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**THE ROLE OF Rhod IN ACTIN DYNAMICS,
RECEPTOR TRAFFICKING AND CANCER CELL
MIGRATION**

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Dedicated to my grandfather...

*Gone though you have, I heard your voice today,
I tried to make out what the words might mean,
Like something seen half-clearly on a screen,
Gone since you have, grief too in time will go,
Or share space with old joy; it must be so,
Rest then in peace, but spare some elation,
Death cannot put down every conversation,
Over and out, as you once used to say,
Not on your life. You're in these lines to stay.
(With permission from, The Guardian*)*

ABSTRACT

The Ras subfamily of Rho GTPases plays an important role in myriads of cell processes, such as actin cytoskeleton dynamics, membrane trafficking and cell migration. Additionally, they also participate in diverse signal transduction pathways that regulate gene transcription, cell survival and cell growth. The ability of these proteins to function as a signaling molecule depends on their capacity to cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. Till date, three Rho family members namely, RhoA, Rac1 and Cdc42 have been well studied as compared to other Rho family members.

This thesis highlights a less studied Rho GTPase, RhoD, which together with Rif constitutes a separate subgroup of the Rho GTPases. Ectopic expression of RhoD and Rif has a dramatic effect on the organization of the actin filament system observed as long flexible filopodia protrusions and the formation of short bundles of actin filaments. Moreover, RhoD has a role in regulating endosome dynamics and is a negative regulator of cell motility. This motivated us to find out more about the signaling pathways downstream of RhoD. We initiated our studies by identifying a number of novel binding partners for RhoD for instance, FILIP1, WHAMM, Rabankyrin-5 and the ZIP kinase. We observed that RhoD together with FILIP1 and WHAMM had a distinct role in actin dynamics, cell adhesion and cell migration as compared to the better studied members of the Rho subfamily. Furthermore, with the same RhoD binding partners, we elucidated another regulatory role of RhoD in Golgi homeostasis and ER-to-Golgi transport measured by VSV-G protein transport assay. Previous studies have identified Rabankyrin-5 as a Rab5 effector. Interestingly, these studies also reported that Rabankyrin-5 localizes to early endosomes and to macropinosomes of epithelial cells. We found that Rabankyrin-5 participates in coordinating Rab5 and RhoD in endosome trafficking. We describe a novel mechanism by which RhoD, Rab5 and Rabankyrin-5 coordinate membrane trafficking events and endocytosis, for instance during the internalization of activated tyrosine kinase receptor, such as the PDGFR β . Finally, we found that RhoD modulates focal adhesion dynamics and actin filament assembly through a novel effector, ZIP kinase. In summary, this thesis sheds light on the less studied Rho GTPase RhoD and provides novel insight into the mechanisms underlying its diverse cellular effects.

LIST OF PUBLICATIONS

This thesis is based on the following papers that are enclosed at the end.

- I. Gad AKB, **Nehru V**[§], Ruusala A, Aspenström P.
RhoD regulates cytoskeletal dynamics via the actin nucleation-promoting factor WASp homologue associated with actin Golgi membranes and microtubules.
Mol Biol Cell. 23 (24): 4807-19.
- II. **Nehru V**^{*}, Yagi H, Sato M, Aspenström P.
RhoD regulates ER to Golgi transport through its effectors Filamin A-binding protein, FILIP1, and WHAMM.
Manuscript.
- III. **Nehru V**^{*}, Aspenström P.
RhoD binds the Rab5 effector Rabankyrin-5 and has a role in trafficking of receptor tyrosine kinases.
Under revision in the journal-Traffic
- IV. **Nehru V**^{*}, Almeida FN, Aspenström P.
Interaction of RhoD and ZIP kinase modulates actin filament assembly and focal adhesion dynamics.
Biochem Biophys Res Commun. 2013 (In Press).

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LIST OF ABBREVIATIONS

Arf	ADP-ribosylation factor
Arp	actin-related protein
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
Cdc42	cell division control protein 42
CRIB	Cdc42/Rac interactive binding
Dbl	diffuse B-cell lymphoma
DH	Dbl-homologous
DNA	deoxyribonucleic acid
DOCK 180	dictator of cytokinesis 180
DRF	diaphanous-related formin
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FHOS	formin homologue overexpressed in spleen
FILIP1	filamin-A interacting protein
FTase	farnesyl transferase
FRL	formin-related gene in leukocytes
GAP	GTPase-activating protein
GDP	Guanosine-5'-diphosphate
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GGTase	geranylgeranyl-transferase I
GTP	Guanosine-5'-triphosphate
hDia	human Diaphanous-related formin
HNSCC	head and neck squamous cell carcinoma
JMY	junction-mediating and-regulatory protein
KDa	kilodalton
MAP	microtubule associated protein
mDia	mouse Diaphanous-related formin
Miro	mitochondrial Rho
MLC	myosin light chain
MLCK	myosin light-chain kinase
MR	mental retardation

NSCLC	non-small cell lung cancer
NPF	nucleation promoting factor
OSCC	oral squamous cell carcinoma
PDGF	platelet-derived growth factor
PH	pleckstrin homology
Ran	ras like nuclear protein
Ras	ras sarcoma viral oncogene homolog
Rho	ras homologous
RNAi	RNA interference
ROCK	rho-associated coiled coil-containing protein kinase
SFKs	src family kinases
SMC	structural maintenance of chromosome domain
TRITC	tetramethyl rhodamine isothiocyanate
WHAMM	WASP homolog associated with actin, membrane and microtubules
WASp	Wiskott-Aldrich Syndrome protein
WAVE	WASP family Verprolin-homologous protein
ZIPk	zipper interacting protein kinase
PAK	p21-activated kinase
MAPK	mitogen-activated protein kinase
MRCK	myotonic dystrophy kinase-related Cdc42-binding kinase
LIMK	LIM domain kinase
BMS	bristol-Meyers Squibb

1. INTRODUCTION

1.1 Synopsis

Just as a skeleton maintains shape and posture of a human body, likewise, all eukaryotic cells also possess a scaffold or cytoskeleton. This is a complex yet vital network of filamentous proteins that transverse through the crowded cytoplasmic environment, giving cells shape and framework in order to accomplish vital cellular processes via the signals emanating from outside the cell to the cell interior.

The Rho subfamily of GTPases is a family of signaling proteins that belongs to the Ras superfamily of small GTPases. They cycle between an active GTP-bound state and an inactive GDP-bound state. This cycling is tightly controlled by sets of proteins namely, guanine nucleotide exchange factors (GEFs) that catalyze nucleotide exchange by virtue of GDP dissociation and GTP association, resulting in the activation of a Rho GTPase. On the other hand, GTPase activating proteins (GAPs) play a role by enhancing the intrinsic GTPase activity that results in the hydrolysis of GTP and thereby inactivating a GTPase (Figure 2) [1,2,3]. This way, the small GTPases can function as molecular switches.

The Rho GTPases regulate diverse cellular processes in their active conformation and they carry out their functions mainly by binding to the effector proteins. They are called “effectors”, because they bind to the GTP-bound form of the GTPases and are able to transduce signals from the activated Rho GTPases to downstream cellular responses. Till date, many effectors for Rho GTPases have been identified. These include protein kinases (i.e., tyrosine kinases, serine/threonine kinases), scaffold proteins and actin regulating proteins (e.g. WASP, formins, etc). It appears that the major role of Rho GTPases is to recruit effector proteins to a particular target site intracellularly where they can execute their functions (Figure 2) [4].

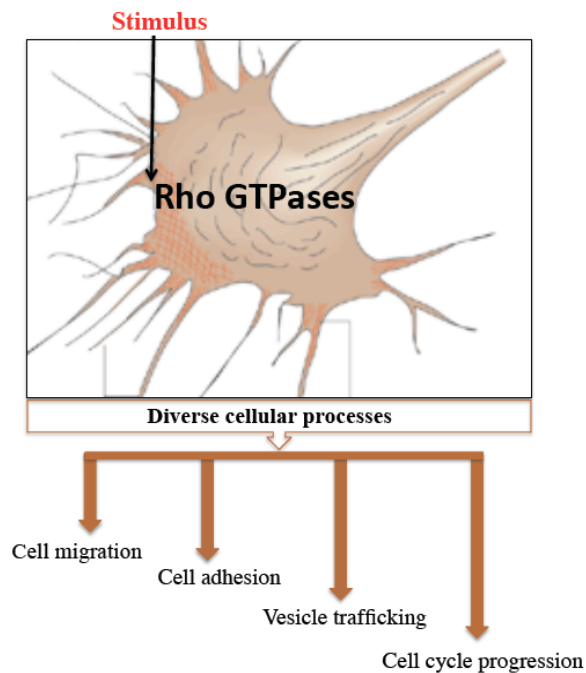


Figure 1. Rho GTPases regulate basic cellular processes [adapted from source 5].

The focus of this thesis is on a less studied member of Rho subfamily of GTPases known as RhoD. Together with the related GTPase Rif, it constitutes a unique subgroup of the classical Rho GTPases. My studies show that RhoD has profound effects via the effector proteins isolated during this thesis work, on the regulation of the actin filament system, ER-to-Golgi transport, receptor trafficking and cell migration.

1.2 The Ras GTPases

The “Ras” stands for **Rat sarcoma**, indicating how the first member of the protein family was identified. In 1964, Jennifer Harvey and Werner Kirsten first observed a preparation of murine leukaemia virus isolated from leukemic rats that induced sarcomas in newly born litters [6,7,8,9,10]. However, the genes involved in inducing sarcomas in rodent litters were shrouded in mystery until 1975, when Edward Scolnick at the National Cancer Institute, USA identified and described the first two *ras* genes, *H-RAS* and *K-RAS* from the seminal studies on the two murine sarcoma viruses identified by Harvey and Kirsten [10,11,12,13,14]. Subsequently, in 1979 the first human *ras* genes were identified and confirmed by three independent research laboratories: one headed by Robert Weinberg at the Massachusetts Institute of Technology, one by Geoffrey Cooper at the Harvard University, and one by Mariano Barbacid in collaboration with Stuart Aaronson lab at the National Institute of Health [10,15,16,17]. The third *ras* gene was identified in 1983 by research team headed by

Robin Weiss from the Institute of Cancer Research, UK and designated as *N-RAS* for its early identification in human neuroblastoma cells [18]. These three genes encoded similar proteins, comprising of around 189 amino acids (approximately 21KDa) of which the first 86 amino acids are identical in sequence including the effector binding site and homologous till amino acid residue 167 [19]. Point mutations in *Ras* genes render them GTPase deficient and thereby constitutively active converting them into an active oncogenes. The most common point mutation occurring in human cancer is a replacement of a glycine for a valine amino acid residue at position 12 of *K-Ras*. The mutation is frequently found in tumor biopsies in patients suffering from pancreatic adenocarcinomas, colon and lung cancers. It is therefore, well-established fact now that about 20-30 % of all human tumors have mutations in *Ras* oncogenes [20,21,22]. The human genome project has established that the *Ras* superfamily consists of over 150 members. These members have evolutionary conserved orthologues present in *Dictyostelium*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* as well as higher eukaryotes [23]. Based on protein sequence and functional similarities *Ras* superfamily has been further subdivided into six subfamilies summarized in (Table 1) [24].

Ras subfamilies	Cellular functions	Members (in humans)
Ras (Rat Sarcoma)	Regulates gene expression	36
Rho (Ras homologous)	Regulates actin organisation, cell cycle progression, gene expression	20
Rab (Ras-like protein in brain)	Regulates intracellular vesicle trafficking, mitotic spindle and nuclear envelope assembly	61
Ran (Ras-like nuclear protein)	Regulates nucleocytoplasmic transport of RNA and proteins	1
Arf (ADP-ribosylation factor)	Regulates vesicular transport	27
Miro (Mitochondrial Rho)	Regulates mitochondrial dynamics	2

Table1. The *Ras* protein superfamily. The *Ras* superfamily members are subdivided based on their protein sequences and functional similarities. It also highlights the role of small GTPases in diverse cellular processes.

