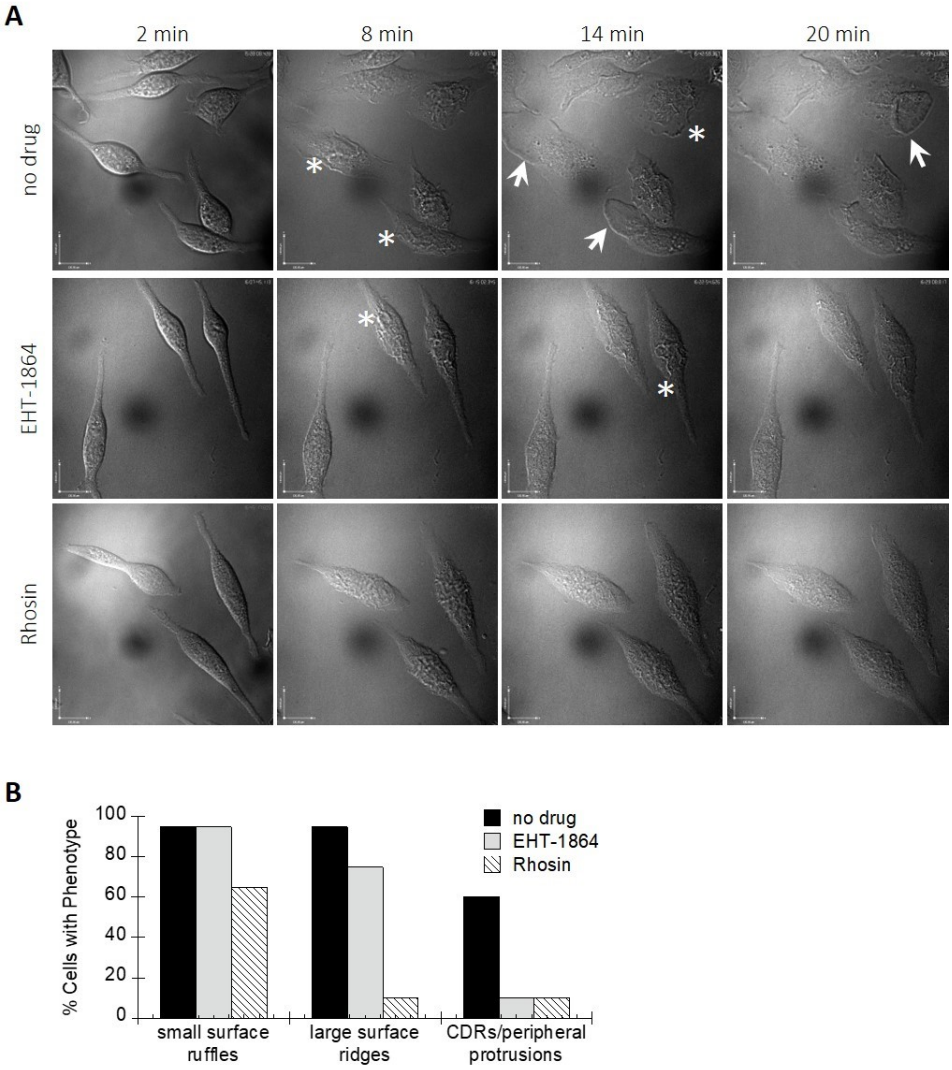
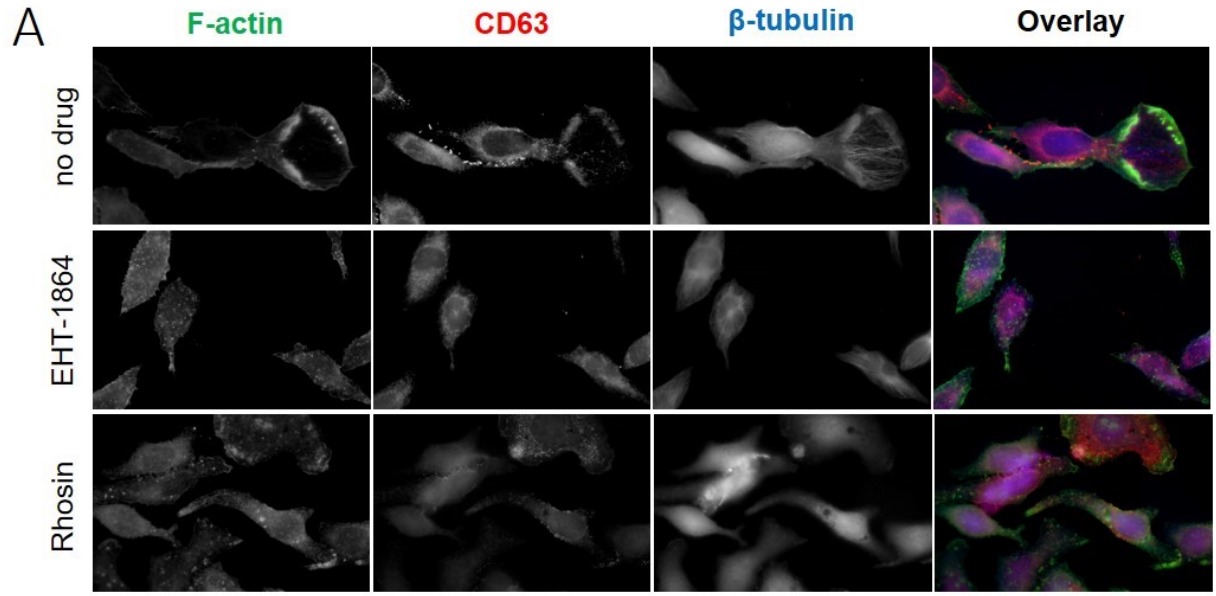


Figure 3.6 Live-cell imaging of stimulated RBL-2H3 cells reveals dynamic membrane ruffling and CDR formation. (A) Representative images from live-cell videos at different timepoints as indicated, post stimulation. RBL-2H3 cells were grown on poly-D-lysine–coated cover slips, IgE-sensitized, then, imaged in brightfield DIC during stimulation with 27 ng/ml DNPBSA. Cells formed small surface ruffles soon after stimulation; then, large surface ridges formed (asterisk), and then, the CDRs resulted in significant peripheral membrane protrusion (arrow). EHT-1864 treatment (middle panels) abrogated the formation of CDRs and peripheral protrusions. Rhosin treatment (bottom panels) reduced both surface ridges and peripheral protrusions. (B) Quantification of cells showing surface ruffles, large membrane ridges, and CDRs/peripheral membrane protrusions when pre-treated with vehicle (no drug), EHT-1864, or Rhosin. n = 20 cells; Bar = 10 μm.


A
F-actin
CD63
β-tubulin
Overlay
no drug
EHT-1864
Rhosin

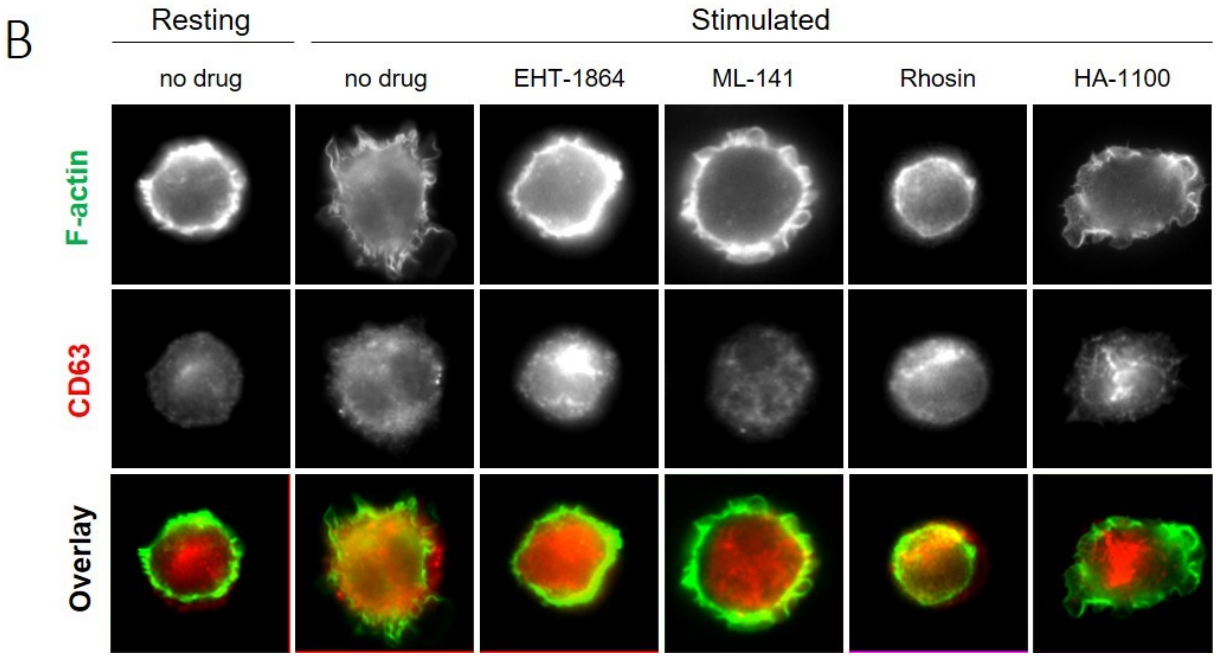
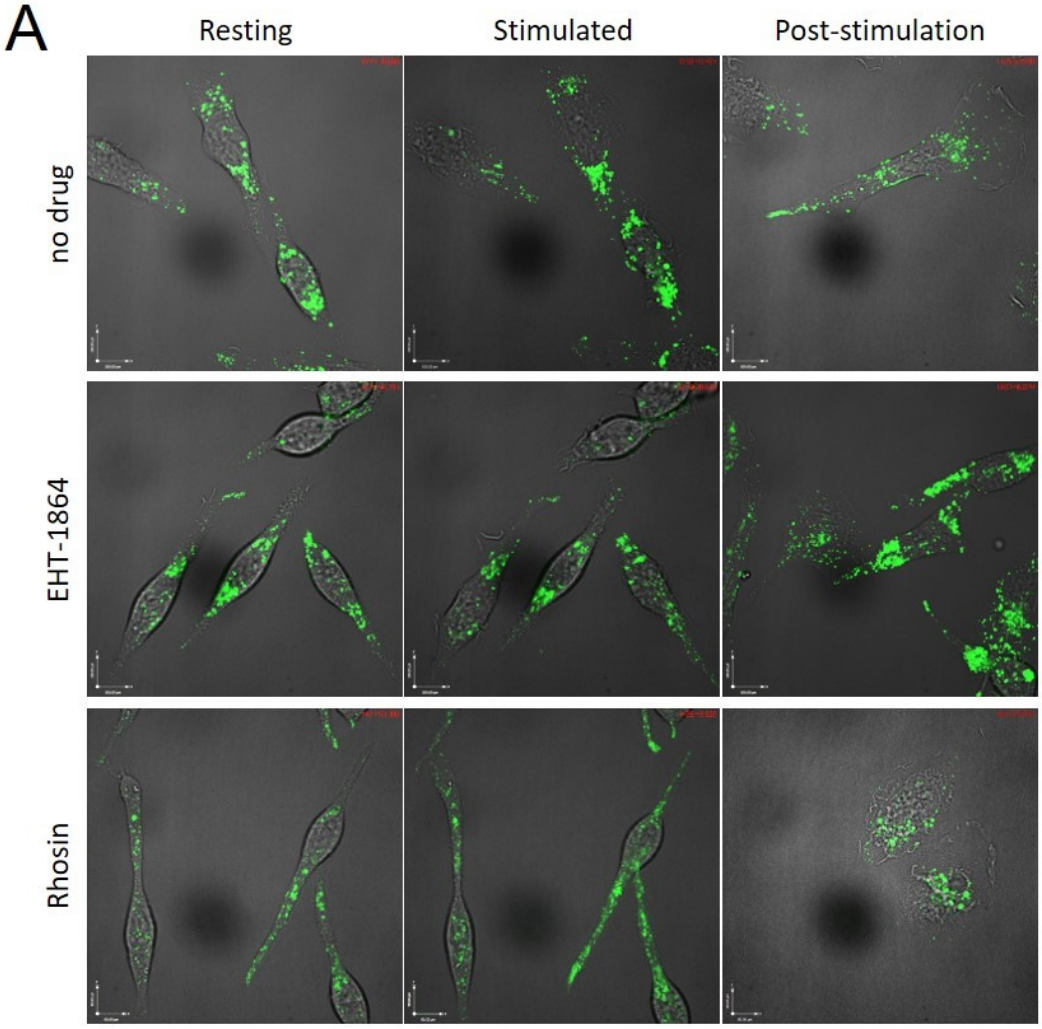

B
Resting
Stimulated
no drug
no drug
EHT-1864
ML-141
Rhosin
HA-1100
F-actin
CD63
Overlay

Figure 3.7. Immunofluorescence microscopy of cytoskeletal changes in stimulated MCs.

Representative images of 3 independent immunofluorescence images of (A) IgE-sensitized RBL-2H3 cells pre-treated with vehicle (no drug), EHT-1864, or Rhosin for 30 min and were Ag stimulated for 10 min, fixed, and stained for F-actin, β-tubulin, and CD63-positive granules. (B) IgE-sensitized BMMCs were pre-treated vehicle (no drug) or the indicated drugs. Cells were Ag stimulated for 10 min, fixed, and stained for F-actin and CD63-positive granules.


A
Resting
Stimulated
Post-stimulation
no drug
EHT-1864
Rhosin

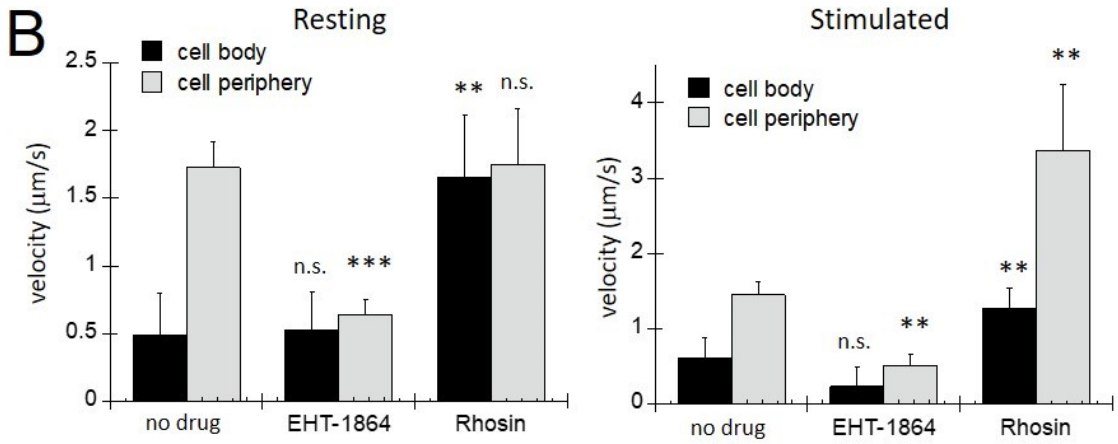

B
Resting
cell body
cell periphery
velocity (μm/s)
2.5
2
1.5
1
0.5
0
n.s.
***
**
n.s.
no drug
EHT-1864
Rhosin
Stimulated
cell body
cell periphery
velocity (μm/s)
4
3
2
1
0
n.s.
**
**
**
no drug
EHT-1864
Rhosin

Figure 3.8. Live-cell imaging of granule movement in stimulated RBL-2H3 cells. (A) Representative images from videos of IgE-sensitized RBL-2H3 cells stained with LysoTracker Green. The movement of granules was tracked during stimulation with 27 ng/ml DNP-BSA. In vehicle-treated cells (no drug, upper panels), small, highly motile granules streamed into peripheral extensions that spread and flattened into large lamellipodia. In EHT-1864–treated cells (middle panels), granules accumulated at the cell body-peripheral extension junctions. In Rhosin-treated cells (lower panels) granules moved in and out of peripheral extensions, and cells did not flatten. Bar = 10 μm. (B) Granule motility in the cell body and cell periphery was quantified before and after stimulation (left and right panels, respectively). Changes in granule velocities were compared between stimulation and resting conditions. EHT-1864–treated cells showed a significant decrease in the motility of granules in the cell periphery, whereas Rhosin-treated cells showed an increase in granule motility. NS, not significant; **P < 0.01; ***P < 0.001; n = 6 cells, with a minimum of 10 granules tracked in each region of interest.

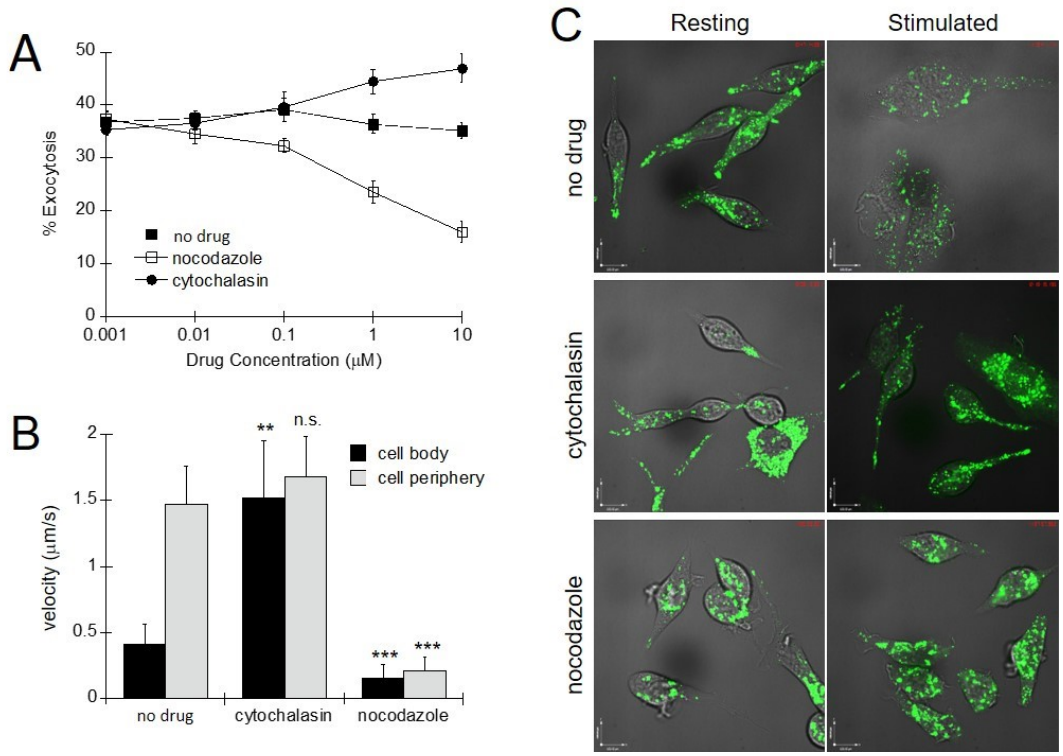


Figure 3.9 Drugs targeting actin and microtubule polymerization affect RBL-2H3 cell morphology and degranulation (A) Exocytosis was measured by determining the levels of β-hexosaminidase activity in extracellular supernatants. Sensitized RBL-2H3 cells were treated with either cytochalasin B or nocodazole for 30 min, followed by 30 min stimulation with 27 ng/ml DNP-BSA. Percentage of exocytosis was calculated as the levels of β-hexosaminidase in the supernatant, divided by the total from 0.5% Triton X-100 lysed cells. Results show RBL-2H3 degranulation was stimulated by cytochalasin and inhibited by nocodazole in a dose-dependent manner. (B) Granule motility was quantified in LysoTracker Green–stained RBL-2H3 cells. Granule velocities were measured in the cell body and cell periphery after stimulation. Comparison of velocities between untreated cells (no drug) and nocodazole-treated cells showed a significant decrease in granule motility, whereas cytochalasin B–treated cells showed increased granule motility in the cell body. NS, not significant; **P < 0.01; ***P < 0.001; n = 5 cells, with a minimum of 10 granules tracked in each region of interest. (C) Representative images from videos 10 min post-stimulation of LysoTracker Green–stained RBL-2H3 cells. Bar = 10 μm.

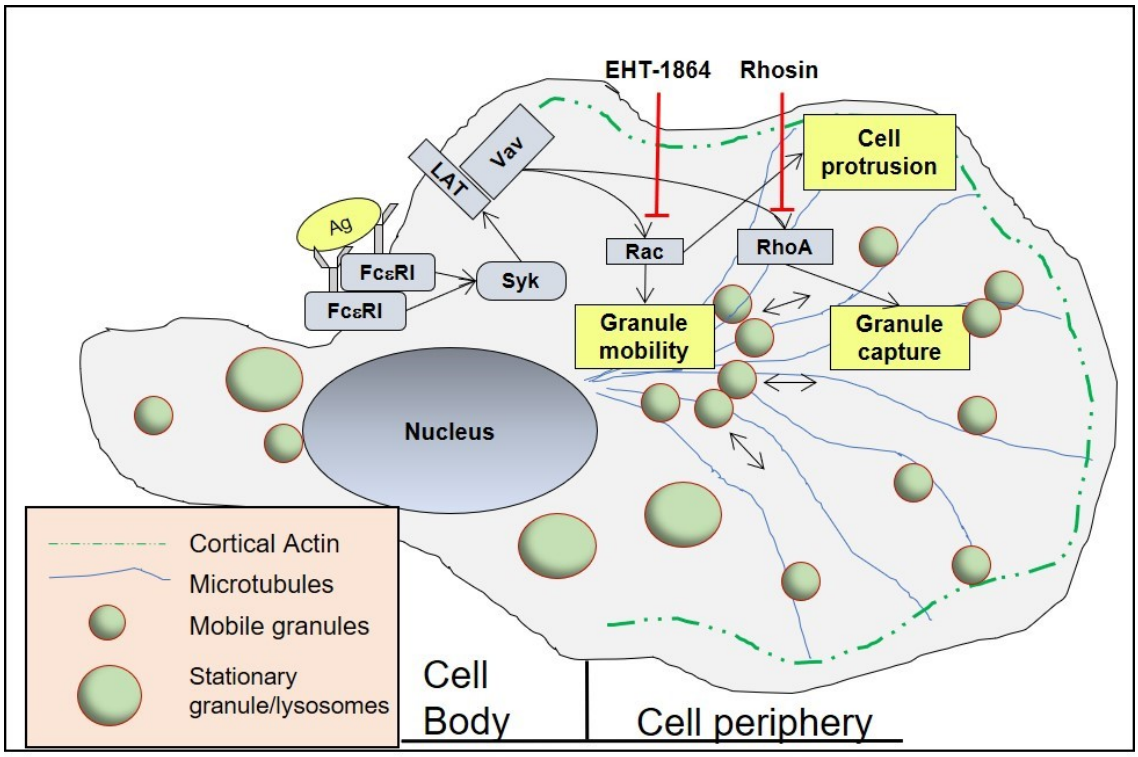


Figure 3.10 The coordinated action of Rho proteins regulates MC degranulation.

Aggregation of the high-affinity IgE receptors on the surface of mast cells by Ag results in downstream signaling to Src and Syk kinases. Syk phosphorylates the LAT adaptor protein, a scaffold that coordinates the activation of multiple mechanisms. In particular, phosphorylated LAT recruits the Rho-GEF, Vav1, which can activate all classes of Rho proteins. Stimulated mast cells undergo morphologic transitions via mechanisms that are coordinated by both Rac and Rho and results in directed granule trafficking for exocytosis. Cell protrusion forms a peripheral degranulation zone, which may be mediated by Rac because it is blocked by the Rac inhibitor EHT-1864. Granules traffic into the degranulation zone, where they are captured for exocytosis, which may be mediated by RhoA because it is blocked by the RhoA inhibitor rhosin.

Chapter 4: Defining the distinction of function of the three mammalian Rho guanine-nucleotide dissociation inhibitors (GDIs) in the regulation of Rho GTPases

Chapter 4: Defining the distinction of function of the three mammalian Rho guanine-nucleotide dissociation inhibitors (GDIs) in the regulation of Rho GTPases

4.1 Abstract

Rho GTPases are negatively regulated by Rho guanine nucleotide dissociation inhibitors (RhoGDIs). RhoGDIs are responsible for spatial and temporal regulation of Rho signaling. Here, we characterized the functional, spatial, and evolutionary properties of the three mammalian RhoGDIs. We performed a phylogenetic analysis to understand the emergence of the three RhoGDIs in vertebrates. While RhoGDI1 is present in all eukaryotes, most vertebrates have at least one additional RhoGDI that can be either RhoGDI2 or RhoGDI3, which suggest that they arose from a single ancestral gene after RhoGDI1 duplication. The emergence of RhoGDI2 and RhoGDI3 from RhoGDI1 also resulted in distinction of their functions. We found that RhoGDIs have distinct binding preferences; while RhoGDI1 bound to all Rho GTPases tested, RhoGDI2 and RhoGDI3 selectively interacted with Rac GTPases. RhoGDIs inhibited nucleotide exchange in a manner that correlates with their distinct binding preferences to Rho GTPases. We also observed that RhoGDIs localize differently in HEK293T and mast cell line, RBL-2H3 cells. In both cell types, RhoGDI1 appears to be cytosolic whereas, RhoGDI2 displays tubule-reticular distribution in the perinuclear region. RhoGDI3 was previously shown to localize to the Golgi membrane while the distribution of RhoGDI2 was more consistent with ER or mitochondrial localization. Our data defines how multiple RhoGDIs contribute to the differential regulation of Rho GTPase function.

4.2 Background

Rho GTPases are a family of highly conserved proteins that act as bimolecular switches to regulate numerous cellular processes. The nucleotide switch is tightly regulated by Rho guanine-nucleotide exchange factors (RhoGEFs) and Rho GTPase activating proteins (RhoGAPs) [118, 119, 125, 200]. RhoGEFs and RhoGAPs work in opposing fashion catalyzing the exchange of GDP to GTP and hydrolysis of GTP, respectively. Rho guanine-nucleotide dissociation inhibitors (RhoGDIs) also regulates Rho signaling [127, 129]. The spatial and temporal regulation of Rho GTPases is critical for distinct cellular effects. Dysregulation in Rho signaling can lead to diseases like cancers.

In mammals, RhoGDIs constitute a family of three members: RhoGDI1 (RhoGDIα or RhoGDIA) a ubiquitously expressed member of the family [129]; RhoGDI2 (RhoGDIβ, Ly-GDI or D4-GDI), which has haematopoietic tissue-specific expression [201, 202]; and RhoGDI3 (RhoGDIγ), a membrane-associated protein that is preferentially expressed in brain, pancreas, lung, kidney and testis [133, 203]. RhoGDIs prevent Rho activation by binding to the switch regions of Rho proteins with N-terminal alpha helical bundles and sequestering the lipid moiety of Rho GTPases in a C-terminal hydrophobic pocket [138] (*see* **Figure 1.6**). They promote the release of Rho proteins from membranes and form cytosolic complexes [204]. Additionally, RhoGDIs play an important role in delivering Rho GTPases to appropriate locations as well as their stability and crosstalk. Therefore, RhoGDIs provide regulation that can govern spatial and temporal Rho signaling.

RhoGDIs are evolutionarily conserved proteins [116, 205]. To predict whether functional specialization may have occurred between different RhoGDIs among vertebrate model systems it is necessary to accurately reconstruct the evolutionary relationships between RhoGDI genes. It is clear from previous studies that invertebrates possess a single ancestral RhoGDI, while mammals and most other tetrapods possess three RhoGDI paralogues [116, 205, 206]. However, the evolution of RhoGDIs from the ancestral invertebrate RhoGDI leading to the RhoGDI repertoire of mammals has not been fully resolved. Specifically, the precise timing of the origin of RhoGDI2 remains uncertain, and previous studies provide conflicting topologies for the relationships between tetrapod RhoGDIs [116, 205, 206].

As discussed in *Chapter 1*, the RhoGDI N-terminus is thought to play a key role in the inhibition of nucleotide exchange [83] as well as the translocation of Rho GTPase to membranes [139]. The N-termini of RhoGDIs may govern the different affinities of RhoGDIs to Rho GTPases [116, 127, 136, 207, 208]. RhoGDI1 binds to RhoA, Cdc42, Rac1 and Rac2 both *in vitro* and *in vivo* [116, 130, 134, 135]; whereas RhoGDI2 binding partners have not been clearly defined. RhoGDI3 was shown to interact with RhoA, RhoB, Cdc42 and RhoG but not with Rac1 or Rac2 [136, 137].

In addition to binding preferences, RhoGDI family members also display distinct sub-cellular localization. RhoGDI3, in particular, was shown to localize predominantly to Golgi membranes [137] whereas RhoGDI1 and RhoGDI2 localization is poorly defined. The biological roles of RhoGDIs and their subfamily members in disease states are not fully explored. Interestingly, RhoGDI knockout mice are available and they display only mild phenotypes [140, 209, 210].