1.3 Molecular regulation and localization of small GTPases

1.3.1. The Rho GTPases

The first gene encoding a Rho protein was isolated in 1985 by Pascal Madaule while working in Richard Axel's group, from a cDNA library obtained from abdominal ganglia of the mollusc, *Aplysia* [25]. It was identified with low stringency cDNA screen and named Rho for Ras homolog (now called RhoA). The protein encoded turned out to share 35% homology with H-Ras [25]. Subsequently, the group of Snyderman identified Rac1 and Rac2 in 1989 [26]. The third member of Rho family CDC42 was first identified in yeast as *S.cerevisiae* cell division-cycle 42 and subsequently by Polakis in 1989 [27]. Human Cdc42 shows 80% amino acid identity with yeast CDC42, 50% and 70% respectively with mammalian RhoA and Rac1 proteins [26]. Till date, 20 members of the Rho subfamily have been identified in mammals including the well studied RhoA, Rac1 and Cdc42 (Figure 3). As with Ras superfamily each Rho subfamily member shares a common highly conserved core G domain that is about 150 amino acids long and is liable for providing essential nucleotide exchange [1,25]. In contrast to the Ras superfamily, most members of the Rho family contain an additional α -helical structure comprising 12 amino acid residue and known as the insert region [1,2,3].

The Rho GTPases are also under the same GTP cycling regime as the Ras proteins but in addition to GEFs and GAPs, they are regulated by guanine nucleotide dissociation inhibitors (GDIs), which target the GDP-bound form of Rho GTPases thereby blocking the GTP/GDP exchange (Figure 2). The Rho GTPases can further be subdivided into classical and so-called atypical Rho GTPases (Figure 3). The latter proteins are not under the same regulatory scheme as the classical Rho GTPases. The atypical Rho GTPases harbor amino acid residues in their active sites, which render them constitutively GTP-bound. This suggests the presence of additional regulatory modes, such as post-translational modifications (i.e., phosphorylation or ubiquitylation) and/or regulation at the transcriptional level.

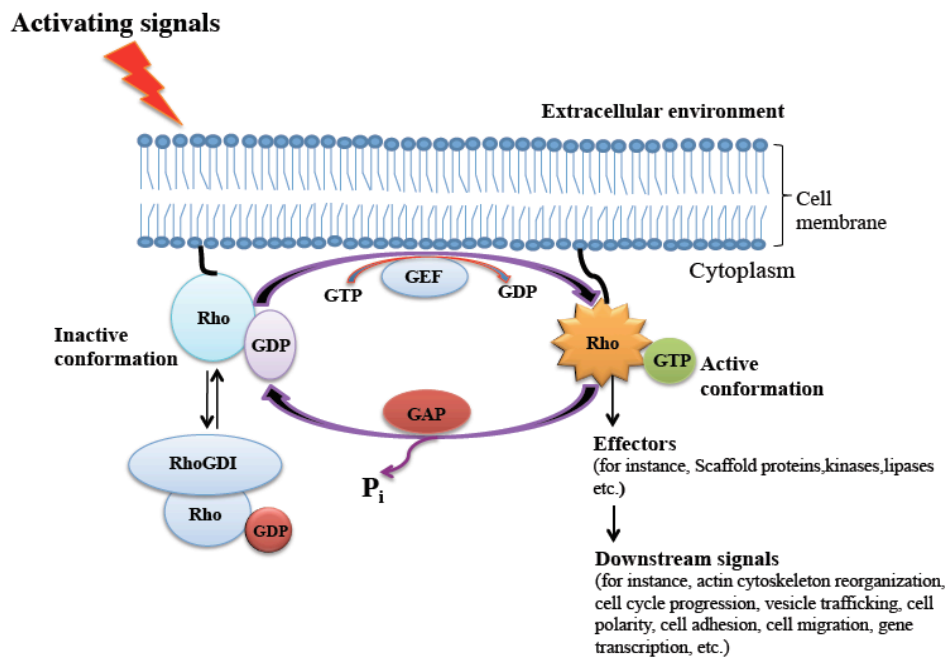


Figure 2. Rho GTPase regulation. Rho GTPases are regulated by GTPase cycle. Small GTPases that belong to classical Rho subfamily cycle between an inactive GDP-bound state and an active GTP-bound form. Active GTPases interact with effector proteins to mediate a cell response [adapted from source 1].

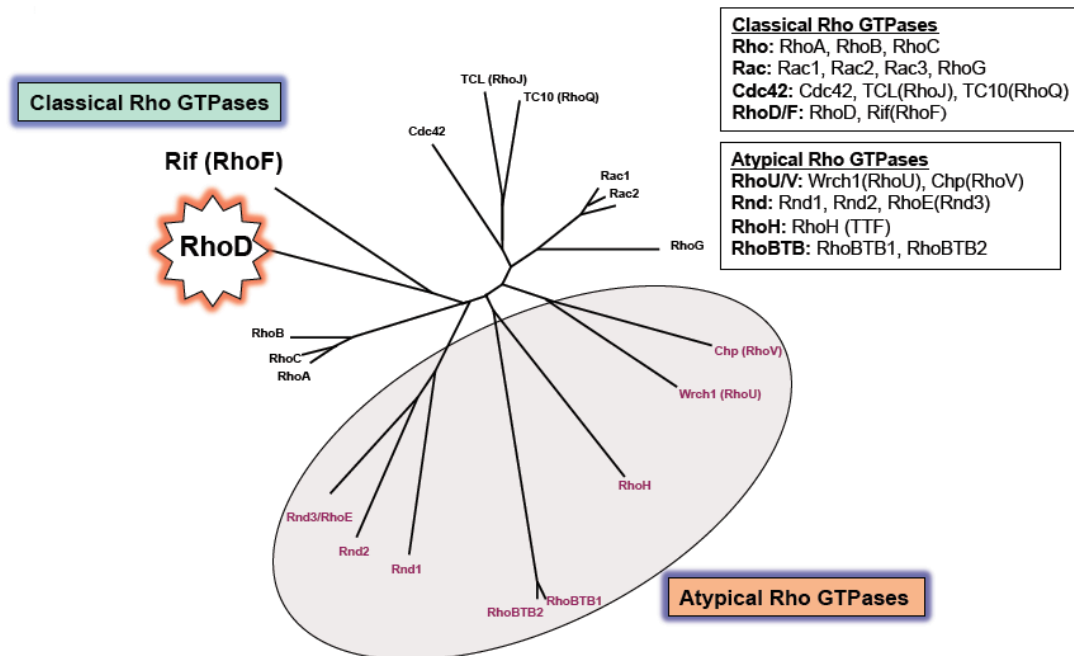


Figure 3. An unrooted dendritic tree represents the human Rho GTPases. The atypical Rho GTPases are encircled and labelled. The 20 members in humans are further divided into 8 families (in box top right) [adapted from source 24].

1.4 Regulation of Rho GTPases

1.4.1a. The RhoGEFs

RhoGEFs have a pivotal role in activating small GTPases by nucleotide exchange. Around 80 RhoGEFs exist in humans [28]. They have been further subdivided into two families, the Dbl family and the DOCK (**D**edicator **o**f **c**ytok**i**nesis) family [29,30,31]. These proteins are controlled by signals emanating from outside as well as inside a cell. The best understood example of RhoGEF is Vav. At the N-terminus of Vav, we find a calponin homology (CH) domain and the catalytic GEF module. On the other hand at the C-terminal region lies the zinc finger domain, proline-rich region, and SH2 domain flanked by two SH3 domains. Upon the deletion of first 66 amino acids from the N-terminus the Vav can be constitutively activated [32]. Vav is involved in downstream signaling of many receptors, for instance EGFR, PDGFR etc [33,34]. When the receptor is activated upon the stimuli, Vav is transiently phosphorylated intracellularly by Src and Syk members of tyrosine kinase family, resulting in stimulation of its catalytic activity [34,35,36,37,38,39].

Dbl RhoGEF family of proteins

The first RhoGEF isolated in mammals was the Dbl. It was isolated in 1985 as an oncogene in NIH3T3 cells by focus formation assay using DNA from a human **d**iffuse **B**-cell lymphoma [40,41]. Eventually, the amino acid sequence revealed similarity to *S. cerevisiae* cell-division-cycle 24 (CDC24) and it turned out to possess a potential to catalyze nucleotide exchange on Cdc42 *in vitro* [40]. A domain was found highly conserved between Dbl and CDC24. Subsequently, it was named as DH (**D**bl **h**omology) domain, constituting the core catalytic domain required for GEF activity [42]. Approximately, 70 proteins containing a DH domain have been identified in humans, however these proteins share little homology amongst each other with an exception of three **c**onserved **r**egions (CR1, CR2 and CR3) each about 10-30 amino acids long [29]. The DH proteins also possess a tandem pleckstrin homology (PH) domain adjacent to DH domain which is pivotal for intracellular targeting of DH-domain, the DH-PH unit is the least structural domain necessary to GEF activity *in vivo* (Figure 4) [43]. The PH domains are well known to interact with phosphorylated phosphoinositides (PIP₃) (Figure 4) [44]. Two possible functional roles have been suggested for PH domain, first, they could directly affect the catalytic activity of the DH domain; second, they could help target GEFs to their correct intracellular location.

An alternative function has also been suggested for the PH domain of Dbs, which is that it participates with the DH domain in Cdc42 binding primarily through interaction involving the switch II motif of the GTPase [45].

In addition to DH-PH domains, most GEFs possess some additional domains that are functionally involved in the regulation of the protein, like SH2 and SH3 domains, serine/threonine, tyrosine kinase domains and PDZ domains. These domains are necessary to link GEFs to upstream receptors and signaling molecules, as well as regulating the catalytic activity of the RhoGEFs [28].

Analysis of the three-dimensional structure of the RhoGEFs Sos1, Trio (DH1) and Tiam show that they are composed of a flattened, elongated bundle of 11 α -helices [46,47,48]. Two of these 11 helices, CR1 and CR3, are exposed on the surface of the DH domain and participate in the formation of the GTPase interaction pocket. GEFs bind to the GDP bound form and destabilize the GDP–GTPase complex while stabilizing a nucleotide-free reaction intermediate [49]. Since the intracellular concentration of GTP exceeds the concentration of GDP with a factor of 10, therefore released GDP is replaced with GTP, leading to activation of the small GTPase. Many RhoGEF members are specific for a particular Rho GTPase. For instance, Fgd1 and Fgd5 are specific for Cdc42 [50]. Other RhoGEFs may trigger the activation of several small GTPases; for instance, Vav1 acts on Cdc42, Rac1 and RhoA, whereas Dbl acts on RhoA and Cdc42 [42,51]. Surprisingly, of the 20 Rho GTPases known, the specificity in activation elicited by most known GEFs has only been investigated for Rho, Rac and Cdc42 subgroups.

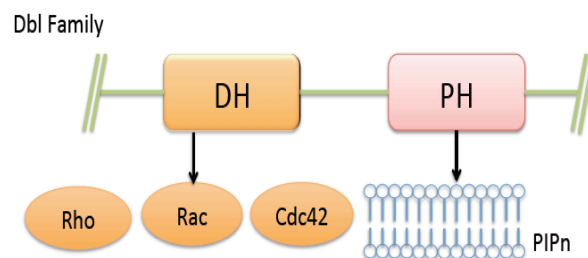


Figure 4. Schematic structure shows Dbl GEF family. Members of Dbl family contain DH catalytic domain adjacent to a phosphoinositide-binding PH domain [adapted from source 52].

DOCK RhoGEF family of proteins

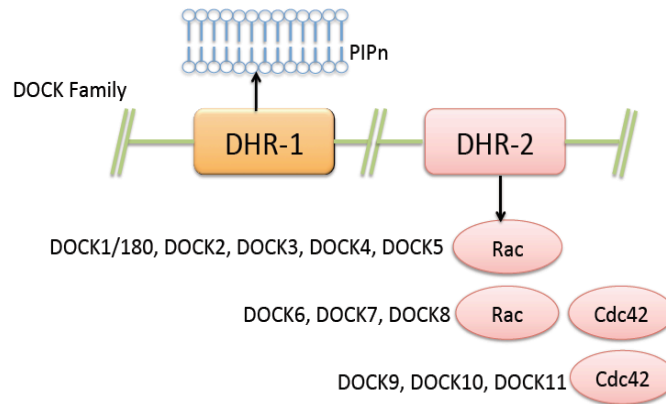


Figure 5. Schematic structure of DOCK family GEFs. The members of DOCK family contain DHR-1 and DHR-2 domain, which are structurally different from the Dbl family but functionally similar. DOCK1/180 till DOCK5 are Rac specific GEFs and DOCK6 uptill DOCK 8 are GEFs for Rac and Cdc42. From DOCK9 till DOCK11 bind nucleotide-free Cdc42 [adapted from source 52].