RhoGDI1 knockout mice studies show progressive impairment of kidneys and reproductive organs [209]. RhoGDI2 knockout mice display subtle alterations in superoxide generation in macrophages during phagocytosis, but no effect on lymphopoiesis [140]. RhoGDI1 and RhoGDI2 double knockout results in defective lymphocyte development and defective chemokine-directed migration. This indicates that RhoGDIs, via Rho GTPases, may play a role in regulating cell behaviour [210]. For example, RhoGDI in complex with Rac1 translocates to the leading edge of the plasma membrane in cells to generate directional movement [211]. Therefore, it is likely that RhoGDI localization impacts spatial and temporal Rho signaling which in turn regulates different cellular processes. However, the specific roles of RhoGDI subfamily members in immune cell functions have not been fully studied. Elucidating the precise functions of each RhoGDI in MC activation and responses may lead to novel, safe and efficacious therapeutics for MC-driven inflammatory disorders.

Here we examined the distinct characteristics of the three RhoGDI family members. The evolution of the three RhoGDIs highlights some of the key characteristics that define this family and their potential distinction of function during the evolution of vertebrates. We demonstrate that RhoGDIs show preferential binding to different Rho GTPases. Additionally, we also demonstrate disparate inhibitory capacities of RhoGDIs in RhoGEF-mediated GTP exchange. We also provide the first evidence for the different localization of RhoGDI1 and RhoGDI2 in HEK-293T cells and RBL-2H3. Using immunofluorescence and sub-cellular fractionation studies, we demonstrate that RhoGDI1 is mostly cytoplasmic whereas, RhoGDI2 appears to be associated with heavier membranes. What roles do RhoGDIs play at these specific sites in the cell, during

MC degranulation is under investigation. Taken together, we establish distinct biochemical, spatial, functional and evolutionary properties of RhoGDI family members.

4.3 Results

4.3.1 Evolutionary relationship of RhoGDIs

The Rho family of GTPases play an important role in numerous cellular processes and thus their functions must be spatially and temporally regulated. RhoGDIs are one class of regulators that are evolutionarily conserved and seemed to have co-evolved with Rho proteins to provide spatial regulation [84, 206, 212]. All eukaryotic cells contain RhoGDI; fungi and invertebrates have a single RhoGDI gene while vertebrates such as fish have two and most tetrapods have three (**Figure 4.1 A**). Phylogenetic analysis of a representative set of animals revealed RhoGDI3 is nearly ubiquitous in vertebrates other than lamprey (**Figure 4.1 B**). However, no RhoGDI2 orthologues were identified in fish which is consistent with previous studies [206]. RhoGDI2 orthologues were found in the genomes of all tetrapods and coelacanth, which is a close relative of tetrapods, which indicates that the origin of RhoGDI2 occurred no later than the common ancestor of coelacanths and tetrapods. This suggests that that RhoGDI3 emerged first during expansion of RhoGDI genes. Further phylogenetic analysis of tetrapod RhoGDI sequences with low amounts of sequence divergence (identified through multiple rounds of phylogenetic analysis), as well as coelacanth sequences, showed significant support for separate RhoGDI2 + RhoGDI3 clades, suggesting that these two RhoGDIs originated through duplication of an ancestral RhoGDI2/3 hybrid gene that was distinct from RhoGDI1 (**Figure 4.1 C**).

4.3.2 Distinction of RhoGDI function

While the N-terminal amino acid sequences of RhoGDIs are highly conserved within orthologous groups, they differ significantly outside of orthologous groups (**Figure 4.2 A**). Alignment of the three human RhoGDIs also depicts a highly conserved C-terminal β-sheet domain and a divergent N-terminal α-helical domain that interacts with Rho protein switch regions [109] (**Figure 4.2 B**). Divergence of the N-terminal sequences suggests that distinction of RhoGDI functions may be determined by the distinct N-termini, resulting in the specialization of RhoGDI2 and RhoGDI3 in particular.

4.3.3 Interaction of RhoGDIs with Rho GTPases

Next we examined if the N-terminus is important for distinction of function of the different RhoGDI orthologs. RhoGDIs are primarily known for their ability to bind Rho GTPases, sequestering their lipid modification and forming soluble cytoplasmic complexes. Previous studies investigating binding affinities of RhoGDIs to prenylated GTPases have revealed contradictory results [208, 213]. RhoGDI2 showed preferential Rho GTPase binding patterns and had reduced affinity to GTPases, compared to RhoGDI1. We believe, this difference may stem from the divergent N-terminal sequences of RhoGDIs, whereas C-terminal interactions with common isoprenyl lipids would be less selective. Therefore, to assess the contribution of the N-terminus of RhoGDI proteins, we analyzed the binding to non-prenylated Rho GTPases. Purified RhoA, Cdc42, Rac1 and Rac2 were incubated with GST-tagged RhoGDIs (**Figure 4.3 A**) immobilized on glutathione agarose. In line with previous studies [116, 130, 134, 135, 137], the three RhoGDIs showed distinct binding preferences. GST-RhoGDI1 did not show preferential

binding; RhoA, Cdc42, Rac1 and Rac2 were bound with similar affinity (**Figure 4.3 B**). GST-RhoGDI2 showed preferential binding to Rac2, a slight interaction with Rac1 and no binding to RhoA or Cdc42 (**Figure 4.3 C**). This is in contrast to previous results showing that RhoGDI2 binds to several Rho GTPases including Cdc42 [208]. GST-RhoGDI3 showed preferential binding to Rac1 and Rac2 (**Figure 4.3 D**). It is important to note that we used a truncated version of RhoGDI3 for these assays which removed the N-terminal 28 amino acid extension. While this allowed for the production of soluble protein, it could impact GST-RhoGDI3 affinities. These results demonstrate the preferential binding partners of the three RhoGDIs and conclude that there is selectivity driven by protein-protein interactions with the common, non-homologous, N-terminal domains.

4.3.4 Inhibition properties of RhoGDIs

Next, we examined the functional differences in RhoGDI with respect to their inhibitory activity of Rho GTPase activation. To do this we used a RhoGEF assay, which measure Rho activation as a fluorescence increase over time due to RhoGEF-stimulated loading of mant-labelled GTP. We used the Dbl homology-plekstrin homology (DH-PH) domains sub-cloned from TrioN as a Rac GEF, which efficiently catalyzes GTP exchange for both Rac1 and Rac2 as shown in kinetic experiments (**Figure 4.4 A**, upper panels *open boxes*). DH-PH domains from the GEFs Dbs and Itsn1 were used to catalyze exchange for RhoA and Cdc42, respectively (**Figure 4.4 A**, lower panels *open boxes*). Addition of RhoGDI1 to RhoGEF assays resulted in the kinetic inhibition of exchange for all Rho proteins (**Figure 4.4A**) with significant inhibition for Rac1 (59% reduction of activity, p=0.0091) and RhoA (44% reduction of activity, p=0.0078) compared to buffer control

(**Figure 4.4 B**). RhoGDI2 showed maximum inhibition of TrioN-mediated Rac2 exchange both kinetically (**Figure 4.4 A**) and in end-point reactions (36% reduction of activity, *p*=0.022) (**Figure 4.4 B**). This was expected since RhoGDI2 showed a strong interaction with Rac2 (**Figure 4.3**). RhoGDI3 showed moderate levels of inhibition of all GTPases with only RhoA being significant (27% reduction of activity, *p*=0.0052). From this study, we concluded that RhoGDIs possess the capacity to inhibit RhoGEF-mediated GTP exchange which is comparable to their Rho binding properties. This may define how they impact Rho signaling in cells.

4.3.5 RhoGDIs display distinct cellular localization

It is generally accepted that RhoGDI1 and RhoGDI2 are cytosolic, while RhoGDI3 was recently demonstrated to be localized to the Golgi membrane [137]. We wanted to examine the subcellular localization of RhoGDIs in HEK293T cells; however, our examination of the specificity of antibodies raised against RhoGDIs showed that, while RhoGDI1 and RhoGDI2 antibodies are specific, RhoGDI3 antibodies detect both RhoGDI3 and RhoGDI1 (**Figure 4.6**). Therefore RhoGDI3 localization was not examined. To verify that immunofluorescence patterns are truly representative of RhoGDI1 and RhoGDI2, the specificity of detection was examined by pre-incubating antibodies with purified GST-RhoGDI1 and GST-RhoGDI2 (*see* **Figure 4.3**). Immunofluorescence signals were diminished only when antibodies were blocked with corresponding RhoGDIs (**Figure 4.6**).

Immunofluorescence microscopy of endogenous RhoGDI1 and RhoGDI2 in HEK-293T cells showed strikingly distinct localization. RhoGDI1 showed predominantly cytosolic staining, while RhoGDI2 showed punctate staining with a clear lack of cytosolic signal (**Figure 4.5 A**).

Exogenous expression of all three RhoGDIs as GFP-tagged forms validated their distinct cellular distribution (**Figure 4.5 B**). These results suggest that, similar to RhoGDI3 [137], RhoGDI2 is also membrane localized, a feature that may have been derived during their co-evolution (*see* **Figure 4.1 A**). However, RhoGDI2 and RhoGDI3 have different subcellular distributions when expressed as GFP-tagged version and therefore associate with different membranes (**Figure 4.5 B**).

Next we further defined the subcellular localization of RhoGDI2 by co-staining cells with markers of intracellular membranes. While there was no overlap between RhoGDI1 and endoplasmic reticulum (ER) or mitochondrial markers (**Figure 4.7 A**), RhoGDI2 showed partial overlap with both (**Figure 4.7 B**). Glut1 localizes to the cell periphery and endosomes which showed no overlap with either RhoGDI1 or RhoGDI2 (**Figure 4.7 C**). This suggests that RhoGDI2 is primarily localized to an intracellular, perinuclear membrane.

Furthermore, we also performed sub-cellular fractionation to biochemically analyze RhoGDI1 and RhoGDI2 pools. HEK-293T lysate was fractionated on sucrose gradients and the proteins in different fractions were TCA precipitated and analyzed by immunoblot (**Figure 4.7 D**). RhoGDI1 was present in the same fractions as the cytosolic marker, GAPDH. RhoGDI2 was in fractions distinct from RhoGDI1 that contained markers of ER and mitochondria, which is in agreement with our microscopic observations. Taken together, our results demonstrate that RhoGDI family members display distinct localization in cells; RhoGDI1 is mostly cytosolic whereas RhoGDI2 is predominantly membrane associated with organelles like ER and/or mitochondria.

4.3.6 RhoGDIs display distinct subcellular localization in RBL-2H3 cells

MCs are hematopoietic stem cell-derived immune cells. The expression of RhoGDI1 and RhoGDI2 in RBL-2H3, a rat basophil leukemia cell line, and BMMCs was confirmed by immunoblot (**Figure 4.8 A)**. We also examined RhoGDI expression at the mRNA level by qPCR (**Figure 4.8 B**). RhoGDI1 and RhoGDI2 were robustly expressed, whereas, RhoGDI3 showed strong expression in comparison to the control, GAPDH. Further work is needed to establish the expression of RhoGDIs at the protein level, during the development of MCs.

Next, we investigated the localization of RhoGDI1 and RhoGDI2 in resting and Ag-stimulated RBL-2H3 cells. Resting RBL-2H3 displayed a uniform cytosolic distribution of RhoGDI1 and a punctate distribution of RhoGDI2, which was seen both in the cell body and peripheral extensions (**Figure 4.9 A**). In FcεRI-stimulated RBL-2H3 cells, RhoGDI1 remained uniformly cytosolic while RhoGDI2 showed a reticulo-tubular distribution in the peri-nuclear region. We confirmed the specificity of staining by performing immunofluorescence imaging with rabbit and mouse IgG isotype controls (**Figure 4.9 B and C**). These results indicate that distinct localization of RhoGDI1 and RhoGDI2 may provide a novel mechanism for the spatial regulation of Rho subfamily members during antigen stimulated MC degranulation.

4.3.7 RhoGDI2 is associated with heavy membranes in RBL-2H3 cells

Next, we investigated the localization of RhoGDI2 in reference to the cellular organelles. We checked whether RhoGDIs colocalize with endoplasmic reticulum (ER) or mitochondria by immunofluorescence. Whilst RhoGDI1 showed cytoplasmic distribution that was distinct from ER marker (calnexin) (**Figure 4.10 A**) and mitochondria marker (Complex II) (**Figure 4.10 B**), RhoGDI2 distribution was comparable to mitochondrial distribution (**Figure 4.10 D**). This agrees

with HEK-293T results (**Figure 4.5 A**). RhoGDI1 also appeared to concentrate in dorsal ruffles that appear on FcεRI-stimulated RBL-2H3s (**Figure 4.9 B** and **Figure 4.10 B; arrowhead**). Whether RhoGDI1 is required for the formation of actin rich dorsal ruffles is under investigation.

Finally, we also performed sucrose gradient subcellular fractionation of resting RBL-2H3 cells (**Figure 4.10 E**). Similar to our earlier results in HEK-293T cells, RhoGDI1 was found mostly in the low density, presumably cytosolic fractions and RhoGDI2 was seen in denser fractions. Mitochondrial membranes, to a certain degree, overlaps with the distribution of RhoGDI2 on these gradients. This indicates that RhoGDI2 may have mitochondrial association. What roles they may play in these specific locations are unclear.

4.4 Discussion

RhoGDIs regulate Rho GTPase expression levels, signaling and crosstalk [116, 130]. The importance of controlling Rho GTPase function is manifested in how aberrant Rho signaling contributes to the severity of diseases such as cancer survival and metastasis [81, 100], Alzheimer's disease [214, 215] inflammation [81] and allergies [155, 216]. The lack of potent drugs that target Rho family of proteins [217] compel studies that look at the naturally occurring inhibitor of the Rho proteins, RhoGDI. Our knowledge of RhoGDI function mostly stems from studies focused on RhoGDI1. However, new studies implicate distinct roles for other RhoGDI family members, which have been discovered in aggressive cancers [136, 218, 219]. Identification of unique and essential roles for RhoGDI2 and RhoGDI3 in cell survival, migration and function, validates them as potential targets for therapeutics in cells with dysregulated RhoGDI signaling. The advantage targeting these proteins is their limited expression in tissues that can result in drugs with better safety profiles.

Our studies into the evolution of RhoGDI proteins in vertebrates revealed that RhoGDI2 and RhoGDI3 could be derived from an ancestral RhoGDI2/3 hybrid gene. RhoGDI1 is a ubiquitous ancestral vertebrate RhoGDI, while the precise timing of the emergence of RhoGDI2 and RhoGDI3 is less clear. Previous studies suggested that the origin of RhoGDI2 predates the evolution of cartilaginous and bony fish, but did not identify RhoGDI2 orthologues in these lineages [206]. Our results, using a more extensive sampling of data from cartilaginous fish, including the whale shark, *Rhincodon typus* [220], also did not identify RhoGDI2 orthologues in these taxa (**Figure 4.1**). Therefore, the taxonomic distribution of RhoGDI2 orthologues could be

explained either by an origin in the ancestor of gnathostomes, coincident with the origin of RhoGDIs classifiable as RhoGDI3, followed by independent losses in the cartilaginous and bony fish, or more likely by a later origin in an ancestor of coelacanth and tetrapods. Interestingly, RhoGDI expansion does not seem to be coordinated with Rho GTPase. The evolutionary history of Rho GTPase has been studied, and vertebrates as well as invertebrates have large numbers of fully diversified Rho proteins [212]. Thus, the evolution of Rho proteins does not seem to be linked to the presence of RhoGDI, which suggests that the expanded repertoire of vertebrate RhoGDIs allows for more complex regulation of existing Rho proteins.

Rho GTPases in complex with RhoGDI structures have been resolved [108]. It is clear that the N- and C-termini of RhoGDI interact with Rho GTPases [108, 109, 213, 221]. The variance in RhoGDI1, RhoGDI2 and RhoGDI3 N-termini (**Figure 4.2**) may play a role in their distinct binding preferences. We investigated whether the N-termini of RhoGDIs plays a role in preferential interactions with Rho GTPase. We used *E. coli* expressed RhoGDIs and Rho GTPases that were not prenylated and hence, the interaction with RhoGDIs would be driven by N-termini. We observed that RhoGDI1 was able to bind to all the tested Rho GTPases, whereas, RhoGDI2 and RhoGDI3 showed preferential binding to Rac GTPases (**Figure 4.3**). This agrees with previous reports and establishes the role of the N-termini of RhoGDIs in their selective interaction with Rho GTPases [108, 137, 208]. Discrepancies between our results and previously reported interactions could also be attributed to post-translational modifications of Rho GTPases and RhoGDIs. PTMs have been demonstrated to alter binding affinities and are potentially a way to regulate Rho signaling [222]. For example, RhoGDIs and Rho GTPases can be phosphorylated by

kinases resulting in both dissociation and stronger association depending on the sites of phosphorylation [153, 223-227].

Further, we also looked at the functional inhibitory properties of RhoGDIs. Previous reports have suggested that RhoGDI1 and RhoGDI2 may have differences in their ability to functionally inhibit Rho GTPases and such a difference may arise due to variance in a single residue [213]. Based on these results, we decided to determine the capacity of the three RhoGDIs to inhibit RhoGEF mediated exchange activity. We observed that in the presence of excess RhoGDI1 (5:1 molar ratio), there was a significant decrease in GTP exchange for all Rho proteins (**Figure 4.4**). RhoGDI2 and RhoGDI3 showed less inhibitory activity in this assay. However, RhoGDI2 was able to inhibit Rac2 GTP exchange which is in accord with its ability to bind Rac2 (**Figure 4.3 C**). Overall The modest levels of inhibition by RhoGDIs are likely due to the fact that Rho GEFs act enzymatically while RhoGDIs are required to form stably bound complexes. As well, regulation of Rho GTPases by RhoGDIs may be influenced by their post translational modification which influences binding [107, 116, 130, 153, 225, 227-229]. This further defines RhoGDI1 as the archetypal member of the RhoGDI family as the most functionally capable while RhoGDI2 and RhoGDI3 have seemed to derive specialized function.

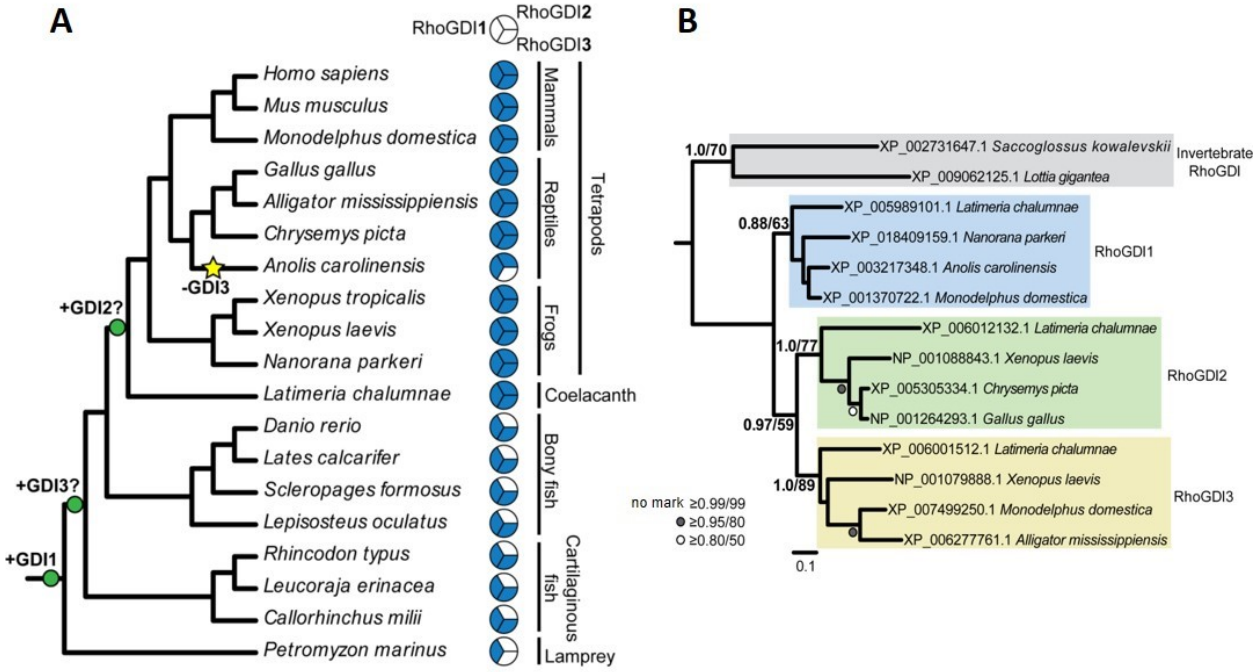
Rho GTPases in complex with RhoGDIs are thought to be cytosolic reserve pools, with upstream signals driving their translocation to distinct membranes, with subsequent dissociation and membrane delivery of Rho proteins [116, 127]. We found that while RhoGDI1 was cytosolic, RhoGDI2 had a predominant membrane-localized pool in HEK-293T (**Figure 4.6**) and RBL-2H3 cells (**Figure 4.9**). This is similar to RhoGDI3, which has been shown to localize to the Golgi

membrane through a distinct N-terminal domain not present in other RhoGDIs [137]. Further work is needed to identify binding partners of RhoGDI1 and RhoGDI2 in RBL-2H3 cells at these specific sites. This could identify Rho subfamily members that is bound to RhoGDI1 and RhoGDI2, as well as any other interacting factors that may have critical role in the selective membrane delivery of Rho GTPases via dissociation from RhoGDIs (e.g., kinases).

Our evolutionary analyses of RhoGDIs (**Figure 4.1 and 4.2**) support the emergence of RhoGDI3, or a RhoGDI2/3 hybrid ancestor in higher vertebrates. Therefore, membrane association may be a feature derived during their co-evolution. Since the localization of Rho GTPases is critical for appropriate regulation of cytoskeleton and other cellular processes, we believe that this distinct localization of RhoGDIs may provide non-redundant regulatory functions. In addition, it was recently demonstrated that the N-termini of RhoGDIs play a role in translocation of complexes to membranes [139] which defines the divergence in this domain as an important feature in the distinction of RhoGDI functions. In addition to the preferential binding capacities of RhoGDIs, distinct localization may also provide basis for non-redundant functions for the three RhoGDI family members. Distinct localization of Rho signaling is critical for cellular function [196, 230]. For example, we recently showed that Rac1 and Rac2 have non-redundant functions in allergen activated mast cells [156]. Rac2 was shown to regulate calcium signaling, whereas Rac1 was required for the regulation of actin dynamics at the plasma membrane. These distinct, non-redundant functions of GTPases could be regulated by different RhoGDIs at specific locations in the cell. Therefore, it remains possible that the discrete locations of RhoGDIs allows them to regulate Rho subfamily members during the activation of MCs.

While we observed that RhoGDI1 was mostly cytosolic and did not appear to colocalize with ER or mitochondria markers, RhoGDI2 did show a striking appearance similar to complex II in HEK-293T and FcεRI-stimulated RBL-2H3 cells (**Figure 4.7** and **4.10**). We confirmed potential mitochondria association of RhoGDI2 by sucrose gradient subcellular fractionation of RBL-2H3 cell lysates (**Figure 4.10**). RhoGDI2 appeared in the same fractions as mitochondria whereas RhoGDI1 is mostly seen in low density fractions. These experiments need further controls for other organelles like Golgi, plasma membrane, granules and lysosomes to understand whether the unique RhoGDI2 distribution can be understood. Rho signaling at these specific locations can contribute to MC degranulation in many ways by the regulation of calcium signaling, granule trafficking or degradation of internalized receptors to prevent hyperstimulation. Further work using knockdown or knockout cells can reveal the complex regulation of Rho signaling by RhoGDIs.

Future work using reduced expression of RhoGDI1 and RhoGDI2 can reveal any defects in the appropriate localization and functions of Rho proteins. Additionally, it can also reveal unique RhoGDI1- and RhoGDI2- signaling that regulates distinct events during MC degranulation (*see model in* **Figure 5.1**). Our work indicates the distinct properties of RhoGDIs in their regulation of Rho GTPases. Further work elucidating the importance of RhoGDIs in MC-mediated inflammatory disorders may validate them as novel, druggable targets to prevent aberrant Rho signaling.


A
RhoGDI1
RhoGDI2
RhoGDI3
Homo sapiens
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Monodelphus domestica
Gallus gallus
Alligator mississippiensis
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Anolis carolinensis
Xenopus tropicalis
Xenopus laevis
Nanorana parkeri
Latimeria chalumnae
Danio rerio
Lates calcarifer
Scleropages formosus
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RhoGDI3
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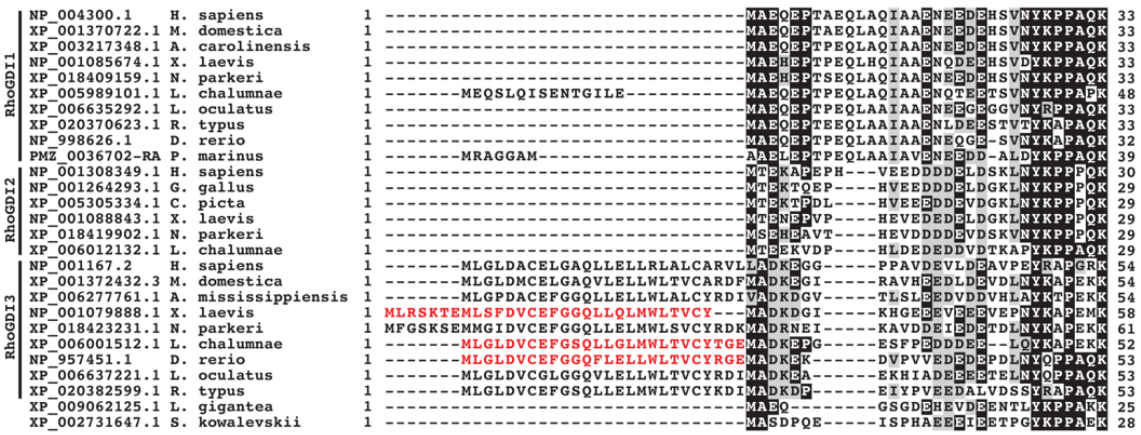
RhoGDI3
≥0.99/99
≥0.95/80
≥0.80/50
0.2

Figure 4.1 Evolution of RhoGDI proteins in vertebrates.

(A) Presence or absence of RhoGDI1, RhoGDI2 and RhoGDI3 orthologues in vertebrate sequence data. The phylogram on the left shows the taxonomic relationships between the vertebrates from which sequence data was analyzed. The Coulson plot on the right shows which RhoGDIs were identified in these vertebrates. Sectors filled in blue indicate identification of at least one orthologous sequence. The latest possible emergence of RhoGDI1, RhoGDI2, and RhoGDI3 are indicated by green dots, although earlier origins are possible. The yellow star indicates loss of RhoGDI3 in *A. carolinensis*. (B) Phylogenetic analysis of vertebrate RhoGDI amino acid sequences rooted on an invertebrate out group. Results support RhoGDI2 and RhoGDI3 arising from a gene duplication after the origin of RhoGDI1. Support values shown for key nodes are MrBayes posterior probabilities (maximum of 1.0 with 0.8 being the minimum significant value) and RAxML bootstrap values (maximum of 100 with 50 being the minimum significant value). Internal nodes are symbolized as in the inset legend. (C) phylogenetic analysis of all identified vertebrate RhoGDI amino acid sequences. MrBayes and RAxML were used to reconstruct the phylogenetic relationships of RhoGDI subfamily of proteins. In several cases, multiple amino acid sequences included here correspond redundantly to a single gene/locus, meaning that the number of amino acid sequences does not correspond to the number of unique gene copies in a genome. Support values shown for main nodes are MrBayes posterior probabilities (maximum of 1.0, with 0.8 the minimum for significance) and RAxML bootstrap values (maximum of 100, with 50 the minimum for significance). Internal nodes are symbolized as inset.