The DOCK gene was first cloned in 1996 as a gene product encoding a 180 KDa protein (hence the name, DOCK 180) interacting with proto-oncogene product c-Crk binding protein [30]. Together, DOCK 180 (nowadays called DOCK1) and its orthologue in *Drosophila melanogaster* and *Caenorhabditis elegans* form an evolutionarily conserved RhoGEF protein family sharing two highly conserved domains known as DHR-1 and DHR-2 (Figure 5) [29,31]. Thus far, 11 DOCK proteins have been identified in mammals and the members of this protein family have been subdivided into 4 subgroups designated as DOCK-A, DOCK-B, DOCK-C and DOCK-D [31]. These proteins control many biologically important cellular processes. Interestingly, both *in vitro* and *in vivo*, the DHR-2 domain of these DOCK proteins has been shown to be sufficient and necessary to trigger guanine nucleotide exchange on GTPases (mainly Cdc42 and Rac1)(Figure 5).

Located upstream of DHR-2 domain is the highly conserved DHR-1 domain present in virtually all DOCK180 related GEFs [29,31]. DHR-1 domain has been shown to effect interaction between phosphatidylinositol (3,5)-bisphosphate and PtdIns (3,4,5)P₃ lipid

signaling *in vitro* as well as *in vivo* [53]. Intriguingly, Rac1 activation is inhibited upon the inactivation of DHR-2 domain of DOCK180 that in turn inhibits cell migration and other cellular processes for instance phagocytosis [31,54,55]. This emphasizes the importance of the DHR-2 domain in the function of the protein.

1.4.1b. The RhoGAPs

Till date, more than 80 RhoGAP proteins have been identified and characterized in humans. Being so many compared to Rho GTPases itself indicates that these proteins are involved in negatively regulating or terminating the signals at the specific location intracellularly. There are considerable evidences to suggest that the activity of Rho GAP within a cell is controlled by diverse means for instance protein-protein interaction, lipid binding, post-translation modification such as phosphorylation [56,57,58,59,60]. The first RhoGAP was discovered in 1989 and known as Bcr (**breakpoint cluster region**) [61]. Biochemically, RhoGAPs interact with the GTP loaded conformation of the Rho GTPases, thereby stimulating their intrinsic GTPase activity up to a 100 fold [62]. The structural determination of the RhoGAP domain has made it possible to decipher exactly how RhoGAPs catalyse the GTPase-activating reaction [63,64]. Crystallographic studies have revealed that the amino acids close to the arginine form a catalytic site involved in direct hydrolyzing GTP reaction to GDP with release of an inorganic phosphate (P_i). Mutation in these residues have also shown to hampers the activity of RhoGAPs [65]. Despite a low degree of similarity in primary structure between the RhoGAP and the RasGAP domains, the tertiary folding, as well as the basic mechanism of RhoGAP domain appears quite similar to RasGAP [62]. The RhoGAP domains are formed of 9 alpha helices and the hallmark is a conserved arginine amino acid residue present in a loop region, the so-called “Arginine finger” [66].

1.4.1c. The RhoGDIs

The RhoGDIs target the GDP-bound form of Rho and Rab GTPases. Their function is to block the exchange by maintaining the GTPases in “OFF-state”. In addition, GDIs also prevent the GTPases from localizing to the plasma membrane. The first RhoGDI identified was RhoGDI-1 (also known as RhoGDI α) [66]. It was shown to be involved in blocking the dissociation of GDP form and eventually binding of GTP to small GTPase RhoB. Till date, only 3 RhoGDIs have been identified: RhoGDI-1, RhoGDI-2

(also known as D4/Ly-GDI,) and RhoGDI-3 (also known as RhoGDI γ). The GDI-1 was initially purified from rabbit intestines and bovine brain cytosol [67]. Subsequently, the cDNAs of RhoGDI-1 was isolated from humans [68]. The RhoGDIs have a very disparate sequence at the N-terminus compared to the C-terminus. The RhoGDI-1 and 2 share around 74 % homology (i.e., 178 amino acid region at the C-terminus) and RhoGDI-3 of around 63% in the same region with GDI-1 and 2 [69].

Out of these three RhoGDIs, 1 and 2 are of cytoplasmic origin and directly involved in controlling the membrane targeting i.e., the membrane association/dissociation cycle and GTP/GDP cycle. The RhoGDI-3 acts in a different manner compared to GDI-1 and 2. Its mode of action is therefore still unclear [68,69].

Mutations in Cdc42 and Rac1 defective in RhoGDI α binding (due to substitution of Arginine 66 with Glutamic acid) are able to target to membranes and induce filopodia or lamellipodia formation [70,71]. Similarly, in null RhoGDI α mesangial cells, transfection of activated Cdc42 or Rac1 mutants exhibited the same spectrum of actin reorganization as the wild-type cells. This suggests that other proteins may assist in the solubilization of nascent Rho GTPases. Moreover, the actin reorganization mediated by Rac1 and Cdc42 do not rely on control by RhoGDI. However, these studies utilized ectopically expressed Rho GTPases, including use of activated mutants evading control by GAPs [70,71]. It appears that the C-terminal hypervariable regions of Rho family GTPases are sufficient to enable delivery to the various resident membrane compartments, rather than their binding to RhoGDI α [72]. Nonetheless, RhoGDI do control the partitioning between the cytosol, membrane compartments and may facilitate the targeting of GTPases to appropriate signaling sites.

1.4.1d. Organization of Rho GTPase signaling pathways

A cell has to respond to myriad of signals. Therefore, well-regulated signaling pathways make sure that cell responds precisely to signals. Proteins involved in signal transduction pathways possess multiple domains that give them a degree of flexibility in order to interact concurrently with variety of other proteins. Rho GTPases are capable of controlling the cell response by interacting with effector proteins [1,2,3]. Based on the upstream signals, the Rho GTPases selectively activate particular downstream effector proteins; thereby organizing downstream signals in a cascade that ultimately leads to a specific cellular response for instance, cell polarity, cell migration

etc. Experimental studies have confirmed the role of GEFs, GAPs in complex with these effector proteins, enabling the control of particular effector protein to form a complex with a specific Rho GTPase [1,2,3]. Therefore, based on the upstream signals, the Rho GTPases efficiently organize signaling pathways downstream.

The original concept of the best studied Rho members RhoA, Rac1 and Cdc42 stated that they regulate stress fibers, lamellipodia and filopodia, respectively [73]. The molecular mechanism underlying these biological responses have been described in some details. For instance, RhoA regulates the formation of stress fibers through the concerted action of Rho-associated protein kinase (ROCK) and Diaphanous-related formins (DRFs). ROCK acts by activating myosin II by phosphorylating myosin-regulating components and promoting actin filament formation via DRFs. Rac1 act via so-called nucleation-promoting factors (NPFs), in this particular case the WAVE complex induces the formation of a weave of actin filament in the protruding lamellipodium at the leading edge of migrating cells. Cdc42, trigger the formation of filopodia via a number of downstream effectors but DRFs seem to have a critical role in the process [74].

1.5 Essential functions of Rho GTPases

1.5.1a. Actin filament system regulation

One of most abundant proteins present in eukaryotic cells is the actin. The polymerization of actin is regulated by variety of actin binding proteins. There are actin-sequestering proteins, such as profilin, which act to keep actin in a monomeric unpolymerized state. Actin filament formation is brought about mainly by two groups of polymerization machineries, the NPFs (most notably, WASP, WAVE and WHAMM) and the DRFs (most notably mDia1 and mDia2) [75]. NPFs bind to the so-called Arp2/3 complex and promote the formation of branched actin filaments, whereas DRFs promote the elongation of actin filaments. The organization of actin is further regulated by proteins that aid in promoting assembly of actin filaments into highly ordered bundles and meshwork. Another important regulatory cue acts through capping of actin filaments, which helps to keep the actin filaments stable. Finally, there are numerous proteins (such as cofilin) that aid in the depolymerization of actin filaments by a severing activity and by promoting actin filament disassembly [2,76].

1.5.1b. Actin polymerization via NPFs and the Arp2/3 complex

Actin regulators trigger formation of new actin filaments by a process known as nucleation. Kinetically, nucleation is rate-limiting step in the actin polymerization due to actin dimer intermediates being highly unstable [77,78]. The actin related proteins were initially purified from *Acanthamoeba castellanii* by affinity chromatography. These proteins had high affinity for the actin sequestering protein, Profilin and stabilized actin for the promotion of filament growth and elongation [79].

The Arp2/3 complex consists of assembly of seven proteins [79,80]. Of these seven proteins, two subunits were actin-related proteins of ARP2 and ARP3 subfamilies (hence, the name Arp2/3 complex). Remaining 5 subunits were initially named by size but have now been named as ARPC1 (actin-related protein complex-1), ARPC2, ARPC3, ARPC4 and p16-ARPC5 (Figure 6). ARPC1 possesses two isoforms in humans namely, ARPC1A and ARPC1B. ARPC1 possesses WD repeats domain whereas ARPC-5 do not contain any common domains. A definitive role of Arp2/3 complex in lamellipodia formation was clarified by Bailly et al., in 2001 by using an antibody against Arp2/3, which inhibited EGF-stimulated lamellipodia formation [81]. The actin filaments in lamellipodia distinctly oriented their barbed ends (i.e., fast growing ends) towards the cell membrane (Figure 6). The Arp 2/3 complex binds to the sides and pointed ends of the pre-existing actin filaments in a manner that contributes to its activation (Figure 6) [82]. Binding to NPFs is also essential in the activation of the Arp2/3 complex. The Arp2/3 complex nucleates actin filament by mimicking actin trimer with two of its subunits binding to actin monomers. The best studied role of Arp2/3 was performed by Welch et al., in 1997 where they showed how bacterium *Listeria monocytogenes* left an arc trajectory in the cytoplasm of platelets cells and comet-like tail rich in short actin filaments with their barbed ends towards the bacterium [83]. The Arp2/3 was subsequently purified from these platelets cells. This meant that the polymerization of actin monomers at the bacterial surface was coupled with propulsion indicating that it provides motile forces at the leading edges [84,85,86].

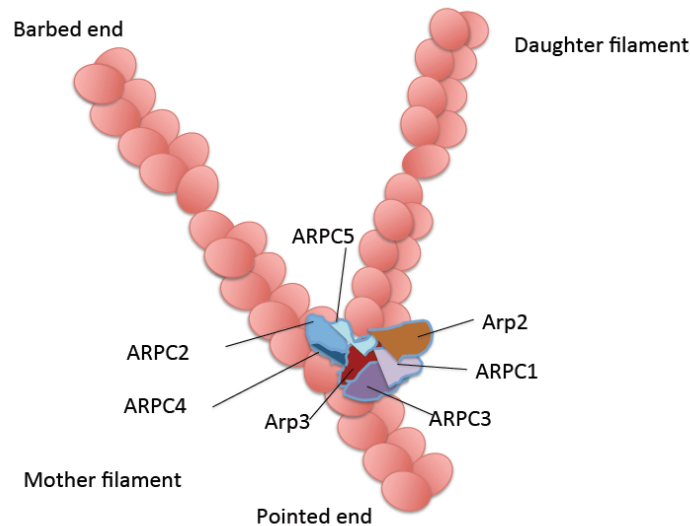


Figure 6. The Arp2/3 complex. The Arp2/3 complex and others actin related proteins (ARPC1-ARPC5) linking two filamentous actins together [adapted from source 87].