A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RhoGDI3
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B

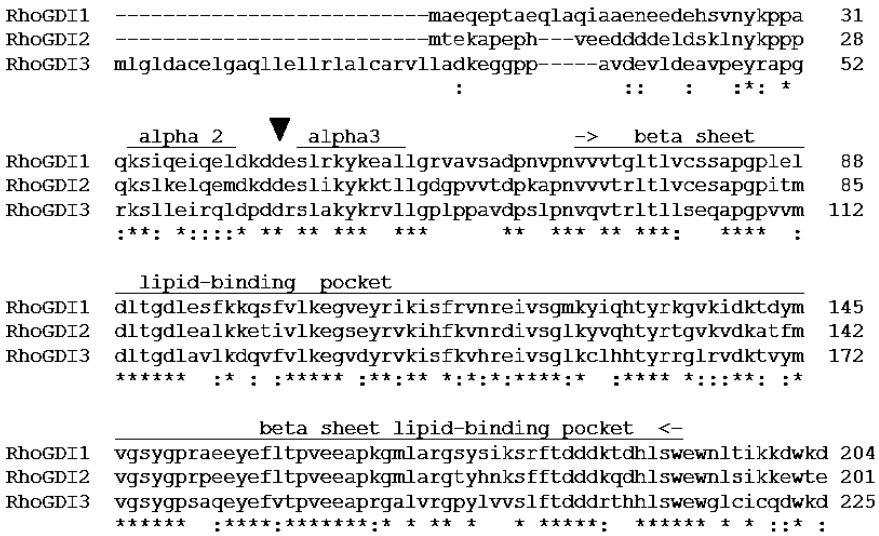

RhoGDI1 ----------maeqeptaeqlaqiaaeneedehsvnykppa 31
RhoGDI2 ----------mtekapeph---veeddddeldsklnykppp 28
RhoGDI3 mlgldacelgaqllellrlalcarvlladkeggpp-----avdevldeavpeyrapg 52
alpha 2
alpha3
-> beta sheet
RhoGDI1 qksiqeiqeldkddeslrkykeallgrvavsadpnvpnvvvtgltlvcssapgplel 88
RhoGDI2 qkslkelqemdkddeslikykktllgdgpvvtdpkapnvvvtrltlvcesapgpitm 85
RhoGDI3 rkslleirqldpddrslakykrvllgplppavdpslpnvqvtrltllseqapgpvvm 112
lipid-binding pocket
RhoGDI1 dltgdlesfkkqsfvlkegveyrikisfrvnreivsgmkyiqhtyrkgvkidktdym 145
RhoGDI2 dltgdlealkketivlkegseyrvkihfkvnrdivsglkyvqhtyrtgvkvdkatfm 142
RhoGDI3 dltgdlavlkdqvfvlkegvdyrvkisfkvhreivsglkclhhtyrrglrvdktvym 172
beta sheet lipid-binding pocket <-
RhoGDI1 vgsygpraeeyefltpveeapkgmlargsysiksrftdddktdhlswewnltikkdwkd 204
RhoGDI2 vgsygprpeeyefltpveeapkgmlargtyhnksfftdddkqdhlswewnlsikkewte 201
RhoGDI3 vgsygpsaqeyefvtpveeaprgalvrgpylvvslftdddrthhlswewglcicqdwkd 225

Figure 4.2 Amino acid alignment of RhoGDIs. (A) N-terminal sequences of mammalian RhoGDI1, RhoGDI2, and RhoGDI3 are similar to that of their respective orthologues from diverse vertebrates, consistent with early origins of their respective specialized functions. The residues shown in *red* are not found in the amino acid sequences to which the accessions refer, but were identified in the genomic DNA assemblies for the respective organisms using tBLASTn, and were adjacent to the RhoGDI3 genes. The figure was produced using BoxShade. (B) Alignment of human RhoGDI1, RhoGDI2 and RhoGDI3 amino acid sequences. N-terminal alpha helices and C-terminal beta sheet lipid-binding pocket are indicated above the sequences. "*" and ":" indicate identical and highly conserved amino acids, respectively. *Triangle* indicates the position of a conserved aspartic acid of RhoGDIs that forms a hydrogen bond with threonine at position 35 of Rac1, Rac2 and Cdc42 or position 37 of RhoA.

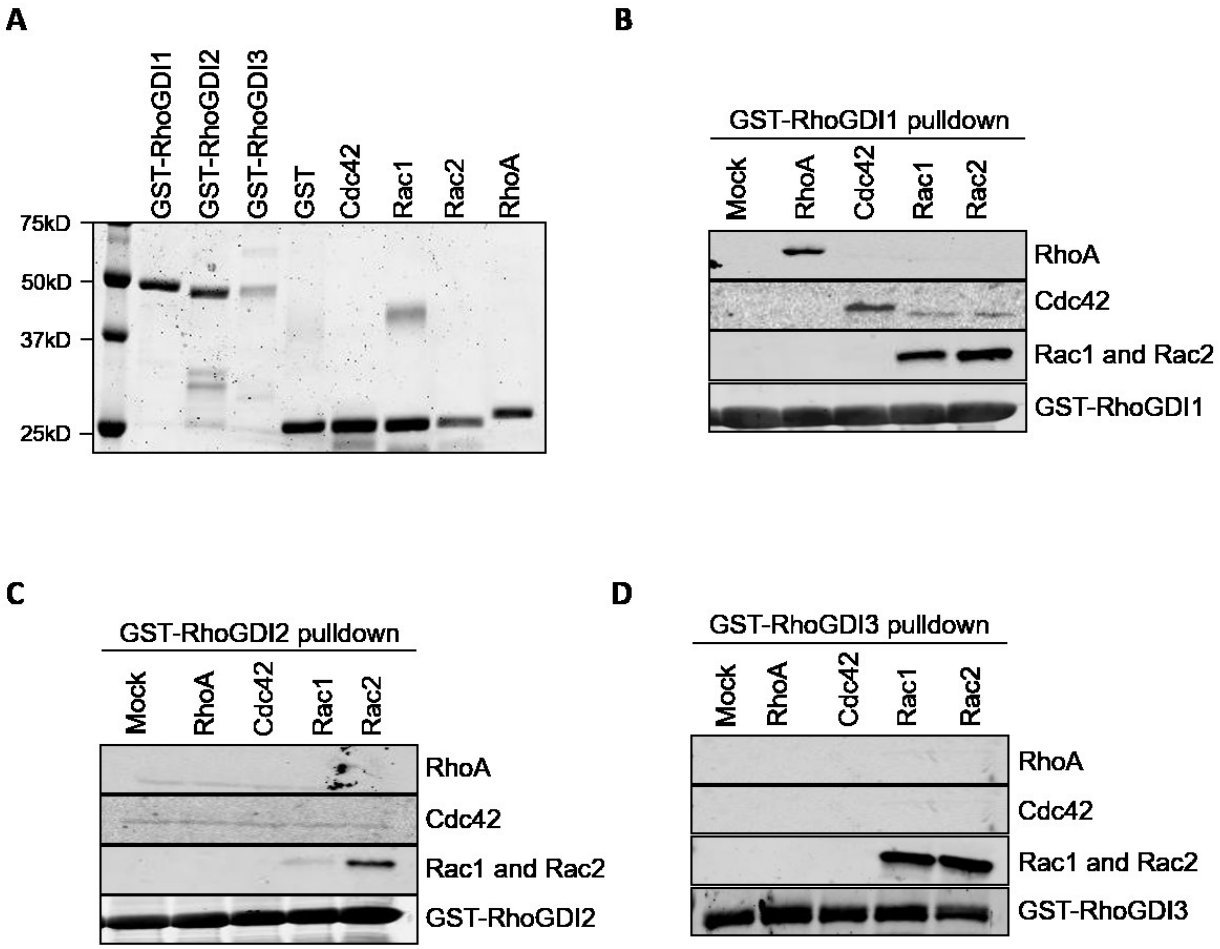
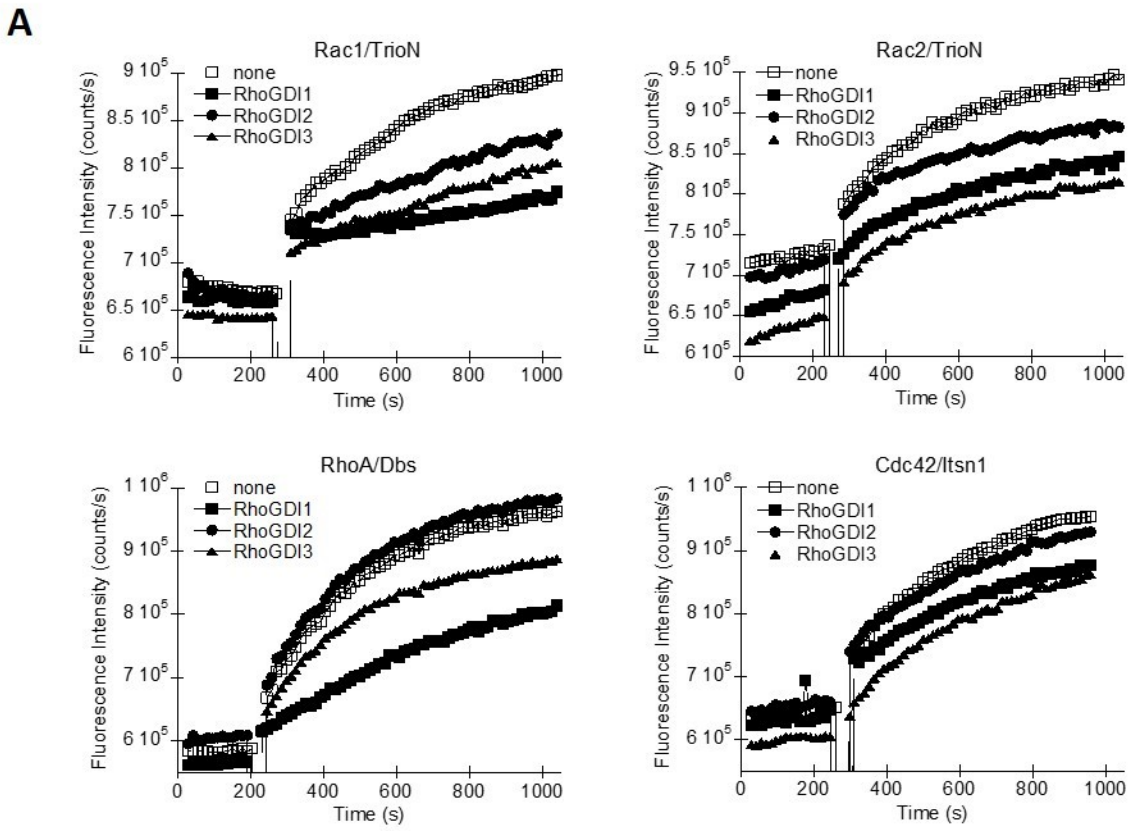

A
GST-RhoGDI1
GST-RhoGDI2
GST-RhoGDI3
GST
Cdc42
Rac1
Rac2
RhoA
75kD
50kD
37kD
25kD
B
GST-RhoGDI1 pulldown
Mock
RhoA
Cdc42
Rac1
Rac2
RhoA
Cdc42
Rac1 and Rac2
GST-RhoGDI1
C
GST-RhoGDI2 pulldown
Mock
RhoA
Cdc42
Rac1
Rac2
RhoA
Cdc42
Rac1 and Rac2
GST-RhoGDI2
D
GST-RhoGDI3 pulldown
Mock
RhoA
Cdc42
Rac1
Rac2
RhoA
Cdc42
Rac1 and Rac2
GST-RhoGDI3

Figure 4.3 Detection of preferential RhoGDI::Rho GTPase interactions.

Purified Rho proteins (non-prenylated) were incubated with GST-tagged RhoGDI immobilized on glutathione beads. (A) Coomassie stained gel showing the purified proteins used in pull-down reactions. (B-D), Immunoblots show the Rho GTPases binding to (B) GST-RhoGDI1, (C) GST-RhoGDI2, (D) GST-RhoGDI3. Blots were probed with anti -RhoA, -Cdc42 and -Rac specific antibodies (Rac antibody detects both Rac1 and Rac2) (n=3). Anti-GST antibody was used to detect GST-tagged RhoGDIs. RhoGDI1 interacted with all the Rho GTPases; RhoGDI2 and RhoGDI3 showed preferential interactions with Rac2, or Rac1 and Rac2, respectively.


A
Rac1/TrioN
Rac2/TrioN
RhoA/Dbs
Cdc42/Itsn1
none
RhoGDI1
RhoGDI2
RhoGDI3
Fluorescence Intensity (counts/s)
Time (s)

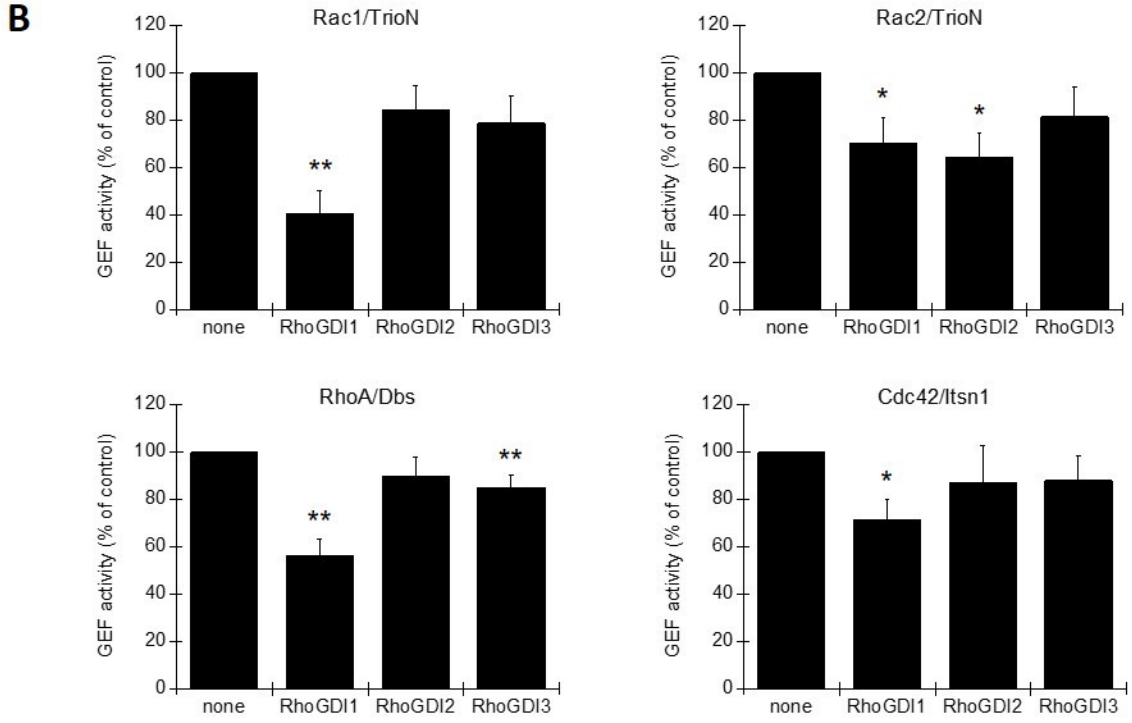

B
Rac1/TrioN
Rac2/TrioN
RhoA/Dbs
Cdc42/Itsn1
GEF activity (% of control)
none
RhoGDI1
RhoGDI2
RhoGDI3

Figure 4.4 RhoGEF assays show distinct inhibitory activities of RhoGDIs (A) Kinetics of RhoGEF exchange activity of TrioN (Rac1 and Rac2 GEF), Dbs (RhoA GEF) and Itsn1 (Cdc42 GEF), done in the presence of RhoGDIs to examine their inhibitory activity compared to buffer control (*none*). Baseline readings were taken for 4 min and then the indicated GEF was added. (B) Relative inhibition of initial exchange rates calculated as the average slope, 5 min after addition of GEF, from kinetic experiments as shown in *panel A*. Data are the average, +/- s.e., of 5 independent experiments. Statistical comparison with reactions in the absence of RhoGDI (*none*) was done by paired Student's t-test (*p*-values: *, $p< 0.05$; **, $p< 0.01$).