Immediately after the Arp 2/3 isolation and its characterization, it became apparent that this complex by itself has weak *in vitro* nucleating ability leading to a conviction that certain cellular factors must be required in order to trigger the nucleation and branching by the Arp2/3 complex. Eventually, many Arp2/3-binding NPFs were isolated. They can be divided into two subclasses: Type 1 NPFs include Wiskott-Aldrich syndrome protein (WASp), neural WASp (N-WASp), WASp family verprolin homologous protein (WAVE also called SCAR), WASp and SCAR homologue (WASH), WASp homolog associated with actin, membrane and microtubules (WHAMM) and Junction-mediating and-regulatory protein (JMY). All these proteins possess verprolin-homology domain (VCA domain or also called WH2), the central (Cofilin homology domain) and the acidic domain consisting of three conserved motifs which allow globular actin binding [88]. The Type II nucleation-promoting factors most notably cortactin lack VCA domains [89]. However, this class of NPFs have acidic domain at their amino terminus which binds directly with Arp2/3 complex [89]. The Cdc42 triggers the activation of Arp2/3 through WASp and N-WASp. Cdc42 interacts with N-WASp directly *in vivo* freeing an intra-molecular and auto-inhibitory interaction thereby exposing C-terminus of Arp2/3 binding and activation site (Figure 7) [2]. Rac1 has been shown to act mainly via the WAVE family of proteins and the so-called WAVE complex (Figure 7). The WAVE proteins are constitutively associated with four additional proteins namely: Sra1/Cyfp1, Nap1/Hem-2, Abi and

HSPC300 [90]. Rac1 binds Sra1 and triggers the dismantling of this inactive complex, thereby letting WAVE to interact directly with Arp2/3 [2,91].

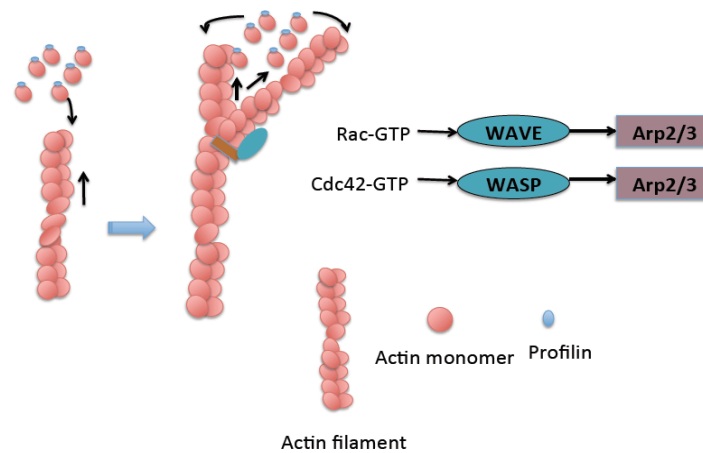


Figure 7. Actin polymerization mediated by the Rho GTPases. Rac1 and Cdc42 activate Arp2/3 via WASP and WAVE in order to initiate a branched filament network [adapted from source 1].

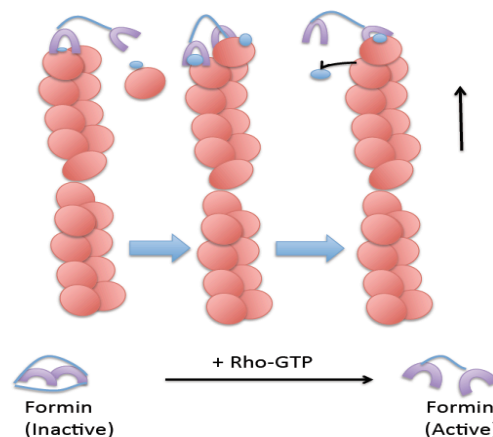


Figure 8. Rho GTPases and formins. Rho triggers the activation of formins that in turn promotes linear elongation of filaments at the barbed ends [adapted from source 1].

Actin polymerization via DRFs

In eukaryotic cells, another mechanism for actin polymerization is through Formin protein family [92]. The formins are a large family of proteins that facilitate the nucleation of new filaments by promoting the interaction between two actin monomers. In addition to nucleation, formins also facilitate the elongation of actin filaments, exclusively at the barbed ends. The formin mDia1 (also known as Dia1, Drf1 for Diaphanous-related formin-1) is the mouse homologue of the diaphanous homolog 1 of

Drosophila. This protein was identified in a yeast two-hybrid screen as RhoA effector and it triggers actin filament elongation (Figure 8). In yeast, there are only two formins known Bnr1 and Bni1 that stimulate the Rho mediated actin polymerization. In mammals, formins function downstream of Rho to form focal contacts and stress fibers. In *Saccharomyces cerevisiae*, they function downstream of Cdc42 to induce actin filament formation in an Arp2/3 independent manner [93,94]. Their function appears to include nucleation of new actin filaments [95,96]. The C-terminal of the yeast formin, Bni1 contains FH1 and FH2 domains. These domains are sufficient to nucleate actin filaments *in vitro* [97,98]. Interestingly, the FH1 and FH2 domains of Bni1 also cap the barbed end of actin filaments, decreasing but not blocking the rate of actin polymerization [99,100,101].

In humans, there at least 15 members of the formin family of proteins but not all of them bind to small GTPases [92]. The interaction of mDia1 with RhoA relieves an autonomous inhibitory interaction making FH2 domain exposed, which then directly binds to barbed ends of actin filaments and functions as the actin polymerization machine. The FH1 domain promotes the interaction of the formin with the profilin/actin complex and brings it to the end of the actin filament to add new G-actin monomer to the barbed end of the filament [2].

ADF/Cofilin

The ADF/Cofilin family of proteins in eukaryotic cells has a role in actin depolymerization. ADF (also known as destrin) was first identified in chick embryo brain tissue as an actin depolymerizing factor (ADF) [102]. These proteins are widely distributed among plants and animals tissues [102,103,104,105]. Cofilin binds to both G-actin as well as F-actin and enhances the rate of polymerization and depolymerization of actin filaments [106,107]. Cofilin engages itself in actin filament depolymerization by triggering monomeric actin disassociation from pointed ends [77,108,109]. Members of cofilin family of proteins depolymerize actin filaments via two mechanisms i.e., via severing and by accelerating the rate of depolymerization from pointed ends. When the ATP bound G-actin is abundantly available in the cell, cofilin enhances actin polymerization through its actin severing activity, by supplying the free barbed ends for continuous polymerization and nucleation via Arp2/3 complex.

Intriguingly, microinjecting the Arp2/3 nucleation blocking antibodies in cells,

significantly inhibited lamellipodia formation but does not inhibit free barbed ends suggesting that there might be an alternative mechanism that takes part in barbed end generation *in vivo*. Microinjection of antibody blocking cofilin in the same cell system inhibited barbed end generation suggesting that cofilin and Arp2/3 work in close cooperation in order to reorganize actin filaments. At the growing barbed ends, Arp 2/3 complex binds to side of ATP loaded filamentous actin thereby triggering the nucleation of newly formed filamentous actin branch. On the other hand, cofilin mediated actin disassembly occurs after the Arp2/3 dissociates from the filamentous actin [110].

1.5.1c. Regulation of cell migration

One of the fundamental and central aspect in the normal development of the multicellular organisms is the cell migration. For vital cellular processes for instance, the formation of tissue during embryonic development, wound repair and inflammatory immune responses, cells need to migrate in a direction to reach to a specific location within a body. Additionally, cell migration also occurs in human diseases such as metastasis, atherosclerosis etc. [111]. One of the characteristics necessary for cell migration is the protrusion at the cell front. Lamellipodia are rich in branched filaments of actin at cell front or leading edges. These sheet like extensions propel the cell body over the substratum. It is well documented that lamellipodia consist of branched actin filament networks formed via the actin-nucleating activity of Arp2/3 complex. Induced by growth factors, cytokines, hormones or extra cellular components, Rac1 is necessary for lamellipodia extensions and upon Rac1 inhibition cells fail to migrate [73,112]. Activation of Rac1 is brought about by tyrosine kinases, G-protein coupled receptors and mediated by phosphoinositide 3-kinase (PI3-Kinase). However, evidences indicate that the Arp2/3 complex is activated by Rac through its target IRSp53. Rac interacts with IRSp53, which in turn interacts through the SH3 domain with WAVE, which then binds to and activates the Arp2/3 complex. Inhibitors of PI3-kinase blocks Rac activation [113,114,115].

Most migrating cells have small foci complex structures that are localized in the lamellipodia and are important for attachments of the lamellipodium to the extracellular matrix. Rac1 is required for focal adhesion complex assembly. Therefore, cells make new focal adhesion sites at the motile edges and degrade the attachments with ECM at the rear end. It is plausible that constant formation of new

interaction between integrins and the ECM at the motile edges of cells maintains activated Rac there. The cell migration speed is dependent on substrate composition, levels of RhoA, Rac1 and Cdc42 activation and it varies with the matrix composition. As the cell lamella moves forward, the focal complexes disassemble and in slowly migrating cells such as fibroblast they can mature into Rho-induced focal adhesions [116]. On the other hand, RhoA regulates the focal complex maturation into focal adhesions through its effector Dia1 and Rho-associated coiled coil-containing protein kinase (ROCK) [117,118,119,120].

Another aspect necessary for cell migration is the cell body contraction. Contraction of cell body and retraction at cell body rear are vital for cell movement. It is dependent on actomyosin contractility and can be controlled by RhoA [121]. For instance, upon RhoA inhibition, macrophages continue to extend, however the cell body fails to translocate [122,123]. It could be due to stress fiber contraction. Stress fibers are the major motor for cellular contraction. Stress fibers are formed of actin and myosin filaments. When the myosin fiber slides past the actin filament, it leads to the shortening of the stress fibers, providing the contractile force for cell motility [124,125]. The stress fibers attach to the integrin, transmembrane receptors via proteins complexes at the so-called focal adhesions. Rho acts via ROCK to affect the MLC by phosphorylating myosin light chain (MLC) and inhibiting MLC phosphatase. MLC phosphorylation is also regulated by MLC kinase (MLCK). The ultimate effect of MLC phosphorylation is cell contractility and stress fibre formation due to enhanced interaction between the actin filaments and MLC [126].

Additional aspect of the cell migration is the directional movement. Cells sense and respond to the guidance cues in their surroundings via filopodia. Filopodia are the finger-like protrusions present at the cell periphery. Cdc42 triggers filopodia formation and is required for directional sensing during chemotaxis in fibroblast cells as well as neurons [112,122,123,106]. By protruding out from cells into surrounding, receptors on filopodia detect changes in the extracellular signals that would then be transmitted back into cells.

1.5.1d. Regulation of membrane trafficking

Members of Rho family GTPases are well known to control many aspects of membrane trafficking, for instance the vesicle transport by endocytosis and exocytosis [127]. This cellular process is vital for the flow of material inside and outside a cell.

Rho GTPases in endocytic pathways

Endocytosis via clathrin

Numerous receptors involved in signal transduction pathways are internalized in cells by clathrin-coated pits. Upon internalization, these transmembrane receptors can be either directed for degradation by ending up in lysosomal compartments, which are highly acidic vesicles or they can be recycled back to cell surface via recycling endosomes [128]. These vital cellular processes are well controlled by actin cytoskeleton as observed in yeast [129]. However, latest results of Schmid et al., 2000, contradict this notion and discuss that the role of actin cytoskeleton may vary based on the cell type used and more importantly the experimental conditions [130]. For instance, in HeLa cells, overexpression of activated Rho or Rac blocks transferrin receptor endocytosis [131]. Additionally, as discussed by Apodaca G et al., 2000 activated Rac blocks endocytosis from both basolateral and apical membranes of epithelial cells that are highly polarized [132,133]. Intriguingly, Rac1 GTPase can bind with synaptojanin 2, a polyphosphoinositide phosphatase involved in clathrin-coated vesicles uncoating [134]. By recruiting synaptojanin 2 at the plasma membrane, Rac1 act specifically by inhibiting the coated pit formation thereby blocking endocytosis. This activity of Rac explains its role in extending the lifespan of activated receptors at the plasma membrane [135].

On the other hand, Rho GTPases also affect certain stages of endosomal trafficking (i.e., stages such as: directing endosome vesicles either to lysosomes or recycling to plasma membrane) [136]. For instance, endogenous and myc-tagged RhoB was shown to localize to endosome compartments and have a key role in endosome trafficking [137]. Ectopic expression of GFP-tagged RhoB localizes in the perinuclear compartment in live cells [72]. However, it is plausible that GFP-tag could lead to mislocalization of RhoB due to protein folding. At the endosome vesicle, RhoB targets serine/threonine kinase, PRK1 to slow down trafficking of the endocytosed EGF receptor from endosome vesicles to pre-lysosomal compartment [132,138]. Another member of Rho family GTPases, RhoD also localizes to endosome. Ectopic expression of activated RhoD/G26V changes early endosomal distribution and motility [139]. Ectopic expression of activated or dominant negative Cdc42 also affect endosome recycling of polarized MDCK cells. However, it is unclear whether the effect is related to actin cytoskeleton [140].