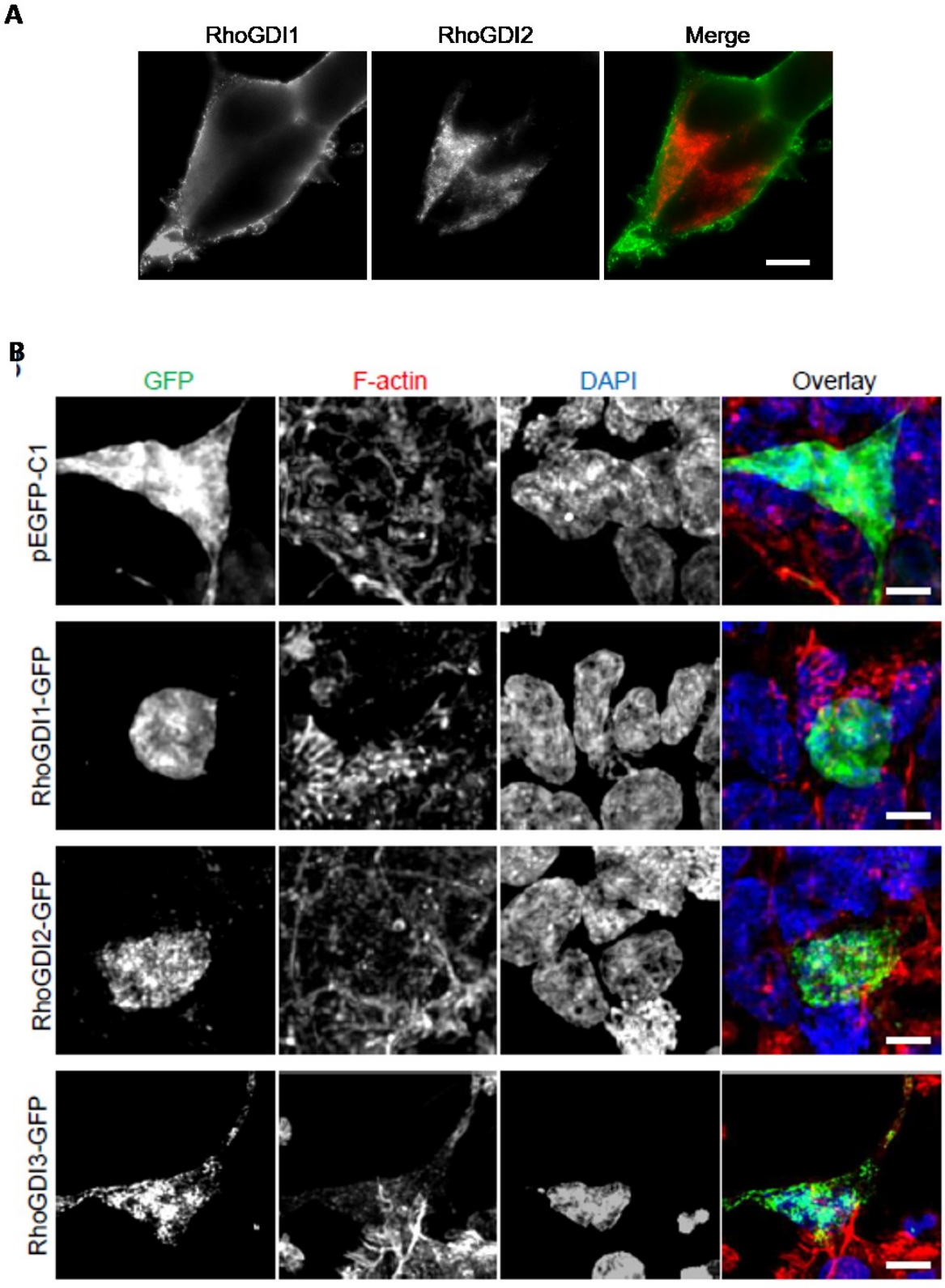
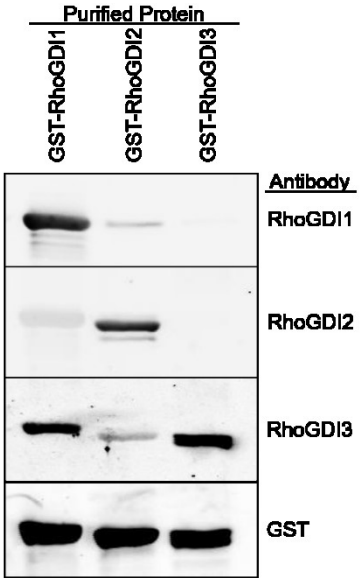
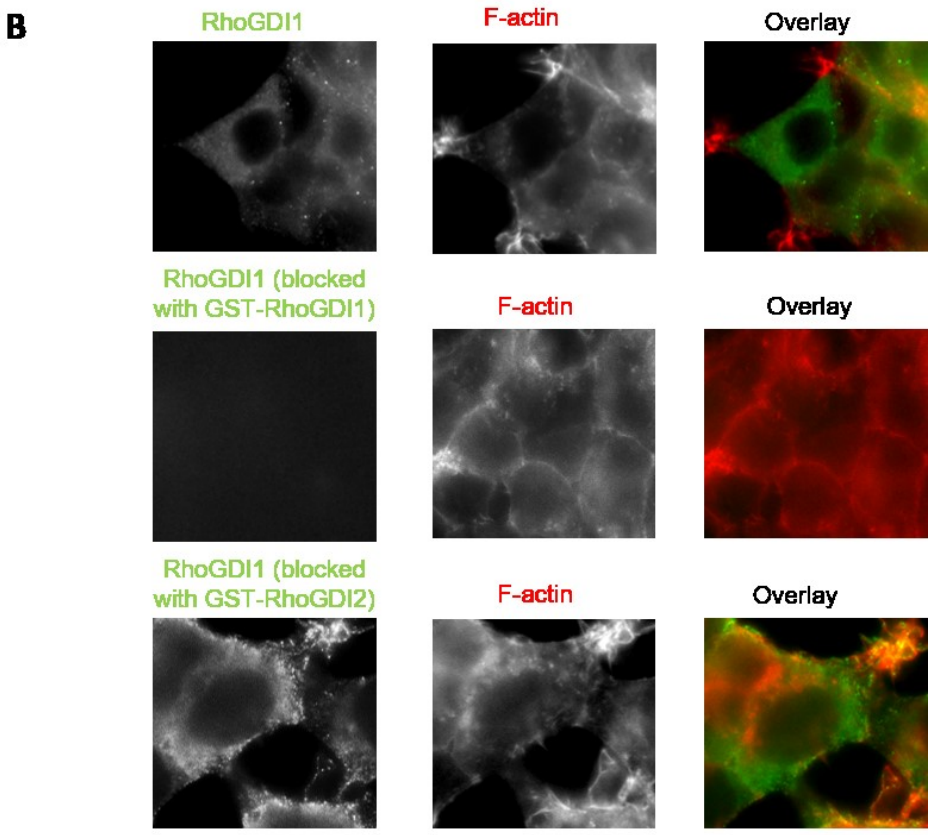

A
RhoGDI1
RhoGDI2
Merge
B
GFP
F-actin
DAPI
Overlay
pEGFP-C1
RhoGDI1-GFP
RhoGDI2-GFP
RhoGDI3-GFP

Figure 4.5 RhoGDI1 and RhoGDI2 display distinct subcellular localization (A) Representative immunofluorescence microscopy images of RhoGDI1 and RhoGDI2 in HEK293T cells (n=6). RhoGDI1 shows diffuse cytoplasmic staining, while RhoGDI2 displays punctate localization in the peri-nuclear region of the cells. (B) Representative confocal images of HEK293T cells transfected with plasmids expressing RhoGDI with C-terminal GFP as indicated. The cellular distribution of RhoGDI are distinct with RhoGDI1 appearing cytosolic similar to GFP alone while RhoGDI2 and RhoGDI3 show distinct membrane association. *Bar* indicates 10 μm.

A


Purified Protein
GST-RhoGDI1
GST-RhoGDI2
GST-RhoGDI3
Antibody
RhoGDI1
RhoGDI2
RhoGDI3
GST


B
RhoGDI1
F-actin
Overlay
RhoGDI1 (blocked with GST-RhoGDI1)
F-actin
Overlay
RhoGDI1 (blocked with GST-RhoGDI2)
F-actin
Overlay

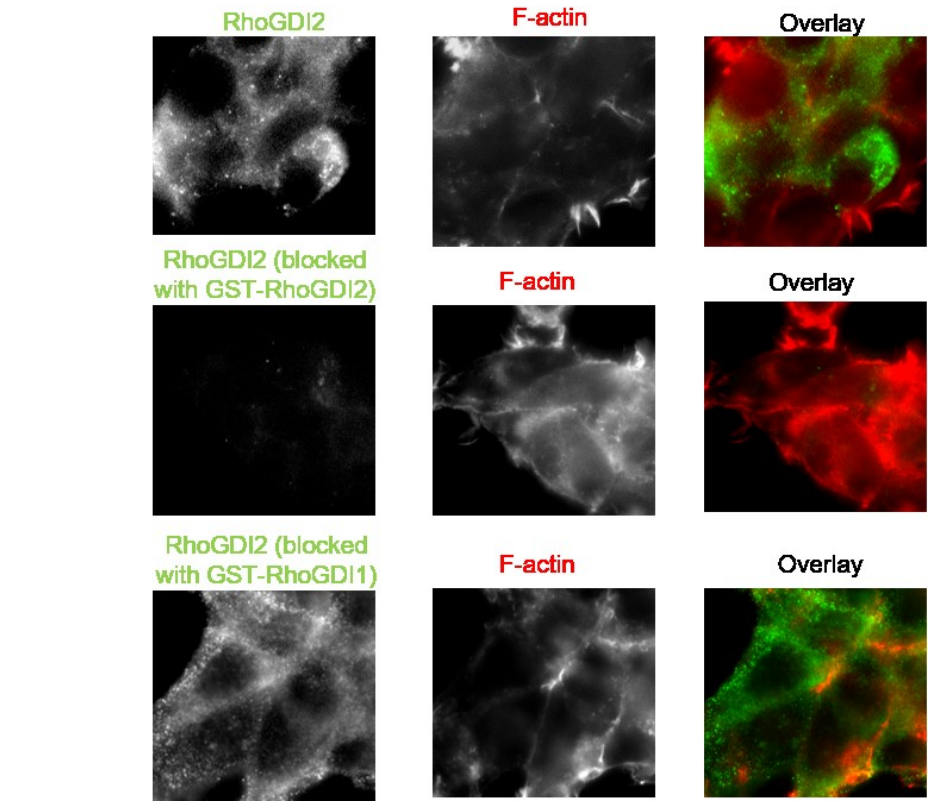
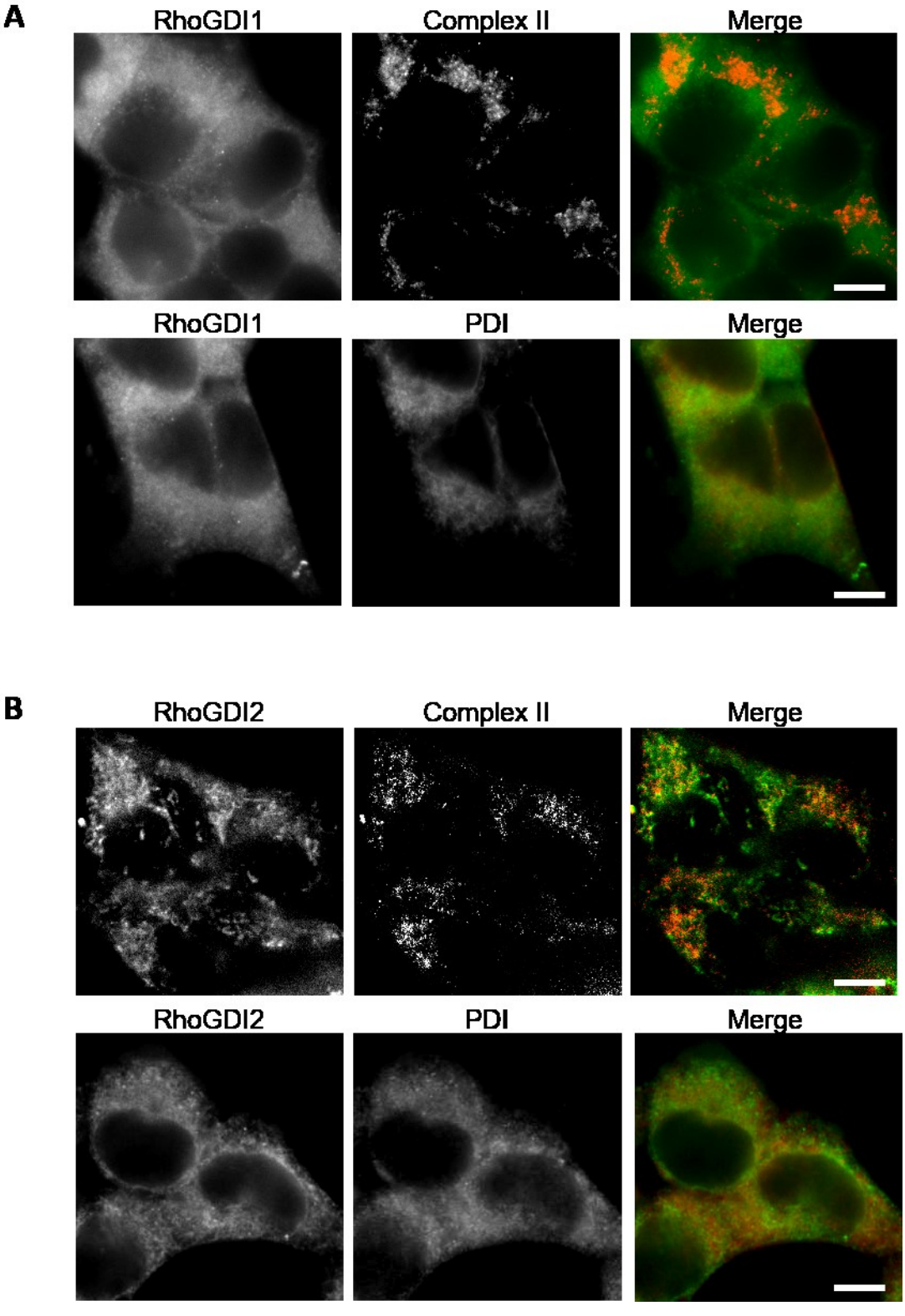
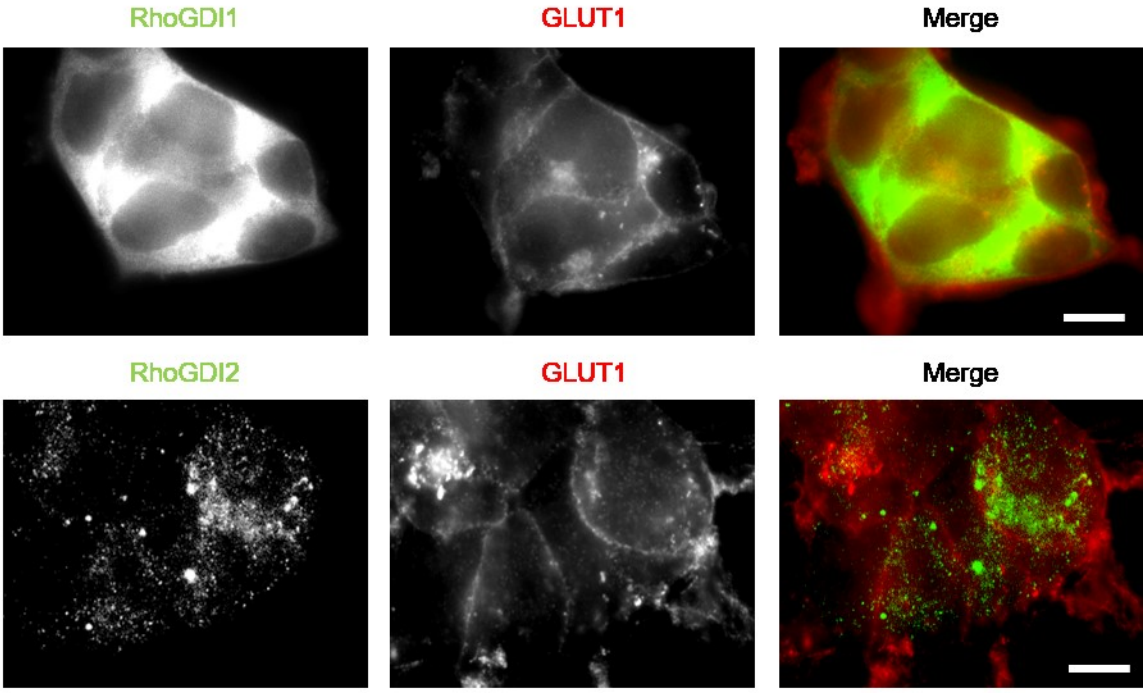

C
RhoGDI2
F-actin
Overlay
RhoGDI2 (blocked with GST-RhoGDI2)
F-actin
Overlay
RhoGDI2 (blocked with GST-RhoGDI1)
F-actin
Overlay

Figure 4.6 Specificity of RhoGDI1, RhoGDI2, and RhoGDI3 antibodies. (A) RhoGDIs were cloned into pGEX-4T1, expressed and purified from E. coli. Immunoblots used commercially available antibodies to RhoGDIs and GST as indicated in *Chapter 2.* (B) Representative immunofluorescence images of HEK-293T cells testing the specificity of RhoGDI1 and RhoGDI2 immunofluorescent labeling. Antibodies used for immunofluorescence detection of endogenous RhoGDI1 and RhoGDI2 were preincubated with either their corresponding protein or with the opposite RhoGDI. Immunofluorescent signals were only blocked when antibodies were preincubated with their corresponding proteins which shows that they specificity of detection (n=3)


A
RhoGDI1
Complex II
Merge
RhoGDI1
PDI
Merge
B
RhoGDI2
Complex II
Merge
RhoGDI2
PDI
Merge

C


RhoGDI1
GLUT1
Merge
RhoGDI2
GLUT1
Merge

D

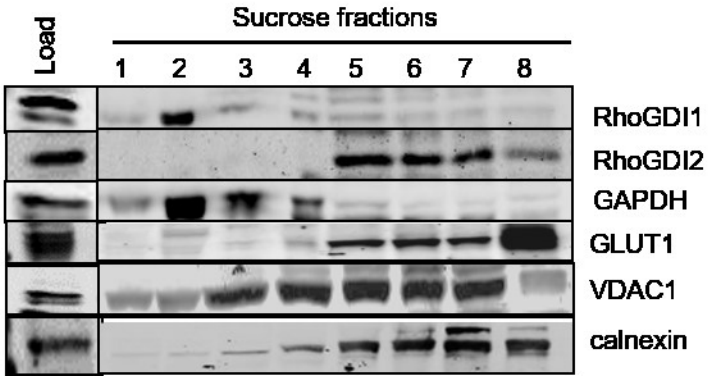

Load
Sucrose fractions
1
2
3
4
5
6
7
8
RhoGDI1
RhoGDI2
GAPDH
GLUT1
VDAC1
calnexin

Figure 4.7 Co-localization of RhoGDI1 and RhoGDI2 with ER and mitochondria markers (A and B) Representative immunofluorescence microscopy images of HEK293T cells stained with antibodies against RhoGDI1 and RhoGDI2, co-stained with (A) ER (*PDI1*), (B) mitochondrial (*Complex II*) and (C) plasma membrane (*GLUT1*) markers (n=3). RhoGDI1 shows cytoplasmic staining and RhoGDI2 displays punctate localization in the peri-nuclear region of the cells. GLUT1 staining at the cell periphery and endosome is distinct from both RhoGDI1 and RhoGDI2. *Bar* indicates 10 μm. (D) Immunoblot analysis of sucrose gradient fractionation of HEK293T cell lysate (n=3). Load represents 10% of input. Blots were probed for RhoGDI1, RhoGDI2, GAPDH (cytoplasmic marker), GLUT1 (plasma membrane marker), VDAC1 (mitochondrial membrane marker) and calnexin (ER marker).

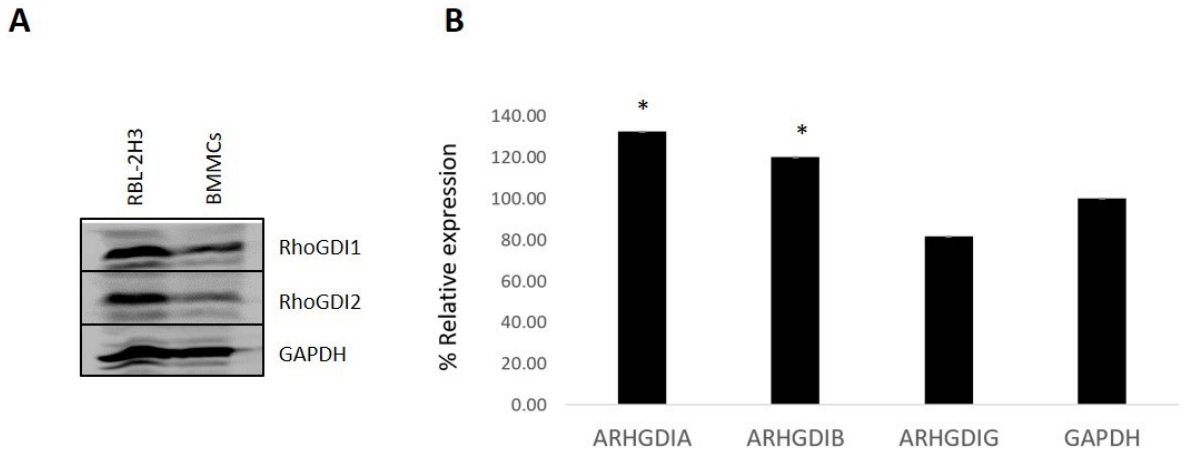


Figure 4.8 Expression of RhoGDIs by RBL-2H3

(A) RBL-2H3 cells and bone marrow derived MCs (BMMCs) lysates were immunoblotted and probed for the expression of RhoGDI1, RhoGDI2 and GAPDH (n=1) (B) A q-PCR analysis was also performed on cDNA generated from resting RBL-2H3 mRNA to identify the mRNA expression of RhoGDIs (n=3). GAPDH was also quantified as an internal control. Statistical comparison with GAPDH was done by paired Student's t-test (*p*-values: *, $p < 0.05$;).

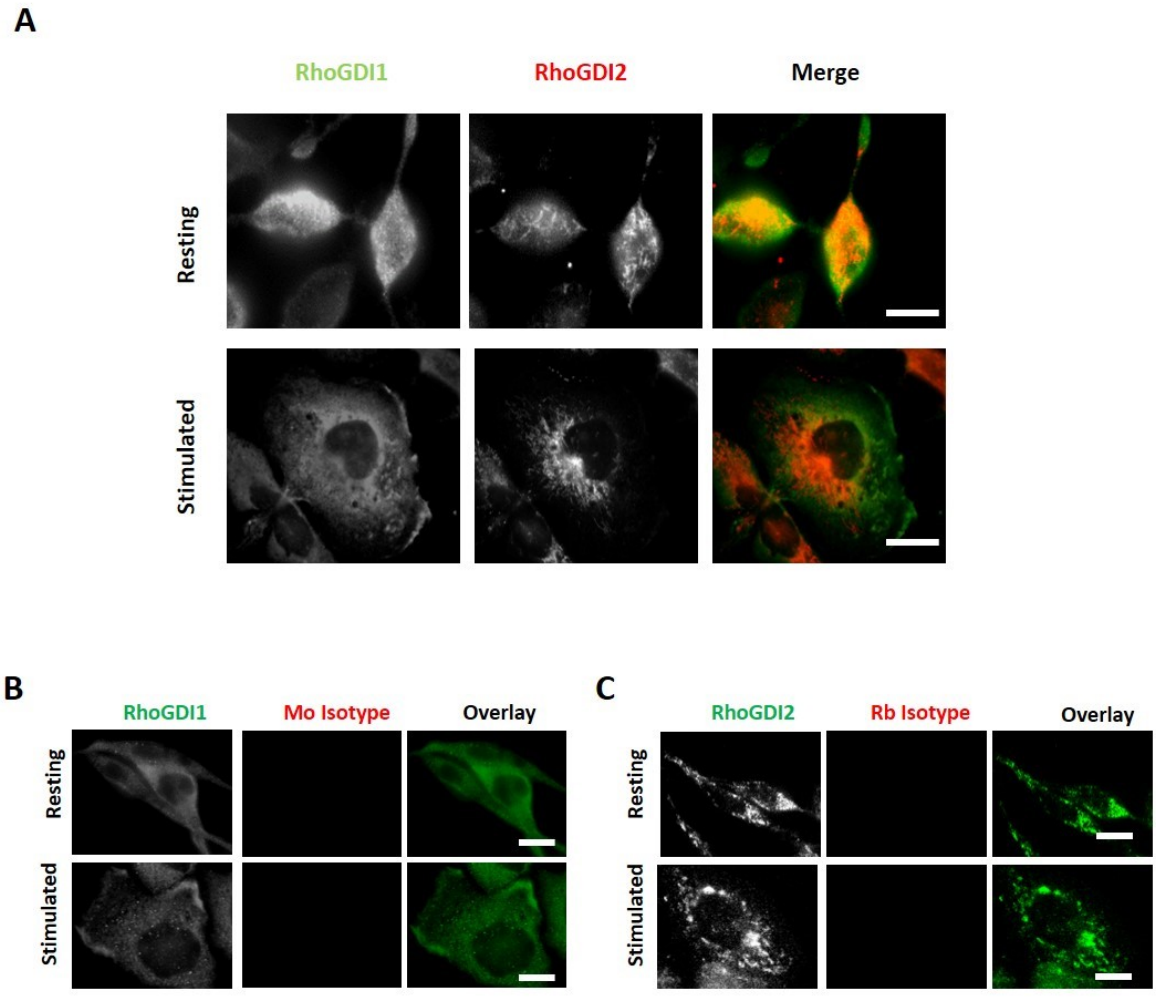
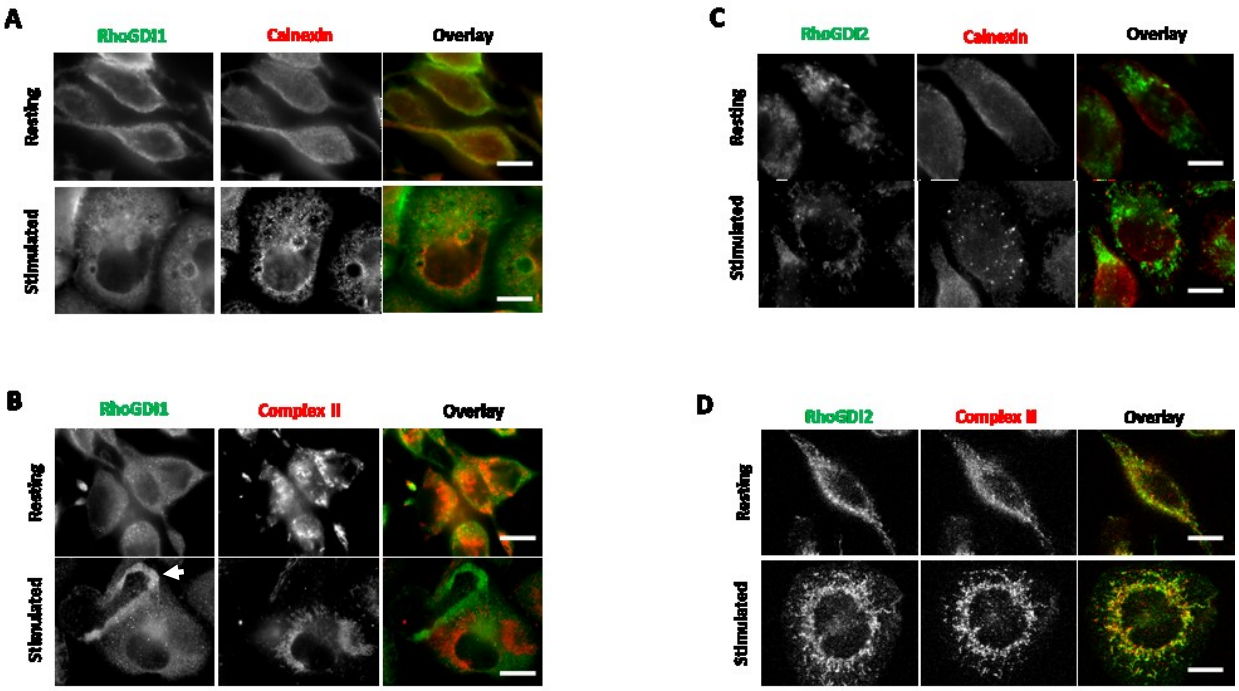


Figure 4.9 RhoGDI1 and RhoGDI2 display distinct localization in FcεRI stimulated RBL-2H3

Representative images of RBL-2H3 that were either left resting or sensitized and stimulated with Ag for 10 min. Cells were fixed in 4% paraformaldehyde and probed for (A) RhoGDI1 and RhoGDI2. (B and C) Fixed RBL-2H3 were probed with mouse and rabbit isotype controls and RhoGDI antibodies. Bar = 10 μm; n=2


A
RhoGDI1
Calnexin
Overlay
Resting
Stimulated
C
RhoGDI2
Calnexin
Overlay
Resting
Stimulated
B
RhoGDI1
Complex II
Overlay
Resting
Stimulated
D
RhoGDI2
Complex II
Overlay
Resting
Stimulated