Pinocytosis

Pinocytosis is a form of endocytosis whereby extracellular fluid is internalized resulting in the formation of cell membrane invagination. Experimentally, it can be studied by tracing cellular uptake of fluorescent molecules from the medium. This form of endocytosis is necessary for a cell in order to uptake nutrients that are necessary for cell homeostasis such cell growth and cell motility. Active variant of Rac has been shown to stimulate pinocytosis. Interestingly, PAK1, a Rac/Cdc42 target is needed for macropinosome formation upon growth factor stimulation [141]. A controversial model has been put forth discussing Rac-stimulated pinocytosis enhances the membrane ruffling and these ruffles fold back forming membrane bound vesicles [142]. Subsequently, this model was accepted since it clearly demonstrated that immature dendritic cells while sampling their surroundings for antigens do pinocytose via membrane ruffling [143].

Phagocytosis

Another form of endocytosis is called phagocytosis. It results by engulfing large particles (i.e., bacteria, virus etc). This form of endocytosis is usually carried out by mature macrophages, neutrophils, and phagocytes cells to clear out the antigens. The mechanism by which phagocytic cells engulf large particles depends on the receptor type present on the phagocytic cell surface. For instance, particles coated with antibody are taken into the cell via Fc gamma receptor [4]. This involves the actin polymerization via Arp2/3 complex mediated by Cdc42/Rac [4]. However, there are no real evidences to indicate that Cdc42/Rac are involved in driving movement of phagosome away from cell membrane although it has been suggested that PI3-K perhaps acts at later steps to regulate endocytosis and not Cdc42/Rac mediated actin polymerization [135].

1.6 Rho GTPases in disease development

1.6.1a. In cancer

After the identification of RhoA, it became apparent that RhoA was not acting as an oncogene in the same sense as Ras. With an exception of RhoH, there are no mutations identified in the genes encoding Rho GTPases in human cancer. Instead, the expression of Rho GTPases seems to be down regulated in cancer particularly in conditions such as

cancer cell migration, tumor invasion and metastasis. For instance, RhoH (also known as TTF) is upregulated in non-Hodgkin's lymphomas, multiple myeloma, as well as large cell lymphoma with the mutations in the 5' UTR region, that indicates the direct role of RhoH/TTF in cancer although precise mechanism is still unclear [144]. Experimentally, tumor derived cell lines and mouse models clearly advocate that the protein levels of master regulators of Rho GTPases (i.e., RhoGEFs and RhoGAPs) have a direct bearing on the initiation as well as tumor progression [144]. Deregulated RhoGEFs and GAPs signaling lead to aggressive cell migration, invasion and metastasis of various tumor cell types caused by variations in the levels of Rho proteins and untimely activation [145]. Altered Rho signaling particularly impacts the cellular scaffold or cytoskeleton whose organization and reorganization supports the motility of cancer cells during the invasive growth [146]. Nowadays, it is generally accepted that Rho GTPases are frequently upregulated in many human cancers, summarized in the Table 2 [144]. The variation in the expression can occur at mRNA or protein level. For instance, experimentally when malignant breast tissue samples were compared to the normal breast tissue samples, it was observed that the levels of Rac1 protein were elevated much higher in the malignant breast tissue compared to the normal breast tissue, indicating Rac1 triggers breast tumor formation *in vivo* [147,148]. In addition upregulation of Rac1b, a splice variant of Rac1 triggers cellular transformation in breast and colon carcinoma cells. *In vitro* studies in mouse fibroblast cells suggest that Rac1b signaling might be involved in cell survival signals via NF κ B [149]. Contrarily, downregulation of Rac1 leads to embryonic lethality *in vivo* [150].

Studies in the mouse leukaemia model have ascribed important roles of Rac3 in cancer progression [151]. In that study, the mice deficient of Rac3 were observed safe against lymphoblast leukaemia induced by crossing with mice expressing a fusion oncogene, BCR-ABL. This suggests that intervention with Rac3 function can be explored as therapeutic target for B-cell lymphomas or blood cancers [151,152].

Altered expression levels of regulators of Rho GTPases also cause cancer development by resulting in the deregulated signals downstream of Rho proteins. For instance, deprivation of Tiam1, a RacGEF, maintains E-cadherin based cell-cell adhesion that leads to epithelial-to-mesenchymal transition [153,154]. The studies carried out in mouse tumor models suggest that the lack of Tiam1 enhances invasion of Ras-induced epithelial skin tumors as well as β -catenin/TCF-induced intestinal tumors [155,156]. Moreover, point mutations in the N-terminal of PH domain of Tiam1, drastically affects

its localization and it has been observed in approximately 10% of samples affected by human renal-cell carcinoma samples [157]. The levels of Tiam1 protein have also been observed to be upregulated in human prostate carcinomas [158].

Rho GTPase	Variation	Cancer/Tumor type
RhoA	Upregulation	Breast, HNSCC*, colon, lung, gastric, bladder and testicular cancer
RhoB	Upregulation or downregulation	Breast (upregulation), lung and HNSCC* (downregulation)
RhoC	Upregulation	Breast and metastatic gastric cancer (inflammatory), pancreatic ductal adenocarcinoma, bladder cancer, NSCLC* and HNSCC
Rac1	Upregulation	Breast, gastric and testicular cancer OSCC*
Rac1b	Alternative splicing	Breast and colon cancer
Rac2	Upregulation	HNSCC*
Rac3	Hyperactivation or upregulation	Breast cancer
RhoG	Upregulation	Breast cancer
Cdc42	Upregulation	Breast and testicular cancer
RhoH/TTF	Upregulation and mutations (5'UTR)	Non-Hodgkin's lymphoma and multiple myeloma (upregulation) and diffuse large B-cell lymphoma (mutation)
RhoE/Rnd3	Upregulation and downregulation	NSCLC (upregulation) and prostate cancer (downregulation)
<u>RHO GTPASE REGULATORS</u>		
Tiam1	Point mutation	Renal-cell carcinoma, prostate carcinoma and breast cancer (upregulation)
LARG	Fusion to MLL	Acute Myeloid Leukemia (AML)
DOCK 180-ELMO1	Upregulation	Glioma
Vav1	Upregulation	Pancreatic adenocarcinoma, neuroblastoma
β -PIX	Upregulation	Breast cancer
RhoGDI α	Downregulation	Invasive ovarian (upregulation) and breast

Table 2. The mutations in Rho GTPases and their regulators with associated cancer types [adapted from source 144].

Upregulation of GDIs has also been well documented to occur during the different stages of cancer progression. RhoGDI1 or RhoGDI α has been observed to be upregulated in colorectal and ovarian cancers [159,160]. Contrarily, downregulation of GDI2 have been observed to promote development of muscle invasive bladder cancer [161]. The RhoGAPs constitute another group of key regulators that contributes to cancer progression. Genomic deletion of DLC-1, a GAP for RhoA and Cdc42 has been found in primary tumours [162]. Deletion of DLC-2 expression has also been found in hepatocellular carcinomas [162,163]. Deletion or downregulation of these GAPs lead to enhanced activation of Rho GTPases and their downstream signaling pathways during cancer progression. The examples mentioned above, it is still unclear about the specificity of most of the regulators of Rho proteins *in vivo* perhaps uncontrolled signals emanating via Rho GTPases are sufficient for the contribution of tumor progression.

1.6.1b. In neurodegenerative disorders

In a developing and well-developed nervous system, Rho GTPases have an important role in neuronal morphogenesis and dendritic plasticity by regulating the actin cytoskeleton at various stages. The Rac1 and Cdc42 have been well studied for their roles to promote the growth and dendritic stability whereas RhoA has been shown to inhibit this growth [164]. Many GEFs and GAPs are expressed in the nervous system making them likely to be involved in the specific Rho GTPases mediated signaling pathways leading to the neuronal processes. For instance, neuropathological disorder, X-chromosome linked mental retardation (MRX) impairs the cognitive function in the individuals suffering from this disease, which affects 1 in 500 males representing 25% of genetically, manifested cases of mental retardation [5]. Upon closer histological inspection of the brain structure it was observed that patients suffering from MRX disease had increased size of hippocampus and certain cerebellar ventricles. In contrast, the cerebral cortex appeared reduce in size compared to the unaffected control tissue samples [165,166]. Additionally, microscopic examination showed thinner and stretched dendritic spines in the affected regions of patients suffering from mental retardation (MR). A closer look at the synaptic contacts revealed that this region had immature dendritic spines cells [167,168]. Synapses at the spines are thought to transmit the majority of functionally excited synaptic communication [169]. It was via positional cloning that ten genes related to mental retardation were identified giving an

insight into mutational basis for study of genetics and biochemistry involved in mental retardation (Table 3).

Out of the genes/proteins enlisted Table 3, three genes/proteins namely, oligophrenin-1, PAK and PIX/Cool-2/ARHGEF6 are directly involved in Rho GTPases mediated signaling pathways observed in neurons [170]. Another example of the involvement of Rho GTPase in neurodegenerative disorder is mutation in the RhoGEF, alsin, which causes the Amyotrophic lateral sclerosis (ALS) [171]. ALS causes a very life-threatening disorder in which motor neurons degenerate with time. It has an important role in neurodegenerative disease [171]. Another key molecule is the Intersectin, for which the gene is located on chromosome 21 in humans and has been studied for its role in neuronal defect, Down syndrome by impairing Rho GTPase mediated signaling using mice model [172].

Apart from the neurodegenerative disorders discussed above, alterations of mitochondrial dynamics and fusion also have been reported to link human neurological diseases affecting a specific area in the brain and nervous system, thereby highlighting the important role of mitochondrial function in maintaining healthy neurons [173]. Interestingly, Miro GTPases are well known to regulate mitochondrial motility along the microtubules in order to migrate to distinct location intracellularly. There is a substantial amount of genetic and chemical evidence indicating the involvement of mitochondria in Parkinson's disease (PD), which is the second most common neurodegenerative disorder in humans, in which there is a continuous loss of dopaminergic neurons in the substantia nigra, symptoms observed are resting tremor, rigidity, bradykinesia and a unsteady gait (Figure 9) [173].

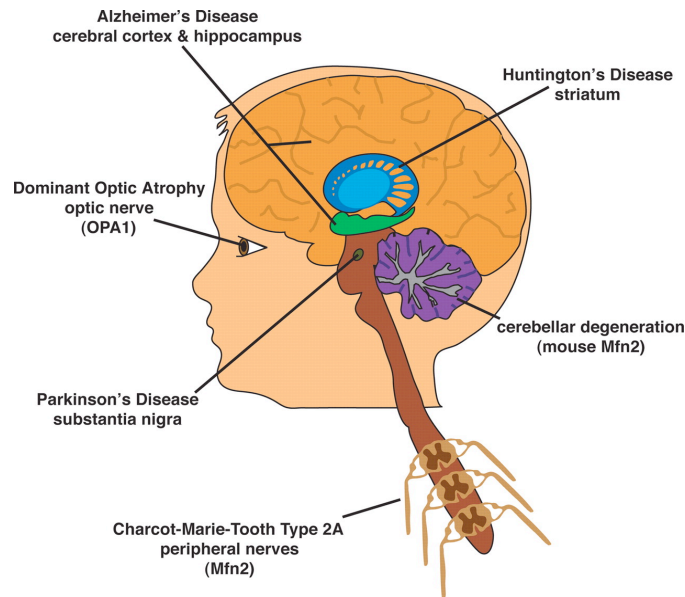


Figure 9. The neurodegenerative disorders related to mitochondrial dynamics defects [adapted from source 173].

Two genes have been identified in patients suffering from Parkinson's disease, Pink1 and Parkin; both have been shown to have a key role in mitochondrial integrity [174]. Pink1 is a serine/threonine kinase and has an N-terminal sequence that targets mitochondria. It localizes both within mitochondria as well as in cytosol. On the other hand, Parkin is a cytosolic E3 ubiquitin ligase with two RING fingers, cysteine and histidine-containing protein motifs that coordinate zinc ions. The studies with Pink1 and parkin in mammals have yielded less success due to the fact that mouse knockouts displayed very little phenotypical changes and did not show the common symptoms of Parkinson's disease [173,175,176,177]. Moreover, additional studies indicate that loss of Pink1 can result in morphological abnormalities in the mitochondria [178].

Deficiency of Pink1 in human dopaminergic neurons or primary mouse neuronal cultures leads to reduced viability accompanied by abnormal and enlarged mitochondria [178]. Experiments with Pink1 double knockout mice (-/-) did not reveal any ultrastructure alterations of mitochondria, except the fact that the mitochondria appeared somewhat larger. Similar results were also observed in the cell line, COS7 where the knockdown of Pink1 also showed increased mitochondrial size through tubulation [173,179].

Genes/Proteins	Functions	Clinical symptoms	Spine/synapse phenotypes
Oligophrenin-1	Rho GAP for RhoA, Rac1 and Cdc42	MR*, cerebellar hypoplasia, epilepsy	Spine length reduced, reduction in mature spines
PAK3	Serine/threonine kinase, effector of Rac1/Cdc42	MR*	Abnormal elongated spines, decrease in mature synapses
α PIX/Cool-2 ARHGEF6	Rho family GEF for Rac1/Cdc42, interact with PAK	MR*	Reduction in large mushroom type spines
FMRP	RNA binding protein (Rac1), interacts with CYFIP, downstream of Rac1	MR*, macrocephaly, long face, long ears, macroorchidism	Long and irregular dendritic spines
MEGAP, WRP, srGAP3	Rho family GAP for Rac/Cdc42	Macrocephaly, growth failure, heart and renal defects, hypotonia and facial abnormalities	Loss of filipodia and dendritic spines
LIMK1	Ser/Thr kinase, downstream of Rac1/Cdc42	Williams syndrome	Decreased spine head size and thicker spine necks
Alsin	GEF for Rac1, Rab5 and Ran	Motor neuron degeneration	Reduced axon growth, increased cell death

Table 3. Shows different Rho GTPases, their regulators and the clinical manifestations when mutated [adapted from source 170].

1.7 Rho GTPases as therapeutic targets

From basic to clinical research, there are considerable evidences suggesting that the pathways downstream of Rho GTPases have role in disease for instance cancer development and progression [180]. Consequently, there has been a profound interest to target specific Rho proteins involved in Rho GTPase-dependent signaling pathways in malignant transformation as potential therapeutic targets. Therefore, varieties of effector protein inhibitors have emerged. However, not all of these inhibitors have entered clinical trials. Nevertheless, it is promising to explore their nature as a drug against different types of tumors [180].

Approximately, 80 GEFs for Rho GTPases are known and their modes of action have been well studied [28,181]. Due to the key roles of these proteins in the activation of a specific Rho GTPase, they were considered targets for drug development (Table 4) [180]. For instance, RhoG, Rac and RhoA are activated by the RhoGEF Trio and an alternate splice variant, Tgat that are considered druggable. In addition, LARG (Leukemia-associated RhoGEF) activates RhoA/B/C have also been considered as a therapeutic target [182,183,184,185]. Trio is upregulated in breast and glioblastoma cancers and it is associated with poor prognostic outcome. Tgat on the other hand is capable of transforming NIH 3T3 cells by virtue of the loss of contact inhibition, anchorage independent growth, tumorigenicity in nude mice and increased invasiveness. Since both Trio and Tgat GEFs are involved in catalyzing the exchange of GDP to GTP, it makes them ideal candidate for drug development by developing an inhibitor molecule that could bind the GTP pocket thereby inactivating the signaling cascade downstream of Rho GTPases (Figure 10) [145,186].

Class	Inhibitors	Applications			
		<i>In vitro</i>	Cells	<i>In vivo</i>	Clinic
Rho GEFs					
Trio/Tgat	TRIAPα	√			
	(bacterial toxin)				
	TRIPα	√	√		
	TRIP ^{E32G}	√	√		
	ITX3	√	√		
LARG		√			
Tiam	NSC23766	√	√		
Rho GTPases					
Rho	C-3 exoenzyme	√	√		
	C3-05/BA-210			√	√
Rac1	EHT1864	√	√		
Rho/Rac	MLS000532223	√	√		
Cdc42	MLS000573151	√	√		
IsoPrenylation inhibitors					
GGTase	Statins		√		Widespread use in clinic to control cholesterol levels
GGTase/FTase	AZD3409				√
	26b	√			

Table 4. Rho GTPases activators and their functional inhibitors [adapted from source 180].

In addition to a central role in controlling actin-related mechanisms, Rho GTPases are also involved in virtually all the cellular processes. This makes them a key ideal targets for drug development. For instance, Rho GTPases are important in cell cycle progression through G1 phase of cell cycle by regulating the expression of cyclin D1 and cyclin-dependent kinase (CDKs) inhibitors p21 and p27 [187,188,189,190]. Intriguingly, very

few compounds have been developed till date that target Rho GTPases and their effectors. Nevertheless, many strategies have been developed to inhibit interactions between GTPases and cognate GEFs in order to block Rho signaling activation (Table 4) [191].

Alternatively, another way of inhibiting GTPase function is by displacing nucleotide binding, nevertheless, questions remain regarding the specificity of this approach [192]. Another approach that could be explored is inhibiting C-terminal modification of Rho GTPases that are necessary for anchoring with the cell membrane. This can be achieved by inhibiting C-terminal modifying enzymes or by limiting the essential supply of lipid for these modifications (for example, isoprenylation), which is possible by using a Statin class of drugs (Table 4) [193]. Statins target Rho function by interfering with the attachment of lipid moieties at their C-terminus. Since, C-terminus lipid modifications are important for correct intracellular localization of Rho GTPases. Statins class of compounds include HMG-CoA reductase inhibitors, which deplete the cellular pool of isoprene precursors as well as prenyl transferase inhibitors (i.e., farnesyltransferase and geranylgeranyl transferase inhibitors (GGTI) [194,195,196].

In conclusion, the examples mentioned above discuss the ability of each Rho GTPase member to trigger the activation of many cellular pathways, making them the likely targets for therapy since they might be a part of critical signaling hubs [197]. Nonetheless, blocking Rho GTPases could affect multiple signaling pathways that might most likely result in dose-limiting toxicities. There are other promising ways for instance targeting kinases by inhibitors (Table 5). The first Rho effector to be targeted as a therapeutic agent was Rho-associated kinase (ROCK1/2) by Y-27632 inhibitor. This fuelled a considerable interest of pharmaceutical companies to invest into drug development using Y-27632 inhibitor (Table 5). The ROCK isoform inhibitors are currently under investigation for many drug discovery programs [197,198]. Using selective inhibitors will allow analyzing biological function [198]. The use of Chelerythrine chloride against MRCK has been shown to be similar to ROCK in inhibiting cancer invasion. Inhibition of p-21activated kinases (PAKs) activity has been tested and inhibitor have been developed by using small molecule group 1-vs 2- selective inhibitors [199]. For future studies, these inhibitors will be useful to study the role of PAKs in disease such as cancer (Table 5) [199].

Kinases	Inhibitors	Applications			
		<i>In vitro</i>	<i>Cells</i>	<i>In vivo</i>	<i>Clinic</i>
ROCK1/2	Y-27632	√	√	√	
	Y-30141				
	Y-30946				
	Fasudi(HA-1077)/Hydro			√	√
	xylfasudil			√	
	H-1152P	√	√		
	Lead compound 14A	√	√		
	Isoquinoline-based compound 35				
	GSK269962A				
	SB-7720770-B	√	√		
ROCK2	SLx-2119		√		
	Indazole piperazine	√			
	Indazole piperidine				
LIMK1/2	BMS compound 3	√	√		
	Pyrrolopyrimidine Compounds	√		√	
MRCK α/β	Chelerythrine chloride	√	√		
PAK4,5,6 and 1	PF-3758309	√	√	√	
PAK	IPA-3	√	√		

Table 5. Kinases inhibitors downstream of Rho GTPases signaling [adapted from source 199]

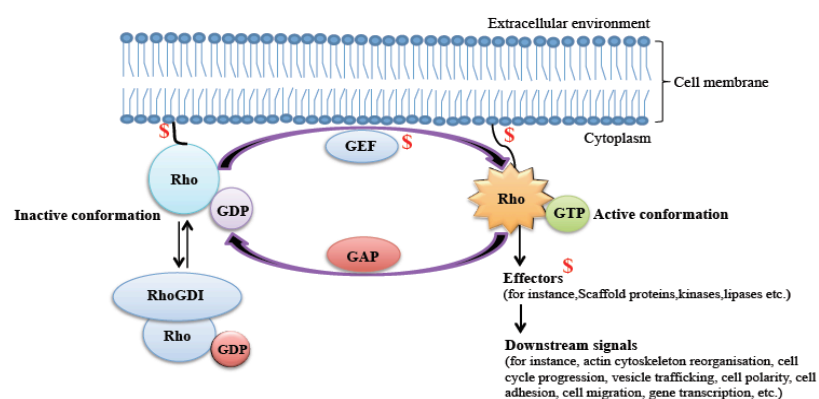


Figure 10. The druggable target sites in the Rho GTP/GDP cycle (\$).

The RhoD and Rif genes are located on chromosome numbers 11(11q14.3) and 12(12q24.31)(Figure 11) [201]. Like most classical Rho GTPases, the RhoD and Rif proteins contain GTP-binding domain and a C-terminal CAAX box. It is considered to be post-translationally farnesylated by geranyl-geranylation; however this has not actually been clearly demonstrated. A recent report suggests that RhoD and Rif possess an elevated intrinsic exchange activity [204]. In this regard, they resemble the atypical Rho members Wrch-1 and the activated Rac1 splice-variant Rac1b. This suggests that RhoD and Rif also act as atypical Rho GTPases, something that also indicate that they are not likely to be regulated by GEFs and GAPs, rather by other means, such as post-translational modifications. Once docked and anchored at the plasma membrane, the RhoD is activated and triggers physiological response to activating signals. Fundamentally, RhoD and Rif share high sequence similarity and domain organization, only noticeable difference being the presence of an extension of few amino acids at the N-terminal of Rif GTPase. However, no particular function has been ascribed to this extension (Figure 12) [201].

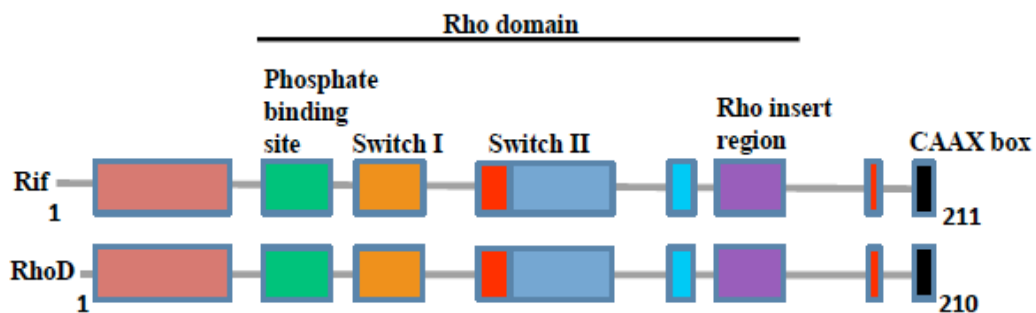


Figure 12. Schematic representation of domain organization of RhoD and Rif [adapted from source 201].

1.8.1b. RhoD/Rif in the regulation of actin cytoskeleton dynamics

The present knowledge and understanding regarding the role of RhoD/Rif in the regulation of actin cytoskeletal dynamics emanates from the experiments where different variants of RhoD/Rif (i.e., wild type, constitutively active and dominant negative) were overexpressed in different cell types. For instance, overexpression of the active variants of RhoD and Rif in PAE/PDGFR β or HeLa cells changed the cell morphology observed as thin and long protrusions of filamentous actin appearing either from the dorsal or the peripheral sides of cells. Similarly, morphological changes have also been observed in

cell lines such as NIH3T3, SHSY5Y and PC-12 [205]. These long and thin filopodia are highly dynamic structures arising from the cells particularly from the leading edges and are rich in linear bundles of filamentous actin cross-linked to each other by actin-binding proteins such as fascin. Filopodia are best known for their important roles in cell-cell interaction, sensing and migration [206,207,208,209,210].

Additionally, RhoD and Rif mediated filopodia's are of 20-40µm in length compared to conventional filopodia, which are of approximately 8-15 µm in length [139,211,212]. The complete understanding of how RhoD is involved in filamentous actin polymerization or filopodia formation has not been completely achieved [212,213]. However, in paper I, we suggest that via the effectors of RhoD, WASP-homolog associated with actin, membrane and microtubules, WHAMM and Filamin-A (FLNa) binding protein, FILIP1 involved in actin polymerization [214]. Interestingly, out of these two-RhoD binding proteins, WHAMM binds to Arp2/3 complex via its c-terminal WCA domain and FILIP1 binds FLNa. Both these proteins act downstream of RhoD in regulating the actin polymerization and cytoskeletal dynamics thereby regulating the polymerization of filamentous actin [214].

1.8.1c. RhoD in the regulation of vesicle trafficking

Studies by Gasman et al., 2003 observed another role of RhoD in regulating the vesicle trafficking or endocytosis [203]. Endosome vesicles are very dynamic structures. These endocytic vesicles need a close cooperation of cytoskeleton (i.e., actin and microtubules) to deliver cargo [202]. For instance, close association of microtubule, microtubule motor proteins and filamentous actin are needed for the correct positioning, internalization and dispatching cargo from early to late/recycling endocytic vesicles within a cell [201]. Intriguingly, members of small GTPases, Rho and Rab subfamilies are involved in regulating endosome motility. For instance, Rac1, RhoA have a key role in receptor uptake. Rab5 GTPase has an important role in stimulating the migration of the early endosome vesicles on the microtubules through kinesin-like motor proteins [201,203]. Also, RhoB has been shown to co-ordinate transport of cargo from late endosome vesicle to lysosomes [138].

Vesicle trafficking needs additional functionally specific proteins known as effector proteins, such as DRFs, which help the members of Rho and Rab GTPases to carry out vesicle trafficking in a well coordinated manner [139]. Examples of effector proteins include, mDia1 that binds to RhoA, mDia2 to Cdc42 and RhoA, FHOS, FRL binds to

Rac1 [203,215].

For shuttling a cargo to a short distance intracellularly, endosomes use actin filaments. Conversely, for a long distance transport endosome vesicles hop on to microtubules. This mechanism has been studied by destroying the microtubule network using microtubule depolymerization compounds such as Nocodazole, Vincristine and Colchicine in cells. Endosome motility was impaired and the endosome vesicles were localized to the cell periphery [202]. Ectopic expression of RhoD, blocks Rab5-dependent effect on early endosomes. Moreover, RhoD induces spherical, scattered and small endosome vesicles compared to Rab5 endosomes. With the identification of RhoD effector protein hDia2C, a great deal of interest arose in how RhoD via hDia2C regulates early endosome motility. Subsequent studies found that hDia2C promotes the alignment of early endosomes on the filamentous actin tracks. However, RhoD-mediated inhibition of endosome shuttling was dependent on Src activity and was reverted by an actin-depolymerizing drug, cytochalasin D [203]. This suggests a mechanism by which RhoD blocks endosome movement in a Src-dependent but actin-independent manner. Fundamentally, the assumption of this study was based on an observation describing the pathway in which RhoD was needed for the c-Src activation on endosomes via hDia2C [203].

Another example is that of Src family kinase members, Fyn that has been observed to localize to vesicles positive for RhoD [203]. RhoD directs Fyn to the cell membrane via the post-translational modification on Fyn (i.e., palmitoylation). Collectively, the observation regarding the localization of RhoD and its role in early endosome motility, suggests that RhoD can control the localization of Src family kinases and thereby regulate the early endosome motility.

2. AIMS

The work in this thesis highlights the role of RhoD, a less studied member of the Rho subfamily of GTPases. The overall aim was to elucidate the biological function of RhoD, and RhoD downstream signaling pathways. At the start of the thesis work, novel RhoD interactors via yeast two-hybrid screening were identified namely: FILIP1, WHAMM, Rabankyrin-5 and ZIP kinase. Furthermore, I wanted to study their potential role in the regulation of actin filament system, cell adhesion, cell migration, protein and tyrosine kinase receptor trafficking.

The specific aims of this thesis are:

- To elucidate the role of RhoD, via its binding partners WHAMM and FILIP1 in the regulation of actin cytoskeletal dynamics, cell adhesion and migration (**Paper I**).
- To clarify the role of RhoD, via its effectors, WHAMM and FILIP1 in the regulation of protein transport from ER to cell membrane via Golgi (**Paper II**).
- To determine the role of RhoD, via the Rab5 effector Rabankyrin-5 in receptor tyrosine kinases trafficking (**Paper III**).
- To dissect the mechanism by which the interaction of RhoD and ZIPk regulates the actin filament assembly and focal adhesion dynamics (**Paper IV**).

3. HIGHLIGHTS OF METHODS

Yeast two-hybrid screen

The *Saccharomyces cerevisiae* strain Y190 (genotype; MATa, *gal4-542*, *gal80-538*, *his3*, *trp1-901*, *ade2-101*, *ura3-52*, *leu2-3, 112*, URA3::GAL1-LacZ, Lys2::GAL1-HIS3cyhr) was transformed with a cDNA that encodes human RhoD/G26V fused to the GAL4 DNA-binding domain (GAL4DB) in the pYTH9 vector [216]. This RhoD construct harbored cysteine-to-serine mutations in its CAAX box, since we reasoned that this would facilitate the nuclear translocation of RhoD during the screening procedure. This GAL4DB-RhoD/G26V-expressing yeast strain was used to screen a cDNA library from human mammary glands.

Protein production and GST pull-down assays

GST-tagged fragments of FILIP1, WHAMM, Rabankyrin-5, RhoD, or GST alone were expressed in *Escherichia coli* and purified on glutathione-Sepharose beads. The pull-down assays were performed described previously [217].

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells, BJ human foreskin fibroblasts stably transfected with hTERT, and SV40 large T antigen (BJ/SV40T) cells, and green monkey COS-1 cells were cultured in DMEM supplemented with 10% (vol./vol.) fetal bovine serum (FBS) and 1% (vol./vol.) penicillin–streptomycin. Porcine aortic endothelial cells stably transfected with the human platelet-derived growth factor β -receptor (PAE/PDGFR β cells) were cultured in HAM's F12 medium supplemented with 10% (vol./vol.) FBS and 1% (vol./vol.) penicillin–streptomycin. All cell lines were cultured at 37°C in an atmosphere of 5% CO₂. The cells were transfected using Lipofectamine or JetPEI reagents, according to the protocols provided by the manufacturers.

Immunoprecipitation

For immunoprecipitation, the transiently transfected cells were lysed on ice in Triton X-100 lysis buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1% aprotinin) 48 h post-transfection. The cell lysates were centrifuged for 15 min at 4°C, and the supernatants were incubated with the primary antibodies for 1 h at 4°C, after which the immunoprecipitates were collected on protein

G-Sepharose for 1 h at 4°C. The beads were washed three times with Triton X-100 lysis buffer and subjected to SDS-PAGE, and the proteins were subsequently transferred onto nitrocellulose. Western blotting analyses were performed with the antibodies as specified in the figure legends; this was followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies. The proteins on the Western blots were revealed using Luminol immunoblotting reagent.

RNAi work

Knockdown of RhoD, WHAMM, FILIP1 or Rabankyrin-5 expression was induced by transfecting the BJ/SV40T cells with RhoD-directed siRNAs or with WHAMM-directed siRNAs, RhoD siRNA, or a nontargeting siRNA using the SilentFect transfection reagent. The cells were incubated for 48 h posttransfection before being processed for the various assays.

Antibodies, reagents, and constructs

All the antibodies, constructs and reagents used in investigating the specific aims mentioned are documented in the articles/manuscripts enclosed.

Immunocytochemistry

The cells were seeded onto coverslips in six-well plates, fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 25 min at 37°C, and then washed with PBS. The cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed with PBS, and blocked in 5% FBS in PBS for 30 min at room temperature. The primary and secondary antibodies were diluted in PBS containing 5% FBS. The cells were incubated with the primary antibodies and secondary antibodies for 1 h each, with washes in PBS between the incubations. The coverslips were then mounted on microscopy slides using Fluoromount-G, photographed using a Zeiss AxioCAM MRm digital camera connected to a Zeiss AxioVert 40 CFL microscope, and processed with the AxioVision software. The cellular effects induced by ectopic expression were determined by microscopy analysis.

Wound closure assay

For the wound closure assay, cells were seeded in six-well plates. The following day, siRNAs were transfected using SilentFect. The cells reached confluency over the next 48 h, and wounds were made in the confluent monolayers with a Gilson P200 pipette

plastic tip. Two to three spots along the wound were marked with a pen under the plate. The wounded areas were photographed directly after the wounding (0 h) and again after 20 h with a Zeiss AxioVert 40 CFL microscope using a 10× objective. The cells that had moved into the wounded areas were counted on the photographs. The field of view was 0.603 mm². The experiment was repeated five times and data from two to three wounds were analyzed for each condition.

Cell viability assay

Cell survival was determined by the calcein AM viability assay according to the protocol provided by the manufacturer. Cells were washed three times with PBS and then treated with 1 mM calcein AM in PBS for 50 min at room temperature; this was followed by analysis of the fluorescence intensity at excitation 490 and emission 520 on a fluorescence plate reader.

Cell adhesion assay

For the adhesion assay, cells were seeded in six-well plates and, the following day, the cells were transfected with siRNAs as described above. After 48 h, the cells were trypsinized and seeded on coverslips precoated with serum. The cells were allowed to adhere for 30 min, 1 h, or 2 h. The cells were then washed with PBS to remove nonadhered cells and fixed in 3% paraformaldehyde for 25 min. The coverslips were mounted and photographed with a Zeiss AxioVert 40 CFL microscope using a 10× objective. Cells attaching to the coverslips under the different conditions were counted on the photographs. The data shown represent quantifications from 5 to 10 random sites at the coverslips and were normalized to the amount of cells attaching at the initial time point.

Golgi transport assay

In essence, the original protocol from Presley et al. was used [218]. In brief, cells were transfected with EGFP-VSV-G alone or together with FILIP1, WHAMM or Rho GTPases. In those cases where the cells had been transfected with siRNAs, the cells were transfected with EGFP-VSV-G after 24 hours. The cells were kept at 40°C after the transfection and the transport of EGFP-VSV-G from the ER to the cell membrane via the Golgi complex was initiated by a transfer of the cells to 32°C.

4. RESULTS AND DISCUSSION

In brief

To decipher the signaling pathways downstream of RhoD, a yeast-two hybrid screening was performed. We used the constitutively active RhoD/G26V mutant fused to the DNA-binding domain of GAL4 as bait to screen a human mammary gland cDNA library fused to the GAL4 activation domain. We confirmed the interaction of RhoD with its potential binding partners by co-immunoprecipitation. We also mapped the precise domain of interaction between RhoD and its binding partners. Subsequently, their functional roles in cellular signaling context were investigated using different tools and strategies (Papers I, II, III, IV). Herein, I will discuss the results that we have achieved.

Serial no.	RhoD interacting protein	Cellular function
1.	FILIP1	Filamin A-interacting protein involved in cytoskeletal function.
2.	Rabankyrin-5	Rab5 effector. Endocytic protein
3.	Death-associated protein Kinase 3 (DAPK3/ZIPk)	Cytoskeletal regulation during apoptosis

Table 6. Shows RhoD binding proteins emanated from the yeast-two hybrid screening.

To elucidate the role of RhoD, via its binding partners, WHAMM and FILIP1 in the regulation of actin cytoskeletal dynamics, cell adhesion and migration

Paper I: RhoD regulates cytoskeletal dynamics via the actin nucleation–promoting factor WASp homologue associated with actin, Golgi membranes and microtubules.

The present notion about RhoD is that it inhibits endosome dynamics and cell motility [203]. Ectopic expression of RhoD leads to dramatic effects on the organization of actin filament system observed as long flexible filopodia protrusions and formation of short bundles of actin filaments [205]. FILIP1 was initially identified in 2002, as a Filamin A (FLNa) binding protein and was shown to have a role in the degradation of FLNa, suggesting that FILIP1 overexpression results in the decreased cell migration [219]. FLNa organizes actin networks by binding with the pre-existing actin filaments to form orthogonal lattices of filaments.

Intriguingly, after identification of FILIP1 as a RhoD-binding protein, a sequence similarity to WHAMM was noticed. More specifically, FILIP1 and WHAMM possess distinct

homology in their domain organization particularly in the structural maintenance of chromosome (SMC) domain. WHAMM does not bind Filamin-A (FLNa) instead it binds to the Arp2/3 complex and functions as an NPF. Previous studies suggest that FLNa is needed for cell motility via the organization of lamellipodia; knocking down of FLNa leads to defective cell migration of neural cells into the ventricular zone [219]. WHAMM localizes to the cis-side of Golgi apparatus [220]. It is clear that WHAMM has the ability to trigger actin polymerization. However, most of its functions seem to be associated with bundling of microtubules and Golgi homeostasis.

Overexpression of RhoD active variant in endothelial cell negatively effects cell migration [139]. Results from our experiments indicate that knocking down of both WHAMM and RhoD decreases cell migration in BJ/SV40T fibroblasts observed in wound closure assay. Furthermore, we observed a significant increase in focal adhesion size in cells depleted of RhoD or WHAMM. Moreover, these cells adhered more firmly to substratum. In conclusion, our data suggests a unique role of less studied member of Rho GTPases subfamily RhoD in cell migration and cell adhesion via its effectors, FILIP1 and WHAMM.

To clarify the role of RhoD, via its effectors, WHAMM and FILIP1 in the regulation of protein transport from ER to cell membrane via Golgi.

Paper II: RhoD regulates ER to Golgi transport through its effectors Filamin A-binding protein FILIP1 and WHAMM

The described role of WHAMM in Golgi homeostasis stimulated us to study the subcellular localization of RhoD in more detail. Previously, RhoD has been shown to localize to early endosome vesicles and cell membrane. We made an observation that endogenous RhoD localizes to the Golgi complex based on the colocalization with the Golgi markers, GM130 and TGN46. We confirmed this by co-expressing RhoD and ArfGAP, a known Golgi apparatus morphology maintenance protein and observed a colocalization between RhoD and ArfGAP. Upon overexpression of active and the dominant negative variants of RhoD (i.e., G26V and T31N respectively) in Cos1 and BJ/SV40T cells, we observed dispersion of the Golgi apparatus. RhoD/T31N had more prominent effect on the Golgi disruption. Similar effects were observed upon overexpression of WHAMM and FILIP1, indicating the presence of a RhoD-dependent signaling pathway in the regulation of Golgi homeostasis.

We measured and quantified this disruption of ER-to-Golgi transport using RhoD/T31N, together with a temperature sensitive mutant of vesicular stomatitis virus coat protein (EGFP-VSV-G). We reasoned that dominant negative variant of RhoD might affect the transport of this virus-derived protein from ER-to-Golgi. In this assay, at 40°C, VSV-G is misfolded and confined to ER. Upon a downshift in the temperature to 32°C this viral protein refolds and funnels through the ER to the plasma membrane via Golgi [218]. A drastic difference in the VSV-G protein transport was observed in the cells overexpressing RhoD/T31N as compared to control cells. Similar effects on the VSV-G transport were observed in WHAMM and FILIP1 overexpressing cells. WHAMM showed a predominant effect on the transport by trapping the VSV-G protein in ER even after 60 minutes. FILIP1 had a weaker effect and delayed the VSV-G transport by holding half of the protein in ER post 60 minutes.

While overexpression of RhoD and its effectors i.e., FILIP1 and WHAMM showed a dramatic effect on protein transport and Golgi disruption, knocking down by siRNA targeting RhoD, FILIP1 and/or WHAMM also affected Golgi homeostasis in BJ/SV40T cells. Knocking down of RhoD, WHAMM and FILIP1 resulted in dispersion of Golgi membranes. Comparatively, WHAMM induced less Golgi dispersion. In conclusion, the work in this paper describes that RhoD, via its effectors, WHAMM and FILIP1 interferes with the protein transport from ER-to-Golgi. Also, it shows that a shift in the balance of RhoD levels and its binding partners interferes with Golgi homeostasis.

To determine the role of RhoD, via the Rab5 effector Rabankyrin-5 in receptor tyrosine kinases trafficking

Paper III: RhoD binds the Rab5 effector Rabankyrin-5 and has a role in trafficking of receptor tyrosine kinases

The data in this manuscript describes the role of RhoD via its novel effector, Rabankyrin-5 in the trafficking of receptor tyrosine kinase (PDGF β). Rabankyrin-5 is a known effector for the Rab5 GTPase and is involved in early endosome and macropinosome motility in epithelial cells. Ectopic expression of Rabankyrin-5 has been well documented to increase macropinosome number and enhance fluid uptake in MDCK epithelial and fibroblasts cells [221]. By knocking down of Rabankyrin-5 in these cells reduces the macropinosome number.

We found that RhoD binds to Rabankyrin-5. Also, Rabankyrin-5 coordinates RhoD and Rab5 in the trafficking of early endosomes. A study by Gasman *et al.* showed that the active variant of RhoD/G26V localizes to early endosome vesicles and has a role in endosome trafficking [203]. In that study, it was observed that ectopically expressed RhoD inhibited Rab5-dependent effects and caused the formation of more spherical, scattered and small endosome vesicles. This RhoD-dependent effect on vesicle trafficking was observed to be independent of Rab5 overexpression, which suggests that RhoD is sufficient to disturb endosomal movement. Our data demonstrates that knocking down of RhoD and/or Rabankyrin-5 affects endocytosis. This was checked by impeding the internalization of receptor tyrosine kinase, PDGFR- β . In conclusion, our study demonstrates that RhoD controls endosome vesicle trafficking and endocytosis, presumably via the novel RhoD effector Rabankyrin-5.

To dissect the mechanism by which the interaction of RhoD and ZIPk regulates the actin filament assembly and focal adhesion dynamics

Paper IV: Interaction of RhoD and ZIP kinase modulates actin filament assembly and focal adhesion dynamics

This study gives an account of RhoD via its effector, **Zipper Interacting Protein kinase (ZIPk)** in regulating actin and focal adhesion reorganization. ZIPk is a serine/threonine kinase implicated in programmed cell death. This protein is also known as death-associated protein kinase 3, DAPK3 and belongs to death-associated protein family (DAPk). Members of this protein kinase family share great deal of similarity in their catalytic (kinase) domain and also cell-death related functions [222]. Close to N-terminus of the ZIPk protein is the kinase domain and it is due to this domain that DAPk, DRP-1 and ZIPk make a subfamily. Outside this region, this subfamily varies in size and structure. Upon upregulation of these kinases, cells undergo morphological changes that lead to programmed cell death by cell rounding and membrane blebbing. ZIPk has been implicated in the control of filamentous actin via myosin regulatory light chain phosphorylation (MRLC). We observed that RhoD interacts with ZIPk in a GTP-dependent manner. Additionally, we also tested the interaction between a point mutant and a deletion mutant of ZIPk (i.e., kinase dead mutant D161A and mutant lacking the C-terminal leucine zipper domain/ Δ LZ) with both the active variant of RhoD/G26V as well as the dominant negative RhoD/T31N. We observed that the ZIPk/ Δ LZ mutant did

not interact with RhoD in a GTP-dependent manner. However, the kinase dead D161A mutant did interact with RhoD in a GTP-dependent manner. Additionally, we found that overexpression of ZIPk induces the reorganization of the actin filament system observed as condensed stress fibres into thick bundles appearing like a star shape, similar to a phenotype described before [223]. Moreover, overexpression of the ZIPk also induces membrane blebbing that was not linked to reduced cell adhesion. Our data shows that both kinase dead mutant (D161A) and the C-terminus deletion mutant (ZIPk/ Δ LZ) did not affect the organization of stress fibres. We also observed that while the ZIPk wild type and its kinase dead counterpart localize to the cell cytoplasm, the Δ LZ mutant localizes in the nucleus of fibroblast cells. This can indicate a role of LZ domain in the localization of ZIPk. Intriguingly, overexpressing ZIPk together with either RhoD wild type or active variant, RhoD/G2V, suppresses the ZIPk-induced stress fibre bundling. The constitutively active RhoD mutant, RhoD/G26V, suppressed ZIPk-induced membrane blebbing, thereby reverting the phenotype to the normal fibroblast cells morphologically. However, the wild-type RhoD, dominant negative RhoD/T31N mutant and a membrane targeting-defective mutant of RhoD failed to suppress ZIPk-induced blebbing. This suggests that the suppressing and the membrane targeting abilities of RhoD are dependent on the GTP-loaded status of RhoD.

After observing that overexpressed ZIPk had a profound effect on stress fibre organisation, we tested the effect of ZIPk on focal adhesion organization. Wild type ZIPk overexpression resulted in a dramatic increase in focal adhesion size. It was only the wild type ZIPk that could increase the focal adhesion size and not kinase dead mutant of the ZIPk (D161A). When ZIPk was coexpressed with active variant of RhoD/G26V, the focal adhesion size was suppressed. Focal adhesion dynamics is related to the activity of focal adhesion kinase (FAK). FAK is activated by integrins via disruption of auto-inhibitory conformation. The phosphorylated tyrosine residue pY³⁹⁷ is positively correlated with the FAK activation. Fibroblast cells ectopically expressing ZIPk resulted in decreased phospho-Y³⁹⁷ and so did the kinase dead D161A mutant. However, the ZIPk/ Δ LZ mutant did not have any effect on phospho-Y³⁹⁷. On the other hand, overexpression of RhoD alone did not change Y³⁹⁷ phosphorylation significantly however, it suppressed the ZIPk-dependent decrease of phospho-Y³⁹⁷. In contrast, the phosphorylation on another tyrosine residue, Y⁵⁷⁶ was not affected significantly upon ectopic expression of either ZIPk or RhoD.

In essence, our data shows that RhoD interacts with ZIPK in a GTP-dependent manner and modulates stress fibers, focal adhesion reorganization and membrane blebbing.

5. FUTURE PROSPECTS

Till date, the best-studied members of Rho subfamily are RhoA, Rac and Cdc42. The other members of Rho GTPases have been less studied and their potential roles in myriads of cellular processes have not been fully explored. The work in this thesis brings forth one of the members of the less studied Rho GTPases subfamily i.e., RhoD.

Our findings with RhoD have unravelled the role of RhoD in the regulation of cell adhesion and migration via novel binding partners i.e., FILIP1 and WHAMM. Additionally, with the same effectors, RhoD also has role in regulating ER-to-Golgi transport and Golgi homeostasis. Our quest to know more about the function of RhoD and effector Rabankryin-5 provides a new understanding and knowledge of how RhoD has a role in the internalization and trafficking of the activated receptor tyrosine kinases. Subsequent work with binding partner, ZIP kinase, gives an insight into how RhoD via its binding partners, ZIP kinase also modulates focal adhesion dynamics and actin filament assembly. In summary, this thesis work contributes to our understanding of complex regulatory networks mediated by RhoD and the associated biological function. With this understanding of RhoD to date, it will be interesting and intriguing to find out additional roles of RhoD via its effectors in cell cycle progression or epithelial-mesenchymal transition, ultimately giving an insight and understanding of the signal transduction pathways mediated by RhoD in metastasis and/or cancer progression.

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AUTHORIZATION LETTER*

Details of communication with, "*The Guardian*", newspaper seeking permission to quote a poem in my PhD thesis.

Reply to my email on Wednesday, January 30, 2013 at 11:32:30 AM GMT+01:00

from:

Edie Reilly

Observer Letters

Letters.Observer@guardian.co.uk

Dear Vishal,

As far as I'm aware you don't need permission to quote the poem. The only time you need permission is if it is going to be reproduced in a publication that will be sold for profit. Therefore, you can quote this poem in your PhD thesis.

Best wishes,

Edie Reilly

Observer Letters

On Sunday, January 27, 2013 at 14:33, I emailed Observer letters, "*The Guardian*".

Dear Sir/Madam,

I am writing a PhD thesis and I would like to quote an obituary poetry that appeared in the "*The Guardian newspaper*" in 2003 written by a poet, Mr. Vikram Seth as a dedication to my late grandfather. I tried to find out the contact details of Mr. Seth, so that I can seek his permission directly but had no luck. I would appreciate, if you could help me in getting his permission so that I can quote his poetry in my thesis.

I look forward to hearing from you.

With regards,

Vishal

PUBLICATIONS AND MANUSCRIPTS