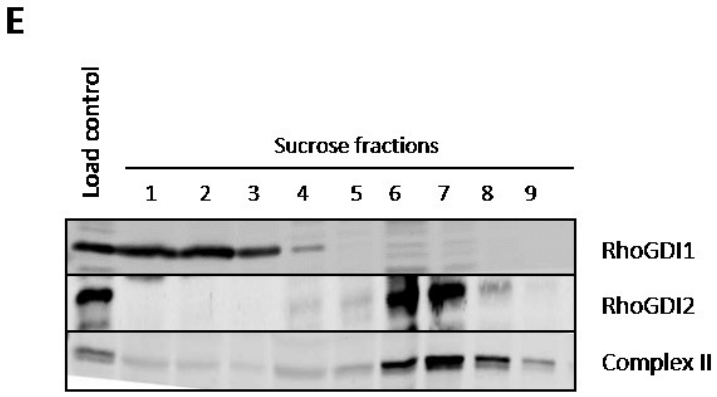

E
Load control
Sucrose fractions
1 2 3 4 5 6 7 8 9
RhoGDI1
RhoGDI2
Complex II

Figure 4.10 RhoGDI2, but not RhoGDI1, may potentially be associated with mitochondria

Representative immunofluorescence microscopy images of fixed resting and stimulated RBL-2H3. IgE sensitized RBL-2H3 cells were left resting or stimulated with Ag for 10 min and fixed with 4% PFA. Cells were probed for RhoGDIs and ER (calnexin) or mitochondria marker (complex II). RhoGDI1 showed cytoplasmic staining and did not colocalize with (A) ER or (B) mitochondria markers. RhoGDI2 showed punctate staining that was similar in its distribution to (D) mitochondria marker (complex II), but not (C) ER marker (calnexin). Bar = 10 μm. n=2. (E) To further assess whether RhoGDI2 is potentially associated with mitochondria membrane, we performed a density centrifugation of RBL-2H3 cell lysates and immunoblotted for RhoGDIs and mitochondria marker (complex II). RhoGDI1 appears in the lighter fractions, whereas, RhoGDI2 appears in the heavier fractions; however, RhoGDi2 does not fully colocalize with complex II.

Chapter 5 Conclusion and Future Directions

Chapter 5 Conclusion and Future Directions

MCs are found embedded in virtually every tissue. They have essential roles in the initiation, development, and regulation of innate and acquired immunity [10, 12, 30]. The vast diversity of immune mediators released by MCs allows them to regulate complex immune responses, making them versatile and robust immunoregulatory cells [15, 26, 231]. They can be activated by numerous pattern recognition receptors [15]. The high affinity IgE receptor, FcεRI, signaling is often associated with allergic and autoimmune responses and is well studied [35, 37, 47, 52, 67, 232]. Activation of FcεRI induces the release of histamine, enzymes, proteases and cytokines by a process known as degranulation or exocytosis. Aberrant MC activation, as seen in allergies, can lead to chronic inflammation, tissue remodeling, organ dysfunction and ultimately death. Therefore, understanding the basic biology of MC activation and degranulation can reveal novel ways for therapeutics.

Downstream of FcεRI, a coordinated signaling pathway consisting of kinases, phosphatases, small GTPases, lipid modifying enzymes, transcription factors and cytoskeletal elements is activated to induce the release of pre-formed immune mediators stored in granules [34, 37, 173, 233]. Additionally, MCs also newly synthesize cytokines and chemokines that take part in the late stage allergic inflammation. Interestingly, the release of pre-formed mediators occurs in a selective manner and depends on the type of immune stimuli. For example, MCs respond to lipopolysaccharide (LPS) by releasing cytokines and chemokines but not proteases [15]. This allows MCs to be, as Galli et al., suggested, ‘tunable’ effectors of the immune system [30]. However, there are still large gaps in our knowledge of MC activation, machinery involved in

mediator release and the multitude of proteins that regulate these. Unravelling this complex network may reveal common pathways essential for degranulation; these pathways can then be strategically targeted to reduce MC-mediated inflammation.

Rho GTPases are activated downstream of FcεRI [160]. They regulate many cellular processes like survival, proliferation, differentiation, migration and exocytosis; however, they are most well known for their role in regulating actin cytoskeleton [85, 87, 222]. There are 20 Rho GTPases expressed by the human genome and they are broadly classified into three main subgroups: Rho, Cdc42 and Rac. Although these subfamily proteins are highly conserved, they have been demonstrated to control distinct events in the cell. For example, Rac1 is needed for neutrophil chemoattractant gradient detection and orientation toward the source [234] whilst Rac2 regulates neutrophil migration, adhesion and even survival [235]. This indicates that Rho subfamily members possess unique and essential roles in immune cell survival and function. Our understanding of Rho functions in immune cells stems from the use of knockout mice and constitutively active and dominant negative mutants. However, as described recently the expression of exogenous Rho proteins can perturb Rho signaling by interfering with the stability of endogenous Rho proteins and preventing crosstalk between subfamily members [130]. This crosstalk is an essential feature that ensures appropriate cell signaling. This is demonstrated during migration where a reciprocal balance between Rac and RhoA exists [130]. Rac1 was shown to directly antagonize RhoA activation to determine cell morphology and migratory behaviour. It is possible that the use of mutant Rho proteins may have masked these subtle but important crosstalk events, which may have distorted our understanding of Rho functions.

By acutely inhibiting Rho activation in FcεRI-stimulated MCs, we identified that there is a balance between Rac and RhoA (*Chapter 3*). After antigen stimulation, they are activated at two different timepoints and they regulate two distinct events i.e., actin-driven morphological changes and microtubule-driven granule trafficking, respectively (*Chapter 3, Figures 3.5-3.8*). Inhibition of either Rho protein significantly affects MC degranulation (*Chapter 3, Figure 3.3*). This, along with previous research showing Rac1 and Rac2 regulate distinct events during MC degranulation [156], confirms that the Rho subfamily members have unique and essential roles in MC activation and function. Therefore, Rho signaling could be a viewed as a druggable target for MC-mediated allergic inflammation to obtain highly selective effects.

The selective activation of Rho proteins at different time intervals after stimulation indicates the tight level of coordination that controls Rho signaling. These involve the well defined three classes of regulators: RhoGEFs, RhoGAPs and RhoGDIs, as well as a multitude of kinases, phosphatases, ubiquitin transferase and acetyl transferases [96, 117, 122, 159, 222, 236]. Therefore, validating efficacious and safe targets to prevent abnormal Rho signaling is an enormous task and requires a systematic approach.

Unlike the other vast families of regulators, RhoGDIs offer a potential way of to alter Rho GTPase function. They are recognized for their ability to regulate spatial and temporal Rho signaling by not just interacting with Rho GTPase and sequestering them in the cytosol, but also by controlling Rho activation, subcellular localization, stability and crosstalk. Intriguingly, RhoGDI subfamily members seem to have non-redundant roles in cancer migration [136, 137, 143-145, 237]. RhoGDIs are frequently upregulated in aggressive cancers but they have

confounding effects on invasiveness depending on the type of cancer. For example, RhoGDI2 overexpression increases invasiveness in gastric cancer [144] but has the opposite effect in bladder cancers [237]. This indicates that RhoGDI functions depends on the cell context and is influenced by other signaling events, eventually, leading to distinct outcomes. Therefore, elucidating RhoGDI family of proteins may validate them as potential targets to prevent aberrant Rho signaling.

In *Chapter 4*, we characterized the functional, spatial, and evolutionary properties of the three mammalian RhoGDIs. Phylogenetic analysis showed that RhoGDI1 is present in all eukaryotes, whereas, RhoGDI2/3 may have emerged as a single ancestral hybrid gene after RhoGDI1 duplication in vertebrates. This may have also conferred RhoGDI2 and RhoGDI3 with distinction of their functions. Remarkably, the duplications of RhoGDIs near the same timepoint in early vertebrate evolution does not correlate with the evolution of Rho GTPase [84, 212]. This indicates that the RhoGDIs may have arisen to regulate complex functions in cell via the regulation of pre-existing Rho GTPases.

In line with these observations, protein binding studies showed that RhoGDI1 bound to Rho, Rac and Cdc42, whereas, RhoGDI2 and RhoGDI3 showed preferential Rac interaction. This distinct binding preferences appear to be driven by the divergent N-termini of RhoGDIs. Further work using C-terminal truncated mutants can characterize the N-terminal driven distinct binding preferences of RhoGDIs. Additionally, the role of post-translational modifications (PTMs) like lipid modifications or phosphorylation in the interactions cannot be ruled out. Co-immunoprecipitation of RhoGDIs at different MC activation states can reveal the dynamic

binding partners of RhoGDIs. These may include different Rho subfamily members, kinases, phosphatases, RhoGEFs and even RhoGAPs [131, 150, 153, 227, 238, 239].

The regulation of association/dissociation of Rho GTPase in complex with RhoGDIs are still unclear. Numerous dissociation factors and mechanisms have been suggested [116], however, not many of these have been characterized in immune cell survival and function. The regulatory network of the association/dissociation of Rho complexes hold enormous promise for therapeutic purposes to prevent unnecessary activation of Rho GTPases and in turn, immune cell functions. Further research on this complex formation and regulation can potentially reveal the following avenues to prevent Rho signaling: 1) drugs that prevent the dissociation of Rho GTPases from their complex with RhoGDIs, akin to brefeldin A, that locks Arf GEF in complex with Arf GTPase [240]; 2) kinases that phosphorylate RhoGDIs to induce dissociation; 3) phosphatases that dephosphorylate Rho GTPases to reduce their affinity to RhoGDIs; 4) dissociation factors like Ezrin, Radixin and Moesin (ERM proteins) that may play a role in appropriate subcellular localization of Rho GTPase [149, 241]; and 5) RhoGEFs and RhoGAPs.

Finally, the distinct subcellular localization of RhoGDIs clearly must play a role in the spatial regulation of Rho signaling. Whether RhoGDI subcellular locations correlates with enrichment of specific Rho proteins in these locations is unclear. However, this may confer unique roles of Rho subfamily members during MC degranulation. Additionally, these distinct subcellular locations of RhoGDIs may also provide avenues for more complex regulation of the balance between Rho subfamily members (see model, **Figure 5.1**). For example, RhoGDI3 regulates pancreatic cell survival and invasiveness via RhoG and RhoB at the Golgi [136]. Further work on

the distinct roles of RhoGDI1 and RhoGDI2 in immune cell functions is needed to validate them as appropriate therapeutic targets that can be used in MC-mediated inflammatory disorders.

Our studies on the activation and regulation of Rho GTPases during MC activation reveals a highly coordinated signaling event that regulates distinct cellular processes. Rac-driven morphological changes and RhoA-driven microtubule extension play a role in creation of degranulation zones and controlled streaming of granules to these active sites. This highlights the unique and essential roles of Rho subfamily members in MC degranulation. Furthermore, we have elucidated the RhoGDI family of proteins in their regulation of Rho GTPase. We identified RhoGDI members possess distinct functional, spatial and biochemical properties. These properties appear to be evolutionarily conserved in vertebrates. We are now investigating the biological roles of RhoGDI1 and RhoGDI2 in stimulated MC responses. Further research on the role of Rho proteins and their regulators in the selective release of immune mediators may reveal how MCs can initiate, orchestrate and resolve complex immune responses.

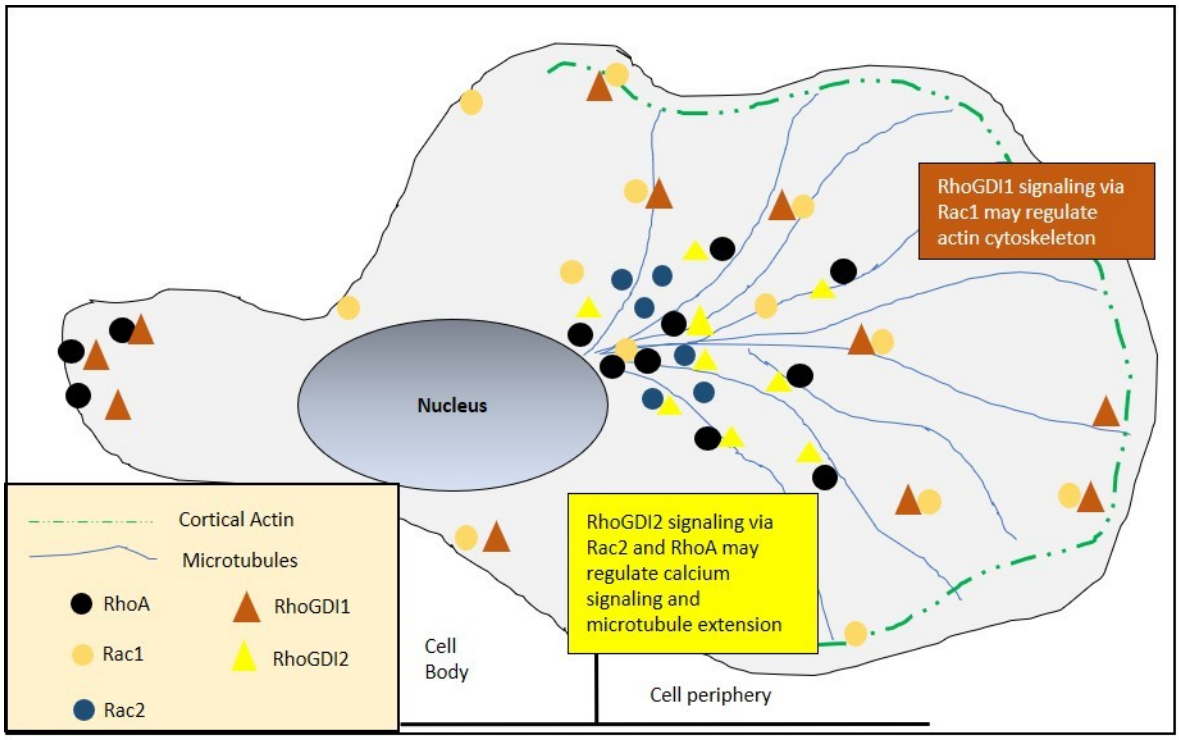


Figure 5.1 RhoGDI signaling regulates MC degranulation via RhoA and Rac

FcεRI-stimulation of MCs results in the redistribution of RhoGDI::Rho GTPase complexes to distinct locations in the cell. Upon antigen stimulation, RhoGDI1 via Rac1 may regulate actin rich peripheral protrusions which are potentially the degranulation zones and both RhoGDIs may regulate Rac2 and RhoA to induce calcium signaling, microtubule extension and granule trafficking.

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R. J. (2006) Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* **442**, 997.

